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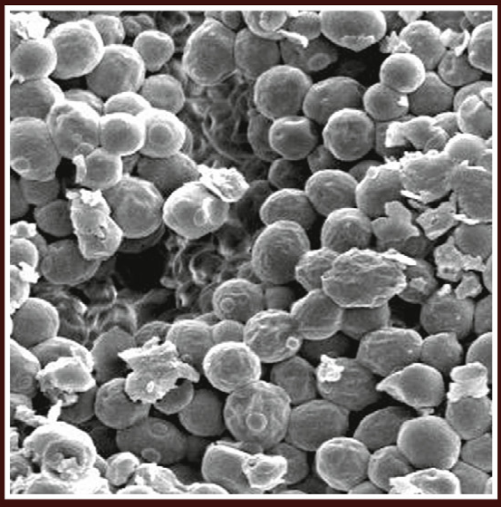

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Managing Microbes, Ensuring Quality and Valorising Waste

Edited by Annie E. Hill

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Edited by

Annie E. Hill

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List of contributors

T. Aiken Data Collection Solutions, Lexington, KY, USA

J.C. Akunna Abertay University, Dundee, United Kingdom

C.A. Boulton University of Nottingham, Loughborough, Leicestershire, UK

S. Davies Briggs of Burton Plc, Burton on Trent, Staffordshire, England, UK

J. Fischer VLB Berlin, Berlin, Germany

J. Forbes Dalhousie University, Halifax, NS, Canada

G.J. Freeman Campden BRI, Nutfield, Surrey, UK

J. Hancock Briggs of Burton Plc, Burton on Trent, Staffordshire, England, UK

A.E. Hill The International Centre for Brewing & Distilling, Heriot-Watt University, Edinburgh, Scotland, UK

R. Hofmann VLB Berlin, Berlin, Germany

M. Hutzler Research Center Weihenstephan for Brewing and Food Quality, TU München, Munich, Germany

F. Jacob Research Center Weihenstephan for Brewing and Food Quality, TU München, Munich, Germany

R. Juvonen VTT Technical Research Centre of Finland, Espoo, Finland

M. Kohlstock Scanbec, Bitterfeld-Wolfen, Germany

J. Koob Research Center Weihenstephan for Brewing and Food Quality, TU München, Munich, Germany

A. Laitila VTT Technical Research Centre of Finland, Espoo, Finland

S.-Q. Liu National University of Singapore, Singapore; and National University of Singapore (Suzhou) Research Institute, Suzhou, Jiangsu, China

A.J. MacIntosh Dalhousie University, NS, Canada

K. Merx Scanbec, Bitterfeld-Wolfen, Germany

K. Mueller-Auffermann Research Center Weihenstephan for Brewing and Food Quality, TU München, Munich, Germany

A.D. Paradh Vasantdada Sugar Institute, Pune, Maharashtra, India

M. Philips Briggs of Burton Plc, Burton on Trent, Staffordshire, England, UK

D.E. Quain International Centre for Brewing Science, School of Biosciences, University of Nottingham, Loughborough, Leicestershire, UK

R. Riedl Research Center Weihenstephan for Brewing and Food Quality, TU München, Munich, Germany

H. Schneiderbanger Research Center Weihenstephan for Brewing and Food Quality, TU München, Munich, Germany

J. Siegrist Sigma-Aldrich, Buchs, Switzerland

G.S. Simate School of Chemical and Metallurgical Engineering, University of the Witwatersrand, Johannesburg, South Africa

G. Spedding Brewing and Distilling Analytical Services, LLC., Lexington, KY, USA

A. Speers The International Centre for Brewing & Distilling, Heriot-Watt University, Edinburgh, Scotland, UK

G.G. Stewart GGStewart Associates, Cardiff, Wales, UK; The International Centre for Brewing & Distilling, Heriot-Watt University, Edinburgh, Scotland, UK

K. Suzuki Quality Control Center, Asahi Breweries, Ltd., Ibaraki, Japan

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Edited by Annie Hill

Preface

The purpose of brewing is to produce beer through the hydrolysis of starch from barley malt, together with wheat, maize, rice, sorghum, unmalted barley, sugar/syrups, and the incorporation of hops. These raw materials are mashed into a sugary nitrogenous fermentable liquid called wort. This medium is converted into an alcoholic, carbonated beverage by yeast. The brewing process is essentially a microbiological/biochemical series of reactions, which involves a number of complementary disciplines including plant breeding and cultivation, chemistry, chemical/civil/mechanical/electrical engineering, and also computer control. Although this volume focuses on the positive microbiological aspects of brewing, it does consider, in considerable detail, microbiological contamination of the process starting with raw materials and it concludes with the quality of the finished beer (fresh and not so fresh) in both small pack containers and on draft.

Although there are many excellent text books on brewing, their primary focus has been the entire process with microbiological aspects being integrated into the syntax. As a consequence, the discussion of fermentation tends to be more biochemical in its emphasis than microbiological. This volume's focus is decidedly microbiological! This applies to both brewer's yeast strains and contaminating microorganisms—bacteria, wild yeasts, and mycelial fungi.

Five chapters are devoted to brewer's yeast and they consider, in appropriate detail, their taxonomy and related areas such as identification and characterization. Wort fermentation and metabolism are discussed and, in particular, the metabolic engineering of these organisms. The fact that brewer's yeast cultures are normally recycled through a number of wort fermentations is emphasized and details of yeast management between fermentations are discussed.

Contaminating fungi, both yeast and mycelial fungi, are discussed in the context of their influence on beer characteristics and quality. It is emphasized, in a number of chapters, that brewing is usually a sterile process (unlike distilling). This is due to the fact that the wort is boiled and in many situations (not all) benefits from the antiseptic properties of hop acids. It is appreciated that often wild yeasts can contaminate pitching yeast cultures and that acid washing does not cleanse the brewing yeast culture of such microorganisms. Also, the stimulation of beer gushing by mycotoxins is discussed.

Most of the remainder of the text focuses on a detailed discussion of contaminating bacteria—both Gram positive and Gram negative that occur in brewing. Sometimes, these bacteria are welcome (e.g., in Lambic beer) but usually this is not the case. This unwelcome contamination can occur on raw materials (particularly malt

and water), during fermentation and maturation and in the final beer. Contamination in all these production stages will influence beer flavor and stability (physical and flavor) and the implications of these bacterial infections are considered in detail.

Graham G. Stewart
Heriot Watt University
Edinburgh, Scotland
November 2014

Introduction to brewing microbiology

It is an exciting time to be a microbiologist! Now that we are in the postgenome era, we have more answers within reach than ever before. More knowledge brings the realization of how much we still have to learn but also the tools to help alleviate risks, solve problems, and manipulate microbes to improve and develop new products and processes.

Central to brewing is of course a microbiological process and as such a brewing microbiologist needs to understand production strain(s), in terms of flavor and aroma profile, physical stability, handling, and conditions required for optimal fermentation. An appreciation of the vulnerability of the process and product to contamination is also required to ensure quality and consistency. A third aspect that has become increasingly useful in brewing microbiology is the exploitation of microbes to add value to byproducts of the brewing process, to reduce cost of effluent discharges, and also to generate energy. Each of these aspects is covered in detail within this volume, but to provide some background:

Brewing yeast

Over 1500 species of yeast have been identified. These are predominantly single-celled fungal microorganisms able to grow in both the presence and absence of oxygen. Of these, there are basically two major strains used in brewing: *Saccharomyces cerevisiae* (ale) and *Saccharomyces pastorianus* (lager), a hybrid of *S. cerevisiae* and *Saccharomyces eubayanus* (Libkind et al., 2011). Ale yeast operates at around room temperature (18–22 °C), ferments quickly, and produces the "fruitiness" characteristic of most ales. Lager yeast works at colder temperatures (8–15 °C), ferments slowly, and utilizes more wort sugars, leaving a cleaner, crisp taste. Ale and lager yeast are the most commonly used worldwide, but the increase in craft brewing has led to a rise in the use of other yeast strains such as *Brettanomyces* spp., which are traditionally used in Lambic beer production.

The discovery and whole genome sequencing of *S. eubayanus* has caught the imagination of both brewers and research microbiologists alike. It was known for some time that *Saccharomyces pastorianus* was a hybrid organism involving *S. cerevisiae* but the other parent(s) were unknown until the isolation of *S. eubayanus*. Genome sequencing has revealed that it is an almost exact genetic match of the non-*S. cerevisiae* subgenome of lager yeast (Libkind et al., 2011). First isolated in Patagonia, it was thought that the parent *S. eubayanus* strain had its origin in South America but recent

surveys have recovered *S. eubayanus* from China (Bing, Han, Liu, Wang, & Bai, 2014). Future studies may uncover the exact parentage and geographical origin.

The increased research intensity over the past few years has led to the realization that, as with *S. eubayanus*, many commercial yeast strains are natural yeast hybrids (Gibson & Liti, 2015). High genetic diversity within yeasts used in the wine industry suggests that hybridization events are common. The ability of yeasts to adapt to changing conditions through hybridization not only confers evolutionary advantage but also presents us with an opportunity to manipulate mating in order to create novel strains without resorting to genetic modification.

Process and product integrity

There are literally millions of food spoilage organisms. However, those responsible for beer spoilage are limited to only a few species of bacteria and "wild" yeast. Beer has a range of properties that hinder microbial growth including low pH, high alcohol concentration, low nutrient level, antiseptic action of hop acids, low oxygen concentration, and carbonation. Its production is a microbiological process though, meaning that the medium into which the brewing yeast is pitched is an ideal environment for the growth of a range of microorganisms. Some wild yeast and bacteria are also able to survive and proliferate in the final beer. Molds can cause spoilage through growth on raw materials but are not regarded as beer-spoilage organisms. Beer-spoilage organisms are defined as those capable of multiplying in beer resulting in product deterioration.

Most brewers now take a proactive approach to beer-spoilage organisms beginning with brewhouse design: use of closed vessels, avoiding dead legs in pipework, and use of cleaning-in-place (CIP). These are all methods that are designed-in to new plants but can also be retrofitted or integrated into existing breweries. A second improvement in tackling spoilage is to carry out ATP testing on brewing liquor, CIP rinse water, and vessel surfaces; this rapid method of microbiological testing does not identify bacteria or yeasts but gives a very quick indication of plant cleanliness and the success of CIP cycles.

Raw materials and final product testing are still predominantly carried out using traditional methods of plating and microscopy, but rapid methods, such as PCR, are increasingly becoming affordable. Improvements in methodology mean that tests previously consigned to research laboratories or dedicated microbiology services are now possible without extensive training or specialized facilities. As equipment and consumables costs fall, we will see further take up in tools to tackle microbial spoilage within breweries and a consequent improvement in product quality and consistency.

Waste valorization

Breweries no longer produce waste; the term "coproduct" has been adopted in recent years to cover all nonbeer outputs such as spent grain and yeast. As our understanding

of microbial metabolism has increased and tools to manipulate specific biochemical pathways have been developed, a range of new applications have been identified, including methods of converting "waste" to either new products or energy.

Brewery waste streams often contain high-value chemicals that can be extracted and reused in other industries. Spent yeast and grain are most commonly used in animal feed and human nutrition, but both can also be used as a flavoring agent, as a source of enzymes and single cell protein, or as a filter element for beverage clarification. Rojo et al. (2014) have discovered that "bagasse," a residue left over from beer brewing, could be used to create a new biomaterial capable of promoting bone regeneration, and which could be used to treat bone diseases, assist in bone grafts, and coat a prosthesis. Yeast may also be used as a substrate for microalgae cultivation and for bioremediation of heavy metals, but more recently it has found use in waste water treatment and biogas production. A number of companies have developed systems to use microbes (both yeast and bacteria) to treat waste-water generating methane, which can be used for power and heat.

As our knowledge grows, we will undoubtedly find further uses for microbes and an even better understanding of how they can contribute to new product development and process design. What is certain is that they will continue to both challenge and reward us.

Annie E. Hill

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In memory of Dr Brian Watt, who led me to choose a career in microbiology.

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Part One

Yeast: properties and management

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Yeast: an overview

1

A. Speers[1], *J. Forbes*[2]
[1]The International Centre for Brewing & Distilling, Heriot-Watt University, Edinburgh, Scotland, UK; [2]Dalhousie University, Halifax, NS, Canada

1.1 Yeast species/strains used in brewing and distilling

The formal classification of brewing yeasts over the past 50 years has changed enough that many brewing scientists (and most brewers!) avoid using the current genus and species to identify their yeast and simply label them as either ale or lager strains. These yeasts are used to produce most beers – that is, either 'ales' or 'lagers'. Ale is normally made with *Saccharomyces cerevisiae* that rises to the top of the fermenter at the cessation of fermentation while lager is made with *S. carlsbergensis*, which settles to the bottom of the tank towards the end of the fermentation. In the past, Barnett, Payne and Yarrow (1983) stated that both types of yeast should be characterized as variants of *S. cerevisiae*. However, the strains differ in their DNA profiles, ability to ferment melibiose, (ale strains lack melibiase activity) and their maximum growth temperature (lager strains do not grow above 34 °C (Webb, 1977)) and for these reasons, Stewart (1990) has argued that the two types of yeast should be classified as separate species.

Additionally, the increasing importance of a third species, *Brettanomyces*, has been recognized following the massive growth of the craft brewing movement in the United States. 'Brets', as they are termed in the industry, are used in various stages in the production of lambic-type beers. They are considered a spoilage yeast in lager and ale fermentations as they produce volatile phenolic flavours and acetic acid due to their ability to produce off flavours by the production of volatile phenols (Libkind et al., 2011), their ability to produce acetic acid (Wijsman, van Dijken, van Kleeff, & Scheffers, 1984) and their ability to over attenuate products below 1 °Plato (Kumara & Verachtert, 1991). Those involved with the wine industry have spent significant amounts of time and money learning to isolate and characterize *Brettanomyces* spp. to develop better methods of early detection and eradication (Conterno, Joseph, Arvik, Henick-Kling, & Bisson, 2006; Dias et al., 2003; Oevelen, Spaepen, Timmermans, & Verachtert, 1977). Despite the large amount of negative attention *Brettanomyces* receives, this interesting microbe has been shown to contribute favourable organoleptic qualities to a number of products and to be of use in several industrial applications.

Belgian lambic beer producers have promoted the unique organoleptic characteristics of *Brettanomyces* species in concert with other microbes for hundreds of years to produce a beer that is crisp, acidic and refreshing (De Keersmaecker, 1996; Oevelen et al., 1977). However, in comparison to ale and lager yeast less is known about *Brettanomyces* species employed in brewing.

Since the early 2000s the advances in molecular biology have added to our understanding of the lager yeasts (Libkind et al., 2011; Walther, Hesselbart, & Wendland,

Brewing Microbiology. http://dx.doi.org/10.1016/B978-1-78242-331-7.00001-0

2014). It appears that a newly discovered and sequenced species, *S. eubayanus*, and *S. cerevisiae* have combined to form the hybrid lager yeast genome. It is hypothesized that materials containing *S. eubayanus* strains were imported from Patagonia to Europe where hybridization events have occurred to form the *S. carlsbergensis* progeny, but more recent studies suggest that the origin of the *S. eubayanus* strain may be Asia (Bing, Han, Liu, Wang, & Bai, 2014).

Two types of lager yeast are in common use in the brewing industry. The first, Group I, the so-called Saaz type (i.e. 'Unterhefe No. 1' isolated by the Carlsberg brewery in 1883) is principally a triplod strain with an almost complete copy of the *Saccharomyces cerevisiae* genome and slightly more than a diploid copy of *S. eubayanus* genome (Walther et al., 2014). These same researchers noted that the Group II lager (i.e. the Froberg type, Weihenstephan WS34/70) has a tetraplod with roughly two copies of chromosomes from *S. cerevisiae* and two from *S. eubayanus*. It has been suggested that the low fermentation temperatures (e.g. as low as 5 °C) that Group I lagers were exposed to may have driven the difference between Group I and II lager yeasts (Walther et al., 2014).

1.2 Yeast cell structure

Yeast is the most important part of the brewing fermentation process. Yeast converts sugar to alcohol, carbon dioxide and other compounds that influence the flavour and aroma of beer. Brewer's yeast is a eukaryote and belongs to the kingdom Fungi. By some scientific classifications, all beer-brewing strains of yeast are placed in the genus *Saccharomyces* (sugar fungus) and species *cerevisiae* (Walker, 1998). However, the brewing industry uses a classification which divides yeast into two types: ale yeast (*S. cerevisiae*) and lager yeast (*S. carlsbergensis*). The distinction is kept so as to separate yeasts used to make ales from those used to make lagers (Briggs, Boulton, Brooks, & Stevens, 2004).

Most of the organisms in the kingdom Fungi are multicellular; however, yeast is a single-cell organism. A single yeast cell measures about 5–10 µm in diameter and is usually spherical, cylindrical or oval in shape (Boulton & Quain, 2001, pp. 5–360). Yeast occurs in single, pairs, chains and clusters (Stewart & Russell, 1998). Figure 1.1 is a simplified diagram of yeast cell structure. The cell wall is a barrier that is mostly composed of carbohydrates surrounding the cell (Boulton & Quain, 2001). It is a rigid structure which is 250 nm thick and constitutes approximately 25% of the dry weight of the cell (Stewart & Russell, 1998). There are three cross-linked layers comprising the cell wall (Figure 1.2). The inner layer is a chitin (a long-chain polymer of an *N*-acetylglucosamine) layer, composed mostly of glucans; the outer layer is mostly mannoproteins while the intermediate layer is a mixture of both the inner and outer layer (White & Zainasheff, 2010).

To reproduce asexually, a yeast cell clones itself, thereby creating a new daughter cell. Cell separation is achieved when the layers of the cell wall separate, leaving the bud scar on the mother cell and the birth scar on the daughter cell (Stewart & Russell, 1998). The bud scar is composed mainly of chitin. The average ale yeast cell will not bud more than 30 times over its lifetime while lager yeast will bud only 20 times before they are unable to bud further (Wyeast Laboratories, 2009).

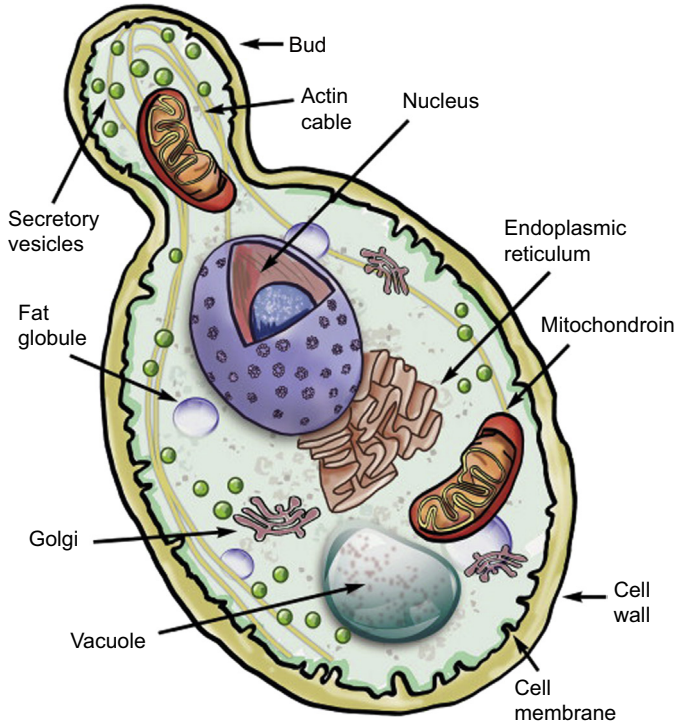

Figure 1.1 Main features of a typical yeast cell (Stewart & Russell, 1998).

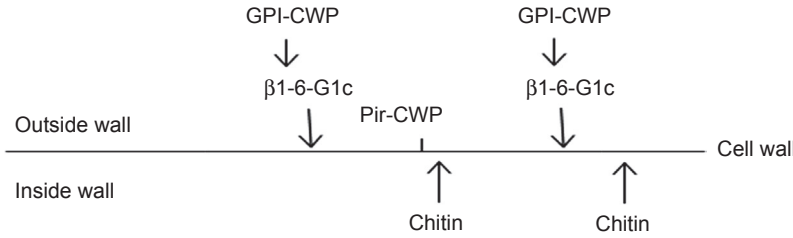

Figure 1.2 Molecular organization of the cell wall of *S. cerevisiae*. GPI-CWP are GPI-dependent cell wall proteins, Pir-CWP are pir proteins on the cell wall and β1-6-Glc are glucan molecules, which are highly branched. Therefore, they are water soluble, which tethers GPI-CWPS to the cell wall (Kils, Mol, Hellingwerf, & Brul, 2002).

The plasma membrane is a semipermeable lipid bilayer between the cell wall and the inside of the cell. There are several distinct roles that the plasma membrane carries out such as to provide a barrier to free diffusion of solutes, to catalyse specific exchange reactions, to store energy dissipation, to provide sites for binding specific molecules involved in metabolic signalling pathways and to provide an organized support matrix for the site of enzyme pathways involved in the biosynthesis of other cell components (Hazel & Williams, 1990). The plasma membrane is quite fluid and

flexible due to its constituents of lipids, sterols and proteins. Additionally, these constituents allow for the creation of a daughter cell.

The formation of double bonds in fatty acids controls their level of saturation. The saturation level determines the ease and extent of hydrogen bonding that can occur between fatty acids (Briggs et al., 2004). Membrane fluidity is necessary for proper membrane function. Lipid bilayers are by their nature fluid and that fluidity is determined by the extent to which the lipids bind to one another (White & Zainasheff, 2010). By controlling the level of saturation in their lipid membranes, yeast cells are able to maintain proper membrane fluidity at different temperatures, which is important during fermentation. Without proper aeration yeast cells are unable to control membrane fluidity through to the end of fermentation which leads to halted fermentations and off-flavours of the final product (White & Zainasheff, 2010).

The cytoplasm is that portion of the cell enclosed by the plasma membrane and excluding other membrane-bound organelles. It is an aqueous colloidal liquid containing a multitude of metabolites (Briggs et al., 2004). The cytoplasm contains intercellular fluid known as the cytosol. The cytosol contains enzymes involved in anaerobic fermentation that enable the cell to convert glucose into energy immediately after it enters the cell (White & Zainasheff, 2010).

The mitochondrion is an organelle where aerobic respiration occurs. Mitochondria consist of a double membrane that is the location of the conversion of pyruvate (a metabolic compound) and the tricarboxylic acid cycle. The nucleus stores the cell DNA and is delineated by a lipid membrane that envelopes the nucleus and is similar to the plasma membrane. The cell uses mRNA to transfer the information out into the cytoplasm for use in protein synthesis (White & Zainasheff, 2010).

The vacuole is a membrane-bound structure that stores nutrients and is also where the cell breaks down proteins. Brewer's yeast vacuoles are large enough to be seen through light microscopy (White & Zainasheff, 2010). The major site for proteolysis is the cell vacuole. Much of the regulation of both specific and nonspecific proteolysis involves the sequestration of target proteins into vacuoles where they are exposed to proteinases (Briggs et al., 2004). The endoplasmic reticulum is a network of membranes and is usually where the cell manufactures proteins, lipids and carbohydrates for membranes and secretion (White & Zainasheff, 2010). Other microbodies are mainly made up by glycogen bodies and lipid granules (Boulton & Quain, 2001).

1.3 Comparison of lager and ale yeast

The distinctions between the yeast used in ale and lager brewing are small. Traditionally, ale yeast were regarded as top fermenters that formed a frothy yeast head on the surface of the fermenting beer, which was skimmed off to be used for subsequent brews, while lager yeasts were bottom fermenters that formed little surface head and were recovered from the bottom of the fermenter (Briggs et al., 2004). Today, this is a less useful distinction as many types of ale yeast now have the capacity to fall out of solution and settle at the bottom of the fermenter (Adams & Moss, 2008).

The optimal growth temperature of lager and ale yeast differs and this is reflected in the different temperatures used for lager fermentations, 8–15 °C, and for ale fermentations, 18–22 °C (Adams & Moss, 2008). Lager and ale yeasts can also be distinguished by the ability of lager strains to ferment the disaccharide melibiose because they have α-D-galactosidase activity, which hydrolyses melibiose to galactose and glucose while ale strains cannot. However, this is of no practical importance since the sugar does not occur in wort (Briggs et al., 2004). Additionally, lager yeast strains can utilize maltotriose more rapidly than ale strains. Lager strains utilize mixtures of galactose and maltose simultaneously, whereas ale strains prefer to utilize maltose (Boulton & Quain, 2001).

1.4 Flocculation

One functional definition of flocculation is that it describes the ability of yeast strains to clump together and fall out of solution. Near the end of fermentation, single cells aggregate into clumps of thousands of cells. Different strains of yeast have different flocculation characteristics. Some strains flocculate earlier during fermentation and subsequently do not attenuate (i.e. finish the fermentation) normally. Flocculating too early results in a beer that is under attenuated and sweet; however, when yeast fails to flocculate entirely, it results in a beer that is cloudy with a yeasty flavour (Speers, 2012).

Flocculation has been studied for many years and the exact mechanism is still debated. Cell wall composition is a key factor in the ability of adjacent cells to stick to each other. Yeast has a thick cell wall made up of protein and polysaccharides with a net negative surface charge due to phosphates in the cell wall (Briggs et al., 2004). The extent of the negative charge depends on the yeast strain, phase of growth, oxygen availability, starvation, generation number, dehydration and cell age. Yeast cells are also hydrophobic due to exposed hydrophobic peptides and lipids (Akiyama-Jibiki, Ishibiki, Yamashita, & Eto, 1997). The primary determinant of flocculation is the yeast strain itself (Speers, Smart, Stewart, & Jin, 1998).

The minute differences in cell wall composition play a key role in flocculation behaviour and determine the degree of flocculation for a strain. Factors that influence the degree of flocculation include the original gravity of the wort, temperature of fermentation, pH of the wort, pitching rate, initial oxygen content, calcium and inorganic ion concentration, and cell age. Additionally, anything that affects the health and growth rate of the yeast affects flocculation (Speers et al., 1998).

The mechanism of lectin-like cell–cell interactions has been established to explain yeast flocculation (Speers et al., 1998). Lectins are a structurally diverse group of proteins that are capable of binding carbohydrates while zymolectin is an anchored yeast cell wall protein that contains one or more mannose binding sites (Boulton & Quain, 2001). This mechanism proposes that specific surface proteins known as zymolectins, which are present on flocculent yeast cells, bind to mannose residues of mannan molecules on neighbouring cell surfaces (Speers et al., 1998). The involvement of this protein–carbohydrate interaction was suggested by Taylor and Orton (Taylor & Orton, 1978), as flocculation can be inhibited specifically by mannose.

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Yeast quality assessment, management and culture maintenance[1]

2

G.G. Stewart
GGStewart Associates, Cardiff, Wales, UK; The International Centre for Brewing & Distilling, Heriot-Watt University, Edinburgh, Scotland, UK

2.1 Introduction

For many decades, brewer's yeast cultures were selected empirically by brewers. A culture had to suit the process and the product, and sometimes the whim of the brewer. However, during the past 40 years or so, research has been focused on understanding the objectives of yeast performance during wort fermentation and to produce beers with the necessary flavour, stability and drinkability. Detailed studies have been encouraged by our increasing knowledge of the microbiology and biochemistry of wort fermentation by yeast (Stewart, Hill, & Russell, 2013). Allied to this is the advent of sophisticated analytical methodology and appropriate instrumentation. Examples are gas chromatography with mass spectroscopy (GC–MS), which is used for the identification and quantification of volatile flavour compounds (Boulton & Quain, 2001). High-performance liquid chromatography (HPLC) has been used to determine wort sugars and dextrins (D'Amore, Russell, & Stewart, 1989), amino acids and small peptides (Lekkas, Stewart, Hill, Taidi, & Hodgson, 2007), and hop constituents (Roberts & Wilson, 2006). Also, thermal energy analysis for N-nitrosodimethylamine (Spiegelhalder, Eisenbrand, & Preussmann, 1979) and bioluminescence for the detection of adenosine triphosphate in biological materials (Boulton & Quain, 2001) are used to detect possible contaminants, and soil that may still be present after cleaning (but not relevant to the content of this chapter). The first two methods (GC and HPLC) are an integral part of yeast strain selection and overall fermentation research.

2.2 Objectives of wort fermentation

The objectives of wort (unfermented beer) fermentation are to consistently metabolize wort constituents into ethanol, carbon dioxide and other fermentation products to produce beer with satisfactory quality and stability. It is also important to produce yeast crops that can be confidently repitched into subsequent brews. This is unlike distiller's yeast strains where the yeast culture is used only once; it is not repitched.

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During the brewing process the overall yeast performance is controlled by a plethora of factors. These include:

- The yeast strain(s) used—ale or lager, flocculent or non-flocculent, top or bottom cropping, etc.;
- The condition of the cultures at pitching and throughout fermentation—viability and vitality;
- The wort sugar spectrum;
- The concentration of free amino nitrogen (FAN) and the category of assimilable nitrogen;
- The concentration of a plethora of ions;
- The tolerance of yeast cultures and individual cells to stress factors such as osmotic pressure, ethanol, temperature, desiccation and mechanical stress;
- The gravity (concentration) of the wort at yeast pitching;
- The wort dissolved oxygen (DO) level at pitching;
- The culture's flocculation characteristics throughout the fermentation;
- The geometry of the fermenters used (horizontal, vertical, conical, spherical, or flat bottom, overall capacity, etc.) and the type of fermentation used—batch or continuous.

All of these factors, individually or more often in combination with one another, permit the definition of the requirements of an acceptable brewer's yeast strain (Stewart & Russell, 2009). To achieve beer of high quality, not only the yeast must be effective in receiving the required nutrients from the growth/fermentation medium (the wort), able to tolerate the prevailing environmental conditions (e.g. osmotic, temperature and ethanol tolerance) and impart the desired flavour to the beer, but the microorganisms themselves must be effectively removed from the fermented wort by flocculation, centrifugation and/or filtration after they have fulfilled their metabolic role.

It has already been discussed that a major difference between brewer's yeast strains and other alcoholic producing yeasts is that brewers recycle their yeast cultures from one fermentation into a subsequent fermentation. It is therefore important to protect the quality of the cropped yeast culture because it will be used to pitch a later fermentation and will consequently have a profound effect on the quality of the resulting beer produced with it. Distillers (e.g. Scotch whisky producers) use a yeast culture only once. This introduces a separate series of selection criteria for a yeast culture that are beyond the scope of this chapter.

2.3 Brewer's yeast species

There are basically three different types of beer: lager, ale and stout (dark beer). In reality, stout is usually a form of ale. The commercial worldwide production volume of ale has always been much lower than that of lager, and over the years this difference has grown worldwide (Stewart, 2013). However, this difference between ale and lager beer volumes has narrowed slightly during the past decade in the USA and is currently 4.6% ale, largely due to the increasing viability of the craft brewing sector.

Although there are several differences between the production methods of these two types of beer, one of the main differences is the characteristics of ale and lager

yeast strains. Consequently, research by many breweries and research institutions on this topic has been extensively conducted (Stewart et al., 2013), and the typical differences between ale (*Saccharomyces cerevisiae*) and lager (*Saccharomyces pastorianus*) yeast strains have been established (Table 2.1).

With the advent of molecular biology-based technologies, gene sequencing of ale and lager brewing strains has shown that they are interspecies hybrids with homologous relationships to one another and also to *Saccharomyces bayanus*, a yeast species used in wine fermentation and identified as a wild yeast in brewing fermentations (Sofie, Saerens, Duong, & Nevoigt, 2010) (Figure 2.1). The gene homology between *S. pastorianus* and *S. bayanus* strains is relatively high at 72%, whereas the homology between *S. pastorianus* and *S. cerevisiae* is much lower at 50% (Pederson, 1995). Research (Libkind et al., 2011) considering the origin of *S. pastorianus* has already been discussed in Chapter 1.

Table 2.1 Differences between ale and lager yeast strains

Ale yeast	Lager yeast
S. cerevisiae	*Saccharomyces uvarum* (*carlsbergensis*) *S. cerevisiae* (lager type) *S. pastorianus*
Fermentation temperature 18–22 °C	Fermentation temperature 8–15 °C
Cells can grow at 37 °C or higher	Maximum growth temperature 34 °C
Cannot ferment the dissacharide melibiose	Ferments the dissacharide melibiose
'Top' fermenter	'Bottom' fermenter

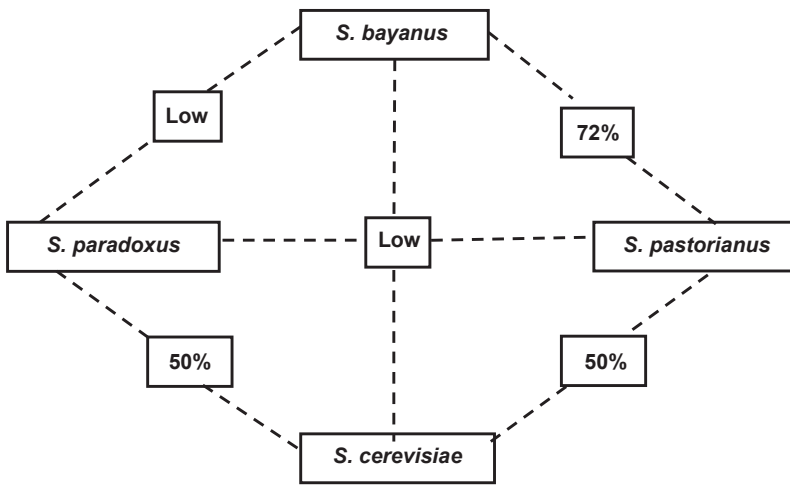

Figure 2.1 *Saccharomyces sensu stricto* group for ale and lager strains.

Most breweries have close control of their yeast strains, and, unlike most distillers, they preserve (maintain) and propagate their cultures themselves on a regular basis.

2.4 Yeast management

The overall process between fermentations is collectively described as yeast management. This process includes strain storage (in a culture collection), propagation, cropping, culture storage, acid washing (if required) and then wort fermentation itself. This latter procedure is not usually regarded as yeast management and will not be discussed here (see Chapters 1 and 3). The use of dried brewer's yeast is becoming popular in some brewing operations and will be discussed later in this chapter (Finn & Stewart, 2002).

It is a normal procedure in many breweries to propagate fresh yeast (particularly lager yeast) every 8–10 generations (fermentation cycles) or less. Prolonged yeast cycles can result in sluggish fermentations, usually due to lower rates of wort maltose and maltotriose uptake, higher levels of sulphur dioxide and hydrogen sulphate, prolonged diacetyl reduction times, and increased flocculation and sedimentation rates.

The long-term preservation of a brewing yeast culture requires that not only is optimal survival important, but it is imperative that no changes in the character of the yeast strain occurs. Many yeast strains are difficult to maintain in a stable state, and the long-term preservation by lyophilization (freeze drying), which has proved useful for mycelial fungi and bacteria, has been found to give poor results with most brewing yeast strains. Storage studies have been conducted with a number of ale and lager brewing strains (Russell & Stewart, 1981).

It is important to emphasize that although considerable information is available about brewer's yeast fermentation per se (e.g. Boulton & Quain, 2001; Sofie et al., 2010; Stewart & Russell, 2009), by comparison, basic detailed information on yeast management processes between wort fermentations has been lacking. Indeed, although the overall fermentation procedures and control have become very sophisticated, yeast management was, until recently, the "poor relation" of the process.

Yeast management can be divided into a number of overlapping procedures:

- Before propagation (the production of yeast biomass) but after fermentation and cropping, most (but not all) yeast strains are stored under standard conditions in a brewery or in an accredited culture collection—sometimes both, for security;
- Yeast propagation (biomass formation) in wort under aerobic conditions;
- Following propagation, the yeast is pitched into wort. This is the first generation (cycle) of a multi-generational procedure;
- At the end of fermentation (attenuation), yeast cropping occurs, followed by storage before repitching. Cropping occurs using the flocculation characteristics of the strain or with a centrifuge;
- To eliminate contaminating bacteria, the yeast slurry can be acid washed. Also, sometimes (but less frequently these days), the yeast slurry is sieved to remove contaminating trub (i.e. coagulated protein–phenol solid material).

2.5 Storage of yeast stock cultures between propagations

The advent of pure yeast strain fermentation dates from the studies of Emil Christian Hansen working in the Carlsberg Laboratory in Copenhagen during the latter decades of the nineteenth century. He isolated four separate strains from the Carlsberg lager yeast culture (Holter & Moller, 1976). He studied these four strains from the standpoint of overall brewery performance, and only one of them proved to be suitable for beer fermentation. This strain, designated as "Carlsberg Yeast No. 1," was introduced into the Carlsberg Brewery in Copenhagen for use on a production scale on 13 May 1883, and pure strain brewing of lager beer can be considered to have commenced from this date onwards (Holter & Moller, 1976). Carlsberg Yeast No. 1 was named *S. carlsbergensis* (Hansen, 1883) and is now known as *S. pastorianus* (Pederson, 1995).

With the advent of the use of pure yeast strain fermentation in brewing, Hansen soon found it necessary to furnish the Carlsberg brewery with production quantities of pure cultures of the single lager strain, and noted that it would be more convenient to develop a specific apparatus for the purpose of large-scale yeast propagation. Consequently, in association with a coppersmith (W.E. Jansen), Hansen developed an apparatus specific for the purpose. At the beginning of 1886, this apparatus was effectively working in the Carlsberg brewery, and it was also working in a number of breweries including that of Heineken.

As a result of Hansen and Jansen's efforts, the practice of using a pure strain in lager production was soon adapted by breweries worldwide, particularly in the USA. Ale-producing regions, however, met this "radical innovation" with severe opposition and scepticism. The method was merely regarded as a means of reducing wild yeast and bacterial infection. It was not until the middle of the last century that the pure ale strain methods were adopted. Indeed, a few ale-producing brewers are yet to adopt this procedure. Anderson (2012) published a paper entitled, "One yeast or two? Pure yeast and top fermentations," which focuses on the reluctance for British ale brewers to introduce pure yeast in the production of top fermentation beers until comparatively recently. Today, yeast propagation equipment is available for large and small breweries "off the shelf" (Nielsen, 2010).

2.6 Preservation of yeast strains

The long-term preservation of a brewing yeast strain requires that not only is optimal survival important, but it is imperative that no change in the characteristics of the yeast strain occurs. Hansen's studies resulted in storage of his strains in liquid nutrient media before propagation. This evolved into many breweries and independent culture collections maintaining their yeast strains on nutrient media solidified initially with gelatine and subsequently with agar. Some yeast strains are difficult to maintain in a stable state and the long-term preservation by lyophilization (freeze drying), which

has proved useful for mycelial fungi and bacteria (Kirsop & Doyle, 1990), has been found to give poor survival results with brewing yeast strains (Kirsop, 1955). However, as will be described later, the use of dried yeast cultures for pitching into wort is increasing in popularity.

Storage studies have been conducted with a number of ale and lager brewing yeast strains (Russell & Stewart, 1981). The following storage conditions were investigated:

- Low temperature as a result of storage in liquid nitrogen (−196 °C). With the advent of −70 °C refrigerators in the 1980s, liquid nitrogen has been largely replaced for this purpose with similar results;
- Lyophilization (freeze drying);
- Storage in distilled water;
- Storage under oil;
- Repeated direct transfer on solid culture media, subcultured once a week for 2 years;
- Long-term storage at 21 °C on solid nutrient medium, subcultured every 6 months for 2 years;
- Long term storage at 4 °C on solid nutrient medium, subcultured every 6 months.

After a 2-year storage period, the wort fermentation tests that included fermentation rate and wort sugar uptake efficiency, flocculation characteristics, sporulation ability, formation of respiratory deficient colonies and rate of survival were conducted and the results were compared to the characteristics of the stored control culture. Low-temperature storage appears to be the storage method of choice. However, there are capital and ongoing cost considerations connected with this method. Storage at 4 °C on nutrient agar slopes, subcultured every 6 months, was the next method of preference to low temperature storage, and this method is simple to perform and relatively inexpensive. Lyophilization and other storage methods revealed yeast instability that varied from strain to strain. Many breweries today store their strains (or contract store them) at −70 °C. Routine subculturing of yeast cultures on solid media every 6 months or so, albeit a less desirable storage method, is still an acceptable method. Freeze drying should be avoided as a storage method (Finn Stewart, 2002), but its use for a pitching culture is becoming increasingly popular.

2.7 Yeast propagation

Yeast propagation is a traditional and well-established process in most large breweries (Nielsen, 2010). Also, some multi-brewery operations propagate their yeast centrally and distribute their yeast culture(s) to individual breweries. Nevertheless, development is constantly ongoing and questions remain to be answered (Stewart et al., 2013). The requirement for a freshly propagated yeast culture is that it is not stressed, is highly vital and viable, and the yeast is free of contaminating organisms. The way to this objective involves a carefully designed sanitary propagation plant with an aeration (oxygenation) system that is able to supply sufficient oxygen to all

cells in the propagation, without causing mechanical stress to the cells, which are in a wort of the appropriate nutrient composition (further details of oxygenation during brewing to follow later).

No matter how much these conditions are optimized, it is still possible to obtain only relatively low cell numbers (approximately 100–200 million cells/mL, equivalent to 2.5–5.0 g dry matter per litre). To avoid losing time during the wait for the yeast to consume all of the wort sugars, a complementary process should be used. This process has been adapted from the baker's yeast propagation process and is conducted in a fed batch reactor, whereby the sugar concentration is maintained at a consistently low level, but not too low, to avoid the yeast growing aerobically and thereby potentially losing some of its fermentation characteristics during the propagation procedure. Consequently, a hybrid process between traditional brewery propagation and the aerobic yeast propagation process used for baker's yeast propagation is the preferred compromise (Boulton & Quain, 1999).

In a brewery, propagation is carried out in a batch reactor, with wort as the medium. This is basically the same medium that will be used later for fermentation into beer. Although wort gravities have been increased for fermentation, weaker worts are still more appropriate for propagation. The propagation medium used to produce yeast for the distilling and baker's yeast industries is usually molasses (sometimes hydrolysed whey), in which the major sugar is sucrose plus a nitrogen source (usually ammonium ions). Also, a fed batch reactor with a continuous supply of dilute substrate and intense aeration (oxygenation) is used to produce distiller's and baker's yeast. When propagation in a brewery is carried out in a batch reactor, the use of wort limits aerobic yeast growth in a concentrated sugar solution, making it difficult to produce theoretical quantities of biomass (Nielsen, 2005). However, the brewing industry has chosen to tolerate this problem, because optimizing yeast growth in a molasses/nitrogen medium could jeopardize wort fermentation properties and lead to poorer beer quality. Also, brewing focuses on strict sanitary conditions to avoid infection (the production of distiller's and baker's yeast is not completely aseptic) and to minimize yeast stress during propagation to avoid the negative effects on fermentation. It is worth repeating that brewer's yeast propagation is based on aerobic conditions and the extensive use of sterile air or oxygen throughout the process. It differs extensively from brewing fermentations in which oxygen is required only at the beginning of the process in order for the lag phase cells to begin to synthesize unsaturated fatty acids and sterols (Figure 2.3), which are important membrane constituents. This synthesis occurs largely from glycogen as the substrate (Figure 2.2). It is interesting to note that oxygen is required only at the following stages in the malting and brewing process:

- During barley germination during malting;
- For biomass formation during yeast propagation;
- At the beginning of fermentation when the yeast is pitched into wort.

At any other point in the process, oxygen can have a negative effect on beer quality, particularly when there is dissolved oxygen in the packaged product, leading to stale characteristics in the beer (Stewart, 2004).

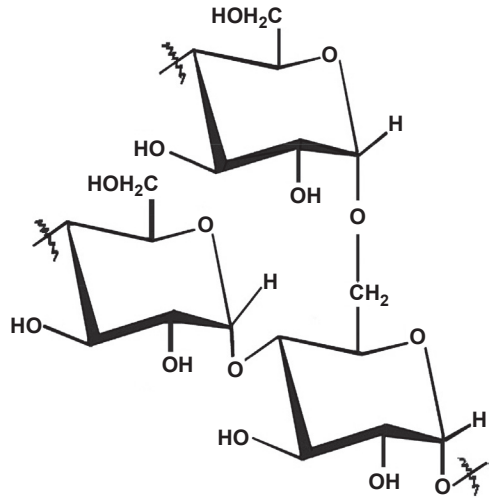

Figure 2.2 Structure of glycogen.

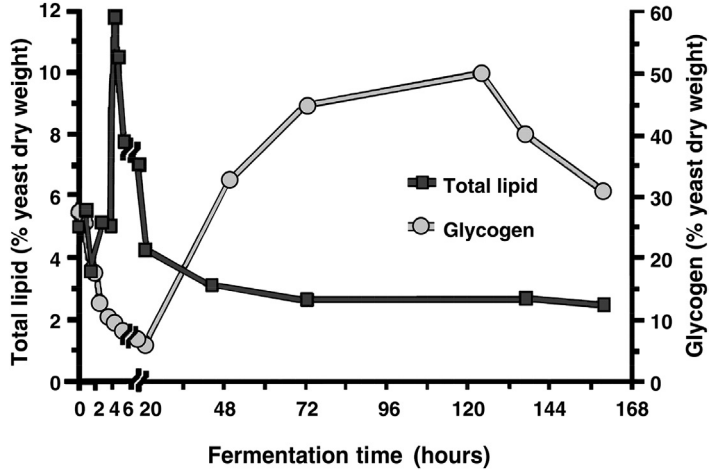

Figure 2.3 Intracellular concentration of glycogen and lipids in a lager yeast strain during fermentation of a 15° Plato wort.

2.8 Yeast collection

Yeast collection (also termed cropping) techniques vary depending on whether one is dealing with a traditional ale top fermentation system, a traditional lager bottom fermentation system, a cylindroconical fermentation system (Stewart & Russell, 2009) or a nonflocculent culture in which the yeast is cropped with a centrifuge (details to follow later). With the traditional ale top fermentation system, although there are many variations to this system, for example, a simple, dual or multi-strain yeast system can be used. The timing of the skimming process can be critical to maintain the

flocculation characteristics of the ale strains. Traditionally, the first skim or "dirt skim" with the trub present is discarded, as is the final skim, usually, with the middle skim being kept for repitching. With the traditional lager bottom fermentation system, the yeast is deposited on the bottom of the vessel at the end of fermentation. This type of yeast collection is essentially nonselective, and the yeast will normally contain entrained trub. The cylindroconical fermentation system has been now widely adopted for both ale and lager fermentations, and the angle at the bottom of the fermenter allows effective removal of the yeast plug.

The use of centrifuges for the removal of yeast and the collection of pitching yeast is now commonplace. There are a number of advantages to centrifuge use; these include shorter process time, cost reduction (after significant initial capital costs), increased productivity and reduced wort shrinkage (Chlup & Stewart, 2011). Care must be taken to ensure that elevated temperatures (above 20 °C) are not generated during centrifugation, and that the design ensures low DO pickup and a high throughput (Chlup, Bernard, & Stewart, 2007). In addition, centrifugation can (under certain circumstances) cause physical damage to yeast cells and, consequently, can negatively affect beer physical stability (haze). This is dependent on centrifuge operating parameters. Hydrodynamic forces and yeast cell interaction within the gap of the centrifuge disc stack create collisions amongst yeast cells, producing kinetic energy, and causing cellular damage. Release of cell wall mannan during mechanical agitation of yeast slurries in conjunction with an increase in beer haze has been well documented (Chlup, Conery, & Stewart, 2007; Chlup, Bernard, & Stewart, 2008).

2.9 Yeast storage

At the end of fermentation, the yeast is cropped for further use, using the flocculating characteristics of the yeast strain or with a centrifuge. However, in this discussion, yeast cropping is considered to be part of fermentation, not yeast management between fermentations. It has already been described that one method of yeast cropping, which is increasing in popularity, is the use of centrifuges, although their use has not been without problems (Table 2.2) (Chlup & Stewart, 2011).

Table 2.2 Yeast condition following centrifugation from a 16° Plato wort fermentation

Yeast exit temperature	16–18 °C	28–30 °C
Viability (%)	95	80
Respiratory-deficient cells (%)[a]	5	25
Glycogen (mg/g dry weight)	18.6	12.2

[a]Respiratory-deficient cells are spontaneous mutants in which there is a deficiency in mitochondrial function (Stewart, 2014).

If a cropped yeast culture is not stored properly, cell consistency will suffer, and it will adversely affect fermentation and beer quality. After cropping, the yeast is stored in a room that is conveniently sanitized and contains a plentiful supply of sterile water and a separate filtered air supply with positive pressure to prevent the entry of contaminants at a temperature of 0 °C. Alternatively, insulated tanks in a dehumidified room can be used. In addition, "off the shelf" yeast storage facilities are available at various working capacities.

Yeast is predominantly stored under 6 inches of beer (sterile water has been used in the past, but its use is unpopular these days). When high-gravity brewing is practised, it is important to ensure that the ethanol level of the storage beer is decreased to 4–6% (v/v) ethanol to maintain the viability and vitality of the stored yeast. As more sophisticated systems become available, storage tanks with external cooling (0–4 °C), equipped with low-shear stirring devices, have become popular. The need for low-shear stirring systems has been shown to be important. With high velocity agitation in a yeast storage tank, the yeast cell surface can become disrupted, intracellular proteases (particularly proteinase A [PrA]) are excreted, and this can result in unfilterable mannan hazes in beer (Stoupis, Stewart, & Stafford, 2002) and poor head retention due to protease hydrolytic activity on foam stability-enhancing peptides (Cooper, Stewart, & Bryce, 2000). There are procedures whereby the yeast is not stored between fermentations. In this case, the yeast is pitched directly from one fermenter to another. This yeast handling procedure occurs with cylindroconical (vertical) fermenters and is termed 'cone to cone yeast pitching'. The procedure was used by some breweries in the 1980s and 1990s, but currently it has limited application because of lack of opportunity and time to conduct quality and contamination studies on the yeast between fermentations.

One of the factors that will affect fermentation rate is the condition under which the yeast culture is stored between fermentations. Of particular importance in this regard is the influence of temperature during these storage conditions on the cell's intracellular glycogen level. Glycogen is the major reserve carbohydrate stored within the yeast cell and is similar in structure to plant amylopectin (Figure 2.2). It has already been discussed that glycogen serves as a store of biochemical energy during the lag phase of fermentation when the energy demand is intense for the synthesis of compounds such as sterols and unsaturated fatty acids (Figure 2.3). Consequently, it is important that appropriate levels of glycogen and trehalose (Figure 2.4) are maintained during storage so that during the initial stages of fermentation the yeast cell is able to synthesize sterols and unsaturated fatty acids and trehalose. Trehalose is a nonreducing disaccharide (Figure 2.4) that plays a protective role in osmoregulation, protection of cells during conditions of nutrient depletion and starvation, and improving cell resistance to high and low temperatures and elevated ethanol.

Storage temperature (Figure 2.5) has a direct influence on the rate and extent of glycogen utilization, as might be expected, considering the effect that temperature has on metabolic rates in general. Although strain dependent, of particular interest is the fact that within 48 h, the yeast stored semi-aerobically at 15 °C has only 15% of the

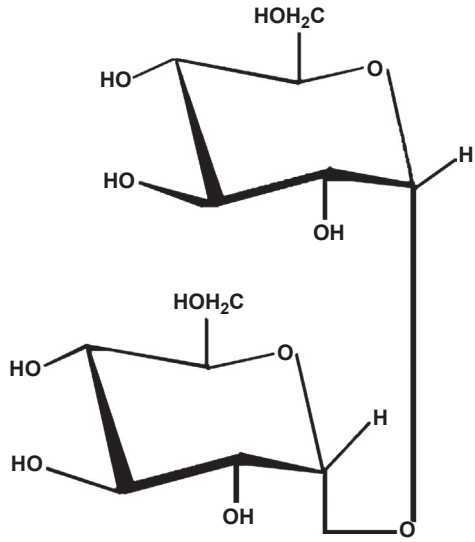

Figure 2.4 Structure of trehalose.

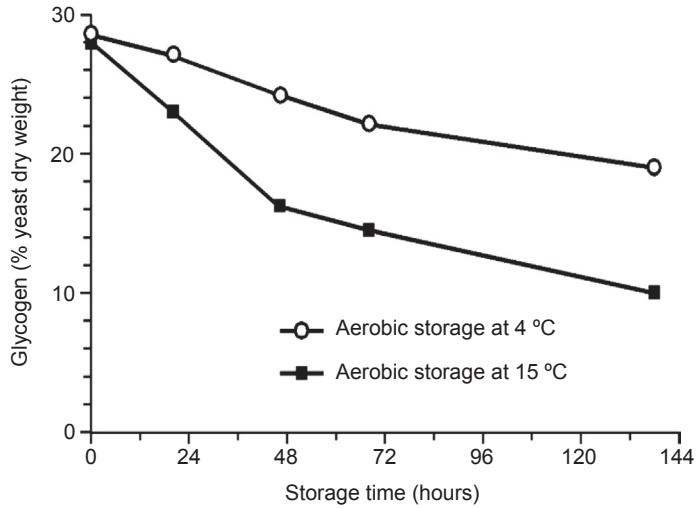

Figure 2.5 Effect of yeast storage temperature on intracellular glycogen concentration.

original glycogen concentration remaining. Glycogen reductions to this extent will have a profound effect on wort fermentation (Figure 2.6). Whereas after storage for 44 h at 4 °C there was 26% of the original glycogen concentration remaining.

The number of times that a yeast crop (generations or cycles) is used for wort fermentations is usually standard practice in a particular brewery. Typically today, a lager yeast culture is currently used 6–10 times before reverting to a fresh culture of the same strain

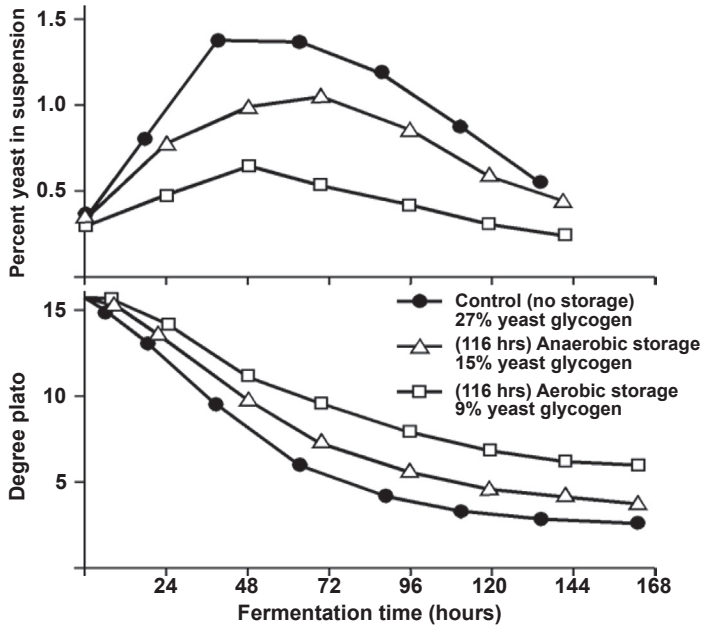

Figure 2.6 Effect of yeast glycogen at pitching on lager fermentation characteristics.

from the pure yeast culture plant. If a particular yeast culture is used beyond the crop specification, fermentation difficulties (fermentation rate and extent are typical examples) are sometimes encountered. An example of this effect is when a brewery increases its wort gravity and adopts high-gravity brewing procedures. A particular brewing operation, over a 15-year period, increased its wort gravity incrementally. To avoid fermentation difficulties, it reduced the number of yeast cycles from a single propagation:

12° Plato wort, >20 yeast cycles
14° Plato wort, 16 yeast cycles
16° Plato wort, 12 yeast cycles
18° Plato wort, eight yeast cycles

These days, some breweries have adopted a larger yeast reuse specification as few as four to six cycles.

The reason why multiple yeast generations can have a negative effect on a culture's fermentation performance is unclear. However, multiple generations will result in reduced levels of intracellular glycogen and an increase in trehalose, indicating additional stress conditions as the cycles progress (Table 2.3) (Boulton & Quain, 2001).

Yeast storage conditions between brewing fermentations can affect fermentation efficiency and beer quality. Good yeast handling practices should encompass collection and storage procedures, avoiding inclusion of oxygen in the slurry, cooling the slurry to 0–4 °C soon after collection, and, perhaps most importantly, ensuring that glycogen levels are maintained because of its critical property at the start of a subsequent wort fermentation.

Table 2.3 Concentration of trehalose and glycogen in lager yeast following one, four and eight cycles after fermentation in 15° Plato wort

	Generations (cycles)		
	One	**Four**	**Eight**
Trehalose[a]	8.8	9.2	11.6
Glycogen[b]	14.6	12.6	9.2

[a]μg/g dry weight of yeast.
[b]mg/g dry weight of yeast.

2.10 Yeast washing

Acid-washing pitching yeast at pH 2–2.2 (with phosphoric, tartaric, hydrochloric, sulphuric or nitric acid solutions), usually during the later stages of storage just before being pitched into wort for fermentation, has been used by many breweries for the past 100 years (and longer) as an effective method to eliminate contaminating bacteria (not wild yeasts) without adversely affecting the physiological quality of the yeast culture as long as the procedure is properly conducted. The acid-washing regimen differs between breweries, with some brewers routinely acid washing their yeast after each fermentation cycle, others acid washing their pitching yeast only when there is significant bacterial contamination, and still others refraining from acid washing completely. Brewer's yeast strains are normally resistant to acidic conditions when the washing is conducted properly. However, if other environmental and operating conditions are modified, then the acid resistance of the culture will vary. Simpson and Hammond (1989) demonstrated that if the temperature of acid washing was greater than 5 °C and/or the ethanol concentration was greater than 8% (v/v), acid washing had a detrimental effect on the culture, causing a decrease in viability and fermentation performance. The physiological condition of the yeast before acid washing is an important factor in acid tolerance, with yeast in poor physiological condition before washing being more adversely affected by acid washing than healthy yeast.

Acid washing primarily affects the yeast cell envelope with the physiological systems associated with both the cell wall and the plasma membrane, subsequently decreasing yeast vitality as measured by the acidification power test (Kara, Simpson, & Hammond, 1988). Research by Cunningham and Stewart (2000) have reported that acid-washing pitching yeast from high-gravity (20° Plato) wort fermentations did not affect the fermentation performance of cropped yeast if it was maintained in good physiological condition. Oxygenation of the yeast at the start of fermentation stimulated yeast growth, leading to a more efficient wort fermentation, and, equally important, in the context of yeast management between fermentations produced yeast that was in good physiological condition, permitting it to tolerate exposure to acid-washing

conditions (phosphoric acid solution at pH 2.2). These data support the findings of Simpson and Hammond (1989), who concluded that yeast in poor physiological condition should not be acid washed.

In summary, the do's and do not's for yeast acid washing listed by Simpson and Hammond (1989) are still appropriate:

Acid washing of yeast can be summarized into the Do's and the Do Not's.

The Do's of acid washing are as follows:

- Use food-grade acid;
- Chill the acid and the yeast slurry before use to less than 5 °C;
- Wash the yeast as a beer slurry or as a slurry in water;
- Ensure constant stirring while the acid is added to the yeast and preferably throughout the wash;
- Ensure that the temperature of the yeast slurry does not exceed 5 °C during washing;
- Verify the pH of the yeast slurry; and
- Pitch the yeast immediately after washing.

The Do Not's of acid washing are as follows:

- Do not wash for more than 2 h—this is very important;
- Do not store washed yeast;
- Do not wash unhealthy yeast; and
- Avoid washing yeast from high-gravity fermentations before dilution. There are a number of options to acid washing brewer's yeast:
  - Never acid wash yeast;
  - Specify low yeast generation (cycle);
  - Discard yeast when there is evidence of contamination (bacteria and/or wild yeast);
  - Acid wash every cycle, as this procedure can have adverse effects on yeast; or acid wash when bacteria infection levels warrant the procedure.

2.11 Yeast stress

During wort fermentation a yeast culture is exposed to a number of stress conditions; the primary stress factor is the use of high-gravity worts (Pratt-Marshall, Brey, de Costa, Bryce, & Stewart, 2002). Under these circumstances, yeast cells are exposed to numerous stresses, including osmotic stress at the beginning of fermentation, due to high concentrations of wort sugars, and ethanol stress at the end of fermentation (Stewart, 1999). Other forms of yeast stress are desiccation (to be discussed later), mechanical stress (details to be provided later) and thermal stress (hot and cold) (Figure 2.7). The yeast is expected to maintain its metabolic activity during stressful conditions not only by surviving these stresses but by rapidly responding to ensure continued cell viability and vitality (Casey, Chen, & Ingledew, 1985).

Stress can have a profound and varied effect on yeast cells, including:

- A negative effect on overall yeast fermentation performance, resulting in decreased attenuation rates, sluggish fermentation and a marked reduction in cell volume with a concomitant loss of cell viability.

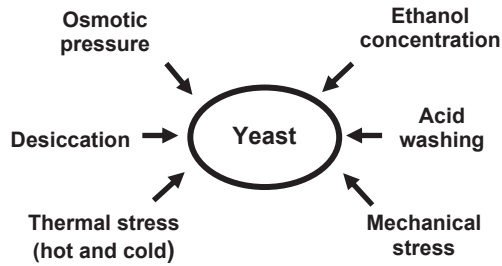

Figure 2.7 Stress factors that promote proteinase A release.

- Cell autolysis can occur with a loss in viability, vitality and cell contents being excreted into the fermenting wort. This has a number of consequences on beer flavour and stability, and especially foam stability.
- Stress can also result in the excretion of intracellular enzymes, particularly PrA, which will also negatively affect beer foam stability (Cooper et al., 1998).

The balance between PrA occurring in the fermenting wort as a result of cell autolysis and/or enzyme excretion of whole cells is still an issue to be examined. Nevertheless, the occurrence of active PrA is important because of its negative effect on beer foam stability.

2.12 Dried yeast

Dried yeast has been used in the baking and distilling industries for more than 50 years (Pyke, 1958). However, its use in brewing is relatively recent (Fels, Reckelbus, & Gosselin, 1998). One of the reasons for this delay is the differences in drying characteristics; ale yeast strains dry relatively well, whereas lager yeast cultures, when dried, have comparatively lower viabilities. The reasons for the drying differences between these two yeast species is still not fully understood, but levels of the storage carbohydrates glycogen and trehalose have been implicated (Gadd, Chalmers, & Reed, 1987). Another reason for the delay in adopting dried yeast in brewing is that this yeast is often contaminated with various bacteria and wild yeasts. However, this problem is not as prevalent today as it was 25 years ago.

The use of dried yeast has several advantages and similarities in comparison to the use of fresh yeast (Fels et al., 1998):

- It is easier to handle and convenient to store;
- It can replace yeast propagation in breweries;
- In some cases it can replace the need for wort aeration at pitching;
- Recent studies have shown that it often has characteristics similar to those of its fresh counterpart with analytical and flavour profiles, rates of fermentation and final attenuation all matching favourably to those of fresh yeast (Debourg & Van Nedervelde, 1999);
- The average viability (determined by methylene violet or methylene blue staining) of dried yeast is 20–30% lower than that of freshly propagated yeast. This problem can be accommodated by pitching according to viable cell numbers.

It has been reported (Finn & Stewart, 2002) that dried yeast samples exhibited different flocculation and haze formation characteristics when compared to fresh yeast samples. The flocculation rate with fresh and dried ale cultures was rapid, with most of the yeast sedimenting out of suspension within the first minute of a Helm's sedimentation test, and with 80% of the culture eventually flocculating out of suspension. The flocculating differences between fresh lager and dried yeast samples were more pronounced than with ale strains. Virtually no flocculation took place within a 10-min test period with the dried yeast samples. This test indicated that the lager dried yeast samples were modified in some way and, as a consequence, exhibited nonflocculent characteristics.

During the studies on flocculation, it was observed that the dried yeast fermentation often left a haze in suspension. Even with the ale yeast fermentation, although the yeast flocculated, a haze remained in suspension. This may have been due, in part, to the number of dead cells pitched into the wort. In addition, the fresh yeast fermentation exhibited a foam head, but a foam head was absent on the dried yeast samples (both ale and lager cultures). PrA and other proteinases are released by dried yeast into the wort in much greater quantities than fresh yeast under similar fermentation conditions (Finn & Stewart, 2002). PrA release into the wort will have an impact on beer foaming characteristics (Cooper et al., 2000; Osmond, Lebor, & Sharpe, 1991). The decreased foam stability is due to the hydrolysis of hydrophobic polypeptides by PrA. Hydrophobic polypeptides are known to be mainly responsible for beer foam stability (Bamforth, 2012). Leakage of intracellular proteinases from living brewer's yeast has been demonstrated (Dreyer, Biedermann, & Otteson, 1983), particularly when they are under stress (Stewart, 1999). Indeed, the addition of dead cells (as could be the case with a dried culture) would greatly increase the levels of PrA.

2.13 Conclusions

During most of the fermentation ethanol production procedures, a yeast culture is used only once for a single fermentation cycle. However, in brewing, the yeast culture is harvested at the end of a fermentation for reuse in a subsequent fermentation. Between fermentations, the cropped yeast is normally stored before being repitched. As a consequence of this procedure, the yeast culture must be carefully managed between fermentations to maintain its quality. The steps in this yeast management procedure have been discussed in this chapter.

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Modelling yeast growth and metabolism for optimum performance

3

A.J. MacIntosh
Dalhousie University, NS, Canada

3.1 Introduction

For thousands of years, the fermentation of cereal wort has challenged our ability to explain, predict and control the behaviour of yeast. Brewing has been described as an art; however, it has often been responsible for driving forward scientific theory and techniques. This chapter discusses some common tools used to describe and predict fermentation behaviour, and how our understanding of this phenomenon has improved over time. This will be accomplished through the use of statistical techniques to fit models to fermentation data. Within the brewing industry, the term 'model' is often applied to any equation (empirical or theoretical) that is fit to fermentation data; it is this definition that will be used throughout this chapter. The advantages and disadvantages of modelling techniques (simple and complex) will be discussed, as will models commonly used by industry. A distinction is also made between theoretical models that attempt to explain behaviour, and empirical models that mathematically attempt to follow expected trends as closely as possible (where the parameters describe only the physical shape of an equation over time). As fermentation is influenced by many parameters, an overview of how some of these affect this process will be discussed. Finally, applications will be discussed, as will several advanced brewing techniques (such as high-gravity brewing) in which predicting behaviour is critical. The chapter will end with a discussion of future trends.

3.2 Parameters influencing yeast growth and fermentation of barley malt

During brewing operations, there are numerous factors affecting the growth of yeast cells and subsequent fermentation of wort. Therefore, when attempting to model and explain this phenomenon, it is imperative to know which parameters affect fermentation and in what manner. A typical fermentation will be affected in many ways, such as the rate of fermentation, the degree to which the media fermented and the ratio of products (and by-products) that are formed. These are affected not only by extrinsic parameters such as temperature and headspace composition, but also by numerous

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intrinsic properties that are covered in other chapters. Most modern prediction methods use models that describe the expected behaviour of the fermentation, while occasionally take into consideration the expected behaviour of a major parameter (such as the original density of the wort, or occasionally temperature).

Brewing fermentations typically use either *Saccharomyces cerevisiae* (ale yeast) or *Saccharomyces pastorianus* (lager yeast), with the latter species producing approximately 90% of the global product (Canadean, 2011). Over the course of 4–20 days, the fermentable sugars within the wort are consumed, and fermentation products (predominantly ethanol and CO_2) are produced. The density of the media (commonly expressed as specific gravity or apparent extract) is often used as an easily measured analogue for the concentration of sugar within the media (although this must be corrected for alcohol concentration). The vast majority of industrial brewing operations use batch fermentations, in which yeast is added (pitched) at concentrations of approximately $12–15 \times 10^6$ cells/mL (Briggs, Boulton, Brookes, & Stevens, 2004). Although continuous industrial fermentations do exist [i.e. Morton Coutts' method used in New Zealand (Virkajärvi and Kronlöf, 1998)], these introduce unnecessary complexity, and often result ultimately in little or no economic return. Continuous fermentations have very different behaviour and characteristics from those described within this chapter.

Over the course of a batch fermentation, the initial concentration of each fermentable sugar will ultimately define the parameters of the fermentation. This parameter is highly dependent upon the malt and mashing style, as every wort will comprise a different configuration of fermentable and non-fermentable sugars. The sugars present in wort (and typical concentrations) are listed in Table 3.1. Although different brewing yeast strains are able to metabolize different sugars, Table 3.1 highlights those sugars most commonly found and metabolized within brewers wort (Stewart, 2006). During brewing operations, the uptake of fermentable sugars by yeast is a highly ordered process; glucose and fructose are consumed first, with any sucrose present being hydrolyzed extracellularly via the enzyme β-fructosidase (invertase) excreted by yeast (Briggs et al., 2004). The presence of glucose in sufficient quantities has been shown to inhibit respiration and the uptake of maltose in brewing yeast. Once the concentration of glucose is sufficiently low, maltose is sequentially utilized by the yeast,

Table 3.1 Typical sugar components of brewing wort

Saccharide	Chemical formula	Typical percent composition[a] (%)
Glucose	$C_6H_{12}O_6$	10–15
Fructose	$C_6H_{12}O_6$	1–2
Sucrose	$C_{12}H_{22}O_{11}$	1–2
Maltose	$C_{12}H_{22}O_{11}$	50–60
Maltotriose	$C_{18}H_{32}O_{16}$	15–20
Higher Saccharides	$H_2O + (C_6H_{10}O_5)_n$	20–30

[a]Typical composition as a percentage of total sugars (Stewart, 2006).

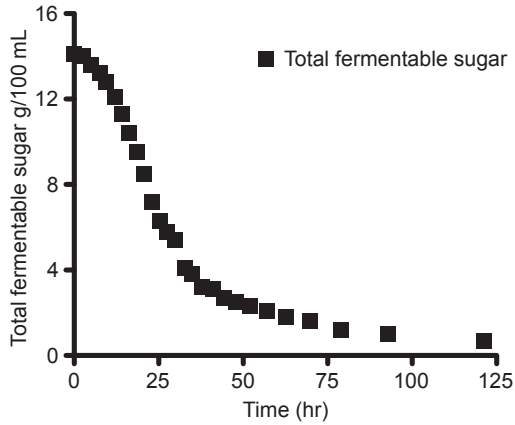

Figure 3.1 Typical attenuation of sugars during brewing fermentations.

followed by maltotriose (Stewart, 2006). Both maltose and maltotriose are hydrolyzed intracellularly into glucose via the enzyme α-glucosidase (maltase) (Briggs et al., 2004a). Most brewing strains cannot metabolize chains of sugar in excess of three glucose units (Stewart & Russell, 1998).

As wort is an aqueous mixture high in fermentable sugars, wort density is often used as an easily measured indicator of fermentation progress. This is directly related to consumption of sugar and subsequent production of alcohol, which results in density attenuation. This decline in density (commonly measured in either units of degree Plato [°P], or specific gravity) observed in fermentations, characteristically follows a sigmoidal (s-shaped) curve (Corrieu, Trelea, & Perret, 2000; Trelea, Latrille, Landaud, & Corrieu, 2001; Speers, Rogers, & Smith, 2003). Similarly, each individual fermentable sugar follows a sigmoidal decline. However, these consumption curves are influenced by a variety of factors, such as yeast state, species and sugar type. Thus, the consumption of total sugar (as shown in Figure 3.1), as well as individual sugar attenuation, is often lagged before consumption and may be symmetrical or asymmetrical.

Although the concentration of sugars will ultimately define the maximum and minimum parameters of any equation that attempts to model this fermentation behaviour, the shape will be influenced by the aforementioned intrinsic and extrinsic parameters. Novel research on how both extrinsic and intrinsic parameters affect fermentation is completed at many notable institutions, as discussed by other researchers mentioned in this text.

3.3 Modelling: techniques and applications

Possibly the most well-known early attempt to model brewing fermentation was completed in 1865 by the chemist Carl Balling. Using beer with original wort extract of 10–14 °P (°P—a measure of density), Balling reported that from 2.0665 g of fermented

extract, the following products were generated: 1.000 g alcohol, 0.9565 g CO_2 and 0.11 g dry yeast matter (Balling, 1845–1865). Upon analysis, it appears as though this formula is a combination of the theoretical conversion of glucose to ethanol and CO_2, combined with empirical assessments of yeast mass generation measured at the end of fermentation. This formula, and associated calculation of original extract (OE), are used worldwide and endorsed by both the European Brewing Convention (EBC Method 9.4) and the American Society of Brewing Chemists (ASBC Beer-6B). However, that is not to say that the formula has remained unchallenged. In the ensuing years since its derivation, the formula has been disputed on multiple grounds. Subsequent researchers have noted that although it is not perfect, the formula is a good approximation that is well known and widely used (Nielsen, Kristiansen, Lassen, & Ericstrøm, 2007). Additionally, several issues with Balling's formula can be corrected for, as summarized by Nielsen et al. (2007). The aforementioned studies have assessed the accuracy of Balling's formula used to model fermentations, specifically the relationship between final density and OE. However, the ratio of fermentation products is known to vary throughout the fermentation. For example, the majority of yeast propagation is completed during the first half of fermentation, whereas the initial CO_2 produced is dissolved within the wort and does not evolve. Modern methods of analysis now allow researchers to predict the parameters of Balling's formula over the entire fermentation and to examine how the product ratios likely change with time. Unfortunately, not every fermentation has each variable monitored in real time; in industrial settings, measurements are usually taken intermittently and when convenient for scheduling purposes. It is hugely impractical to precisely monitor every aspect of fermentation performance and to understand how this will affect the overall fermentation, and real-world concerns of profitability and scheduling often supersede that of rigor. Therefore, assumptions must be made that allow the brewer or researcher to roughly predict fermentation behaviour. Often, only one or two parameters are actually monitored to observe whether, or how, a fermentation deviates from previously completed fermentations that had identical (or similar) initial parameters.

Throughout the brewing process, sugars are metabolized into alcohol and carbon dioxide, resulting in wort density attenuation. When plotted with respect to time, this decline follows a sigmoidal curve, from an initial sugar concentration of anywhere from 10 to 20% (or higher when using high-gravity brewing techniques) to 2–4% over the course of a typical fermentation. Mathematical models can be fit to this data allowing brewers to predict, assess and more accurately compare fermentations. Within the brewing industry, there are several models that can be applied, each with advantages and disadvantages. Some models are theoretically derived, whereas others are fully or semi-empirical. In modelling sugar attenuation, brewing researchers use simpler models; however, these may not accurately characterize real-world fermentations (particularly at the onset and latter half of fermentation). With a limited number of data points, important trends can be missed, and small errors in measurement can greatly affect alcohol and extract calculations. With the development of computer-aided modelling, scientists have applied nonlinear fitting techniques to model and to more precisely determine interpolated values of variables (Speers et al., 2003). Since then, advances in other scientific fields have introduced novel models that may be more adept at modelling the patterns observed during fermentation.

3.3.1 Modelling fermentations

Modelling total sugar consumption has many advantages, such as predicting the final density/sugar content (Defernez et al., 2007), approximating the time until completion (Speers et al., 2003) and phenotyping the yeast strain. Nonlinear models are already promoted for use in various analytical methods within the brewing industry, such as the "nearest neighbour" and "predictive modelling" techniques (Trelea, Titica, et al., 2001), in which easily measured parameters are related to others. The most common functions used to predict density decline in brewing fermentations are the logistic model (Speers et al., 2003; ASBC Yeast-14, 2011), the regularized incomplete β-function (IBF) (Trelea, Latrille, et al., 2001) and the modified Gompertz function (Gibson, Bratchell, & Roberts, 1988). Differences in reported and predicted density can significantly influence the decision-making process in large breweries and can make comparing metrics (such as fermentability of grain) very problematic. Because of this, using the correct function for a given application is often of the upmost importance. The following section discusses several commonly used sigmoidal models; the first—Richard's model—is based upon theoretical principles (Richards, 1959), but is not often used in the brewing industry. The second—the incomplete β distribution—is an applied empirical distribution that is often reported in the brewing literature (Trelea, Latrille, et al., 2001). The final—Gompertz's model—is an empirical model that is widely used in microbiology (Gibson et al., 1988) to describe growth curves (analogous to consumption curves). Each model is described in detail below.

3.3.2 Logistic models

The logistic model is a family of nested equations that describe a sigmoidal curve. As additional parameters are introduced, the curve gains more flexibility to fit data. However, the ideal number of parameters for a particular dataset must be statistically determined via an F-test. The four-parameter logistic function (4P logistic model) is a sigmoidal curve that is often used to describe changes in a population, as it effectively models autocatalytic behaviour (Eqn (3.1)). This curve is commonly used in the brewing industry to model the decline in apparent extract. The 4P model is the basis of ASBC Yeast-14 (ASBC, 2011) and is used to assess malt for premature yeast flocculation behaviour and to compare the fermentability of yeast strains. The generalized logistic model is a five-parameter variant of the logistic model (Eqn (3.2)) that expands the theoretical basis to an asymmetrical curve (Richards, 1959), required for modelling sugar attenuation. The five-parameter (5P) logistic model has not previously appeared within brewing literature; however, it is commonly used in other fields for applications such as population growth modelling and dosage calculations (Gottschalk & Dunn, 2005). The generalized logistic curve is equal to the symmetrical 4P logistic when the parameter $s = 1$:

$$P_{(t)} = P_e + \frac{P_i - P_e}{(1 + e^{-B(t-M)})} \tag{3.1}$$

where P_i is the initial asymptotic density value (in °P) for the density attenuation regression, B is a function of the slope at the inflection point, t is time, P_t is the

density at time t, M is the time at point B, and P_e is the equilibrium asymptotic density value.

$$P_{(t)} = P_e + \frac{P_i - P_e}{(1 + se^{-B(t-M)})^{1/s}} \tag{3.2}$$

where s is a variable that modifies the point of inflection (M).

As shown in Figure 3.2, the consumption of sugar follows an asymmetrical sigmoidal curve. As the consumption of sugar is analogous to yeast growth, the use of a common biological growth curve should work well for this application. The Gomertz model (GM) is an empirical model named after Benjamin Gompertz (1825) that is widely used in the field of microbiology to predict the growth curves of bacteria (Buchanan, Whiting, & Damert, 1997). This model is a special case of the generalized logistic formula, and describes a sigmoidal curve in which the latter half of the curve approaches the asymptote more slowly than the initial half. This model is often used when one expects an asymmetrical curve, such as when working with microorganisms. A modified version of the Gompertz curve mentioned in the brewing literature to describe density attenuation is described in Eqn (3.3) (Speers et al., 2003):

$$P_t = P_e + (P_i - P_e) \cdot e^{\left(e^{-B(t-M)}\right)} \tag{3.3}$$

where P_i and P_e are the upper and lower asymptotes, respectively, M is the time of the inflection point of the curve, B is the consumption rate factor, and t is the time at $P_{(t)}$.

This version of the Gompertz model is an empirical model, not one derived from theory (Speers et al., 2003). An advantage of this model is the low number of parameters (four) required to fit the model while still allowing for an asymmetrical shape; this is particularly advantageous with a limited number of data points. However, in

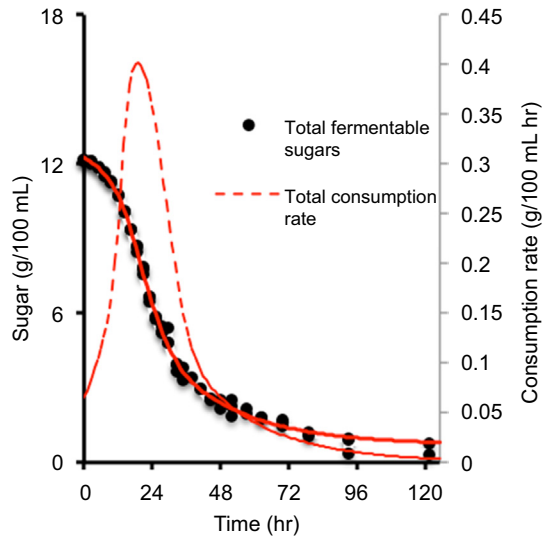

Figure 3.2 Total fermentable sugar data and total sugar consumption rate as calculated by the summation of each individual sugar consumption rate.

testing of data from more than 50 industrial brewing fermentations, Speers et al. (2003) showed that the 4P logistic model fit the data more accurately than the modified Gompertz model.

3.3.3 The incomplete β-function (IBF)

The IBF can be used to describe an asymmetric curve as described by Eqn (3.4). The full name for this equation is the regularized incomplete β-function; however, the name is often shortened in literature to the incomplete β-function. The IBF has been modified for describing the attenuation of extract by Treala, Latrille, et al. (2001) and has been used by several researchers (i.e. Defernez et al., 2007) to model and predict the end parameters of fermentation. Equation (3.5) is the aforementioned modified version of the IBF with two additional terms (P_i and P_e) added to fit experimental data (describing the upper and lower boundaries of the sugar consumption curve). With the additional variables, the IBF can be used to describe brewing fermentations quite well. However, as with the modified Gompertz model, the fit is purely empirical, and the shape parameters do not describe biological functions.

$$IBF = \frac{\beta(x{:}\propto,\beta)}{\beta(\propto,\beta)} = \frac{\int_0^x u^{\propto-1}(1-u)^{\beta-1}du}{\int_0^1 u^{\propto-1}(1-u)^{\beta-1}du} \tag{3.4}$$

where β and α are shape parameters and:

$$P_t = P_i - (P_i - P_e) \cdot IBF(x \cdot t{:}\propto,\beta) \tag{3.5}$$

3.3.4 Additional models

Although the 4P logistic, IBF and Gompertz models are all discussed within brewing literature, there are many additional models designed to describe sigmoidal curves outside of this area. The fields of predictive microbiology, medical science and biology all offer many models that may be useful in describing sugar attenuation. Many additional models are available; however, those previously described are most prevalent in the brewing literature. It is noteworthy that a review of the literature will reveal many unequal versions of the logistic model all that effectively describe sigmoidal curves.

3.3.5 Application of models

Each model was applied to sugar attenuation data taken from multiple brewing fermentations (assessed using high-pressure liquid chromatography). Three techniques: Akaike's (corrected) Information Criterion, comparison of the coefficients of determination (r^2) and absolute residual sum of squares (RSS) were used to compare the fit of each model. Ideally, the data would adhere to a simplistic, theoretically derived formula such as a low parameter symmetric model. Unfortunately, the variability in both shape and lag time for each individual sugar necessitated a more flexible

model. Table 3.2 details the fit of each model through examination of the residuals, coefficients of determination and absolute residual sums of squares. As shown in Figure 3.3, each sugar was found (not surprisingly) to follow a sigmoidal attenuation. Noteworthy is that although the attenuation of glucose was immediate, the attenuation of other sugars (maltose, glucose, maltotriose and fructose (Figures 3.4–3.7), respectively) were delayed (lagged) to varying degrees.

Table 3.2 Residual analysis for each sugar attenuation modelled using the modified Gompertz, IBF and 5P logistic models

	Modified Gompertz		IBF		5P logistic	
Sugar	Residual pattern	r^2 – RSS	Residual pattern	r^2 – RSS	Residual pattern	r^2 – RSS
Glucose	Random[a]	0.998–0.308	Pattern	0.996–0.678	Random[a]	0.998–0.299
Fructose	Random[a]	0.987–0.015	Random[a]	0.989–0.013	Random[a]	0.988–0.014
Maltose	Pattern	0.979–3.473	Pattern	0.991–1.436	Random[a]	0.996–0.660
Maltotriose	Pattern	0.986–0.210	Pattern	0.994–0.084	Random[a]	0.996–0.061

[a]The heterosecdacity caused by know variance inherent to the assay was common to all models. IBF, incomplete β-function; RSS, residual sum of squares.
MacIntosh, A. J. (2013). *Carbon dioxide generation, transport and release during the fermentation of barley malt*. PhD dissertation, Dalhousie University.

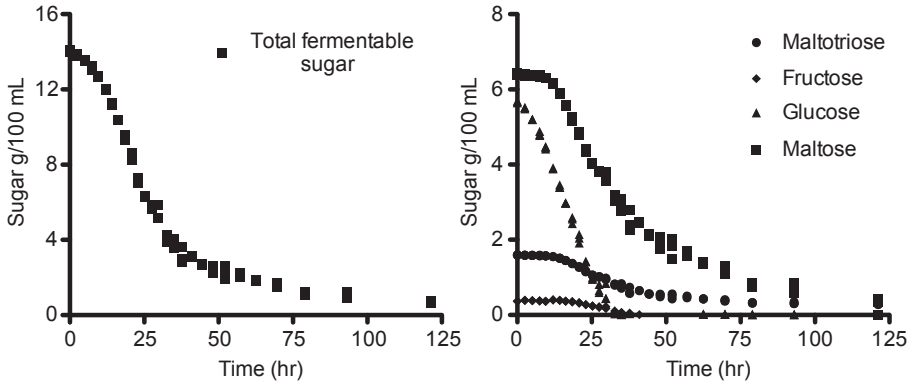

Figure 3.3 Raw total (left) and individual (right) sugar attenuation values taken throughout an experimental fermentation.

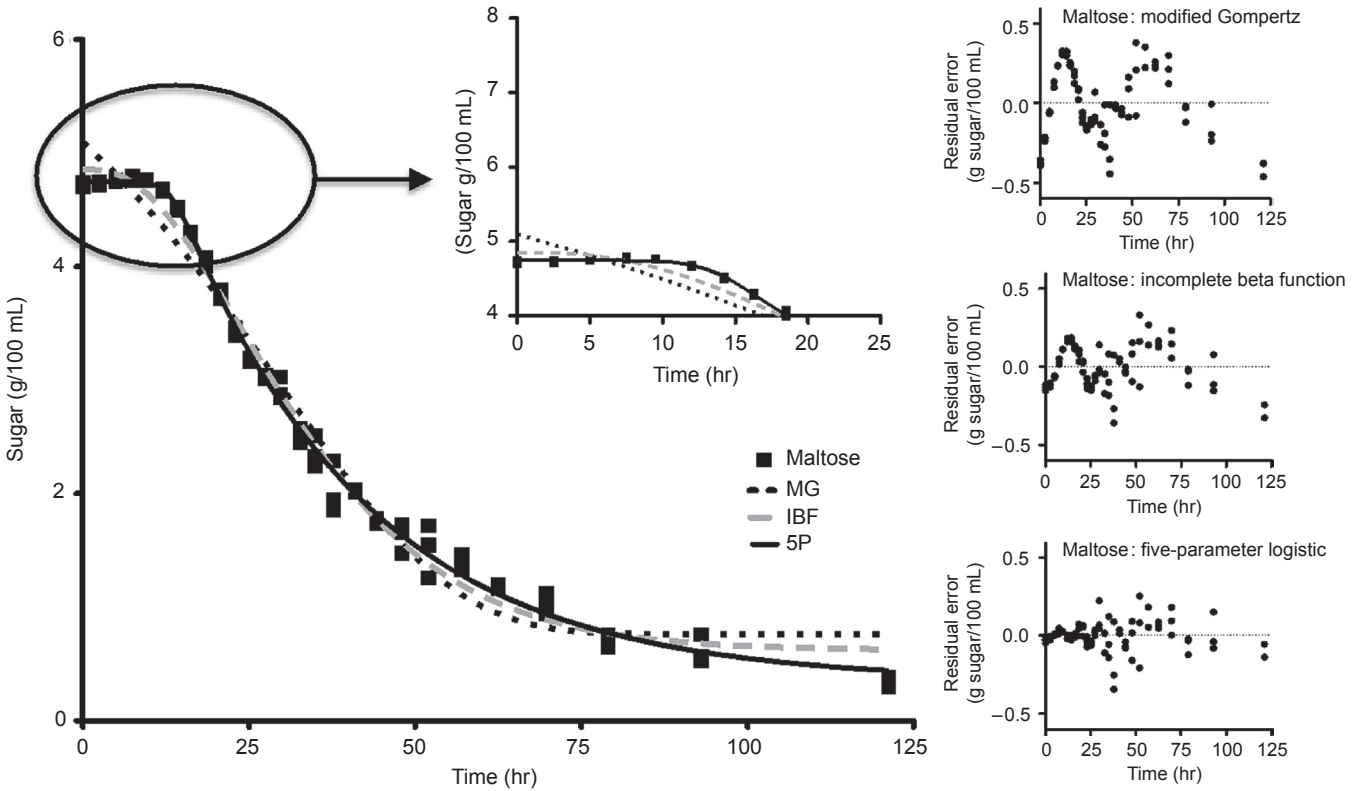

Figure 3.4 Modelled maltose attenuation data (MG, modified Gompertz; IBF, incomplete β-function; 5P, five-parameter logistic). The residuals for each model are depicted on the right.

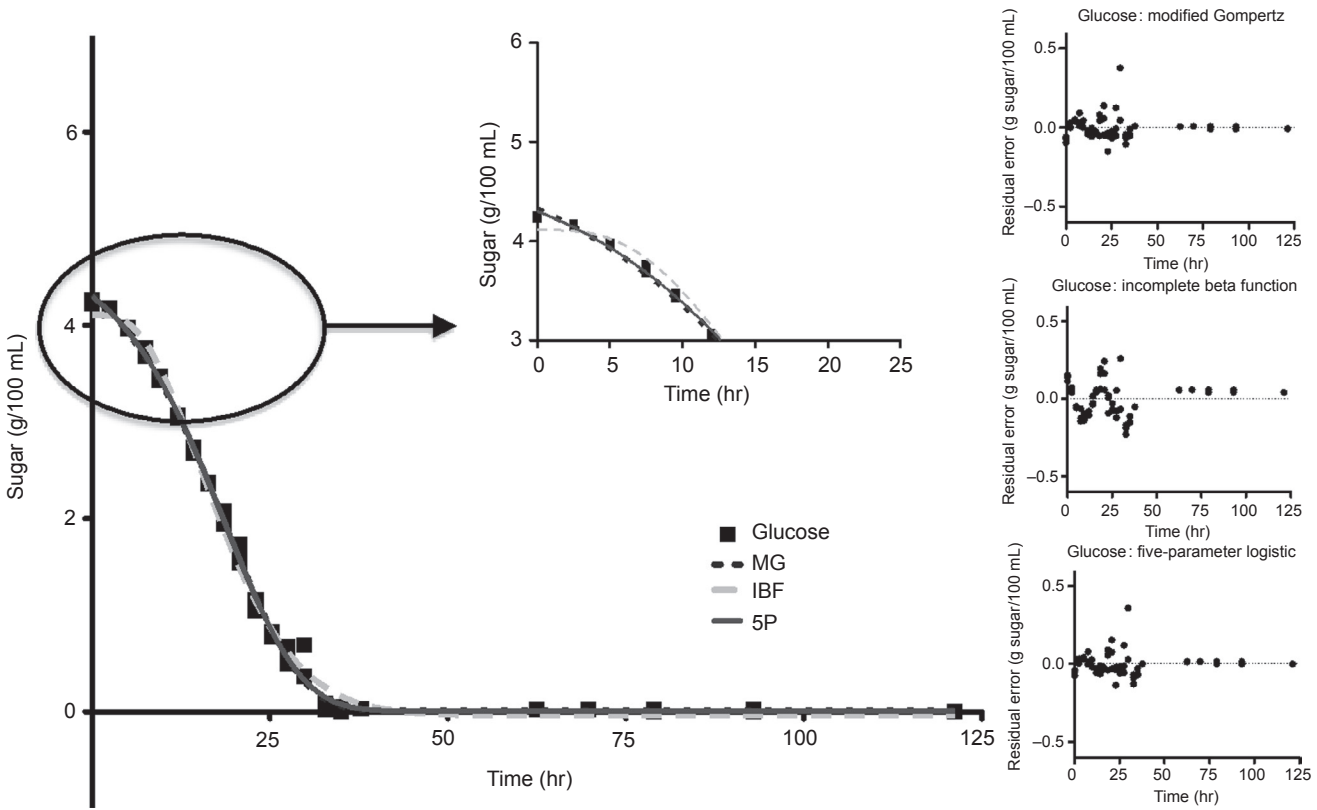

Figure 3.5 Modelled glucose attenuation data (MG, modified Gompertz; IBF, incomplete β-function; 5P, five-parameter logistic). The residuals for each model are depicted on the right.

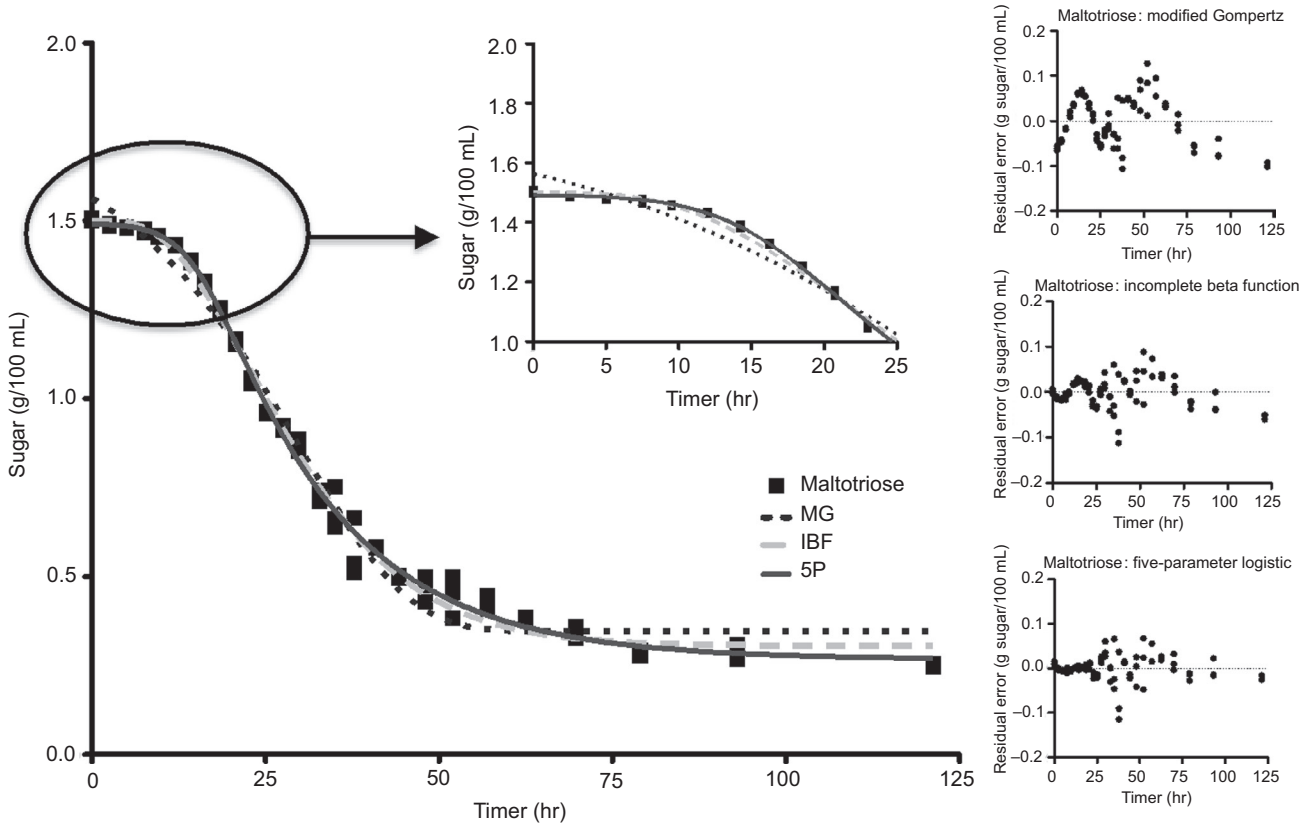

Figure 3.6 Modelled maltotriose attenuation data (MG, modified Gompertz; IBF, incomplete β-function; 5P, five-parameter logistic). The residuals for each model are depicted on the right.

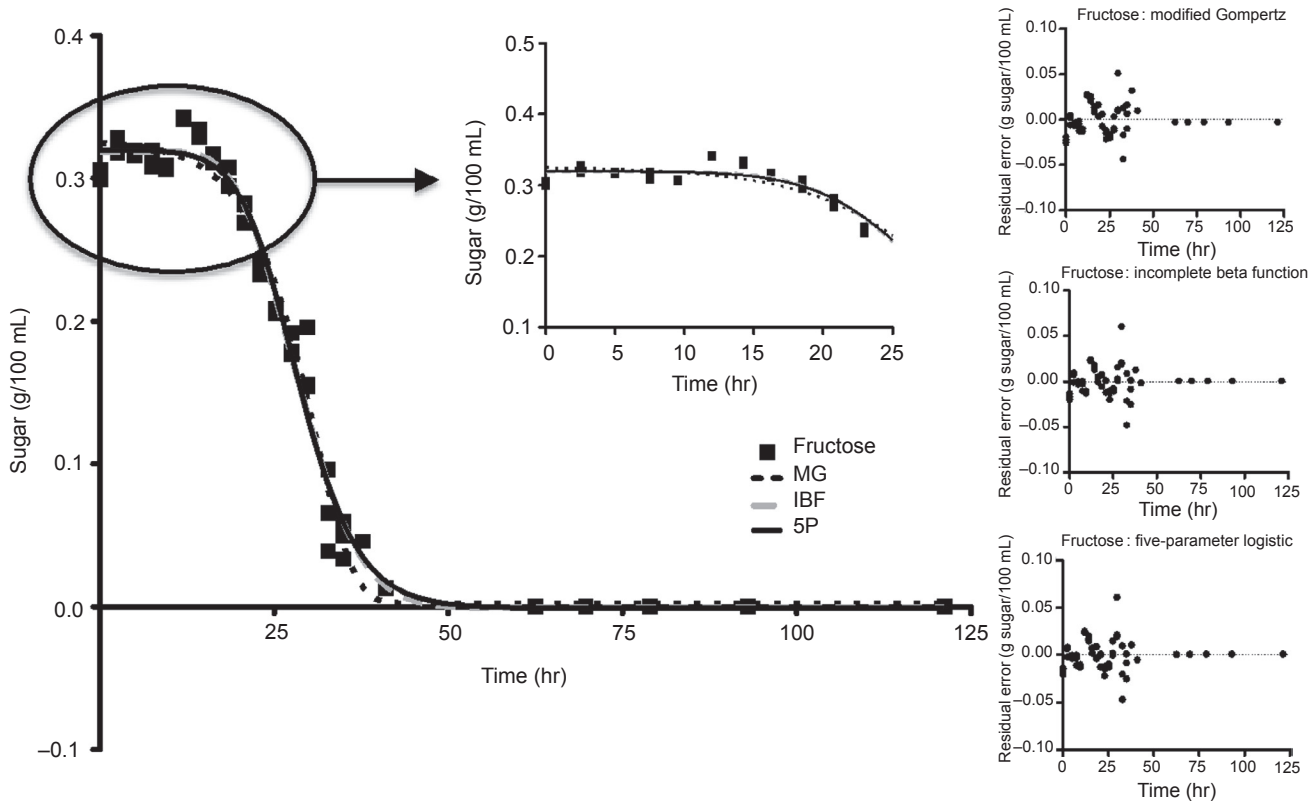

Figure 3.7 Modelled fructose attenuation data (MG, modified Gompertz; IBF, incomplete β-function; 5P, five-parameter logistic). The residuals for each model are depicted on the right.

The Gompertz model, although widely used for modelling the growth of many organisms (Buchanan & Cygnarowicz, 1990), has limited potential in modelling brewing fermentations. This model fits sugar attenuation well, provided that the sugar does not undergo consumption 'lag'. However, as we can see with maltose and maltotriose (Figures 3.4 and 3.6, respectively), this model deviates from the data near the beginning of fermentation, creating a trend in the residual error. Therefore, care should be taken when using this model, as it may fail to adequately describe brewing data. Next, although the IBF describes a versatile sigmoidal curve, it is an expanded mathematical distribution that is not designed to model biological behaviour. The limitations of this approach are apparent when modelling consumption data for sugars without a lag period. This is especially evident with glucose attenuation data (Figure 3.5), in which the derivative of the curve (the rate of consumption) at time zero, will be zero by definition.

The theoretical basis behind the logistic model is that the primary variable (sugar concentration or density) will have an autocatalytic effect upon the rate of change. Although this is shown to be likely true (as attested to by the sigmoidal shape of the sugar curves), the non-symmetrical nature of the data alludes to additional factors beyond substrate consumption that slows attenuation during the second half of fermentation (such as alcohol concentration). Therefore, the semi-empirical logistic model, which allows for asymmetry within the curve (i.e. 5P logistic), produced the most accurate fit, conforming to the actual shape of the attenuation curves. Additionally, the biological significance of the parameters described by Richard's curve provides a means of comparison between trials. As evidenced by the lack of pattern in every residual chart, this model can be used to accurately describe sugar attenuation in brewing operations. That being said, the 5P logistic model may suffer from 'over-parameterization' should the number of data points fall sufficiently low (the exact number depends upon when the samples are taken), which may often be the case in an industrial setting. This can be assessed by comparing the 4P logistic to the nested 5P using an F-test.

In summary, each model has advantages and disadvantages with respect to a particular situation. For example, the logistic models and IBF require numerous parameters to accurately model asymmetrical fermentations, whereas the IBF and Gompertz models each show pattern residual deviation under specific circumstances (with and without consumption lag). Furthermore, in specific circumstances, the simpler Gompertz model was chosen by statistical rigor to be superior. This example illustrates the importance of understanding the capability and limitations of a chosen model for brewing applications, as the differences reported by each model are significant at scale.

3.4 Advanced fermentation techniques

Compounding the importance of proper modelling and fermentation prediction is the phenomenon of high-gravity brewing. This increasingly common brewing technique can be simply described as the brewing of wort with a higher than normal specific

gravity (the result of additional sugars). The resulting product is diluted or blended, allowing greater capacity within the brewery (more beer is produced without the need to expand fermentor capacity/plant size). Although the use of this technique poses unique challenges to the brewer, this procedure often has potential to deliver large economical benefits, while also granting a great deal of control over the final product. In addition, although the main economical benefits of this technique come from the increased capacity without the need for additional equipment, there are a few other benefits to this procedure. There can be expected a slightly higher ethanol yield per unit sugar, as the number of yeast produced during fermentation is lower than would be expected from multiple fermentations. Moreover, the necessity of diluting post fermentation allows the brewer to blend the high ethanol liquor into a variety of brands and to achieve greater consistency within the final products.

There are, however, numerous considerations that the brewer must understand before committing to high-gravity brewing. Some of the drawbacks include greater stress upon the yeast (greater osmotic stress, ethanol content, etc.) and difficulty in matching the flavour of low-gravity (and undiluted) beer, often due in part to extensive use of adjuncts in high-gravity worts). Therefore, when contemplating high-gravity brewing, yeast selection and care is critical, as is quality control. Other noteworthy considerations include additional demands on the kettle (with higher carbohydrate loads), longer fermentations (partially yeast dependent) and potentially reduced foam stability (Stewart & Russell, 1998).

3.5 Future trends and sources for further information

Like most industries, the brewing industry will ultimately follow the will of the consumer. For many years, this led to the development of light beer (one of the most popular products in North America). However, a perceived lack of choice has (among other factors) contributed to the recent success of craft markets in North America. Looking forward, the modern brewer must be keenly aware of consumer perception, especially in an increasing global market, in which nearly all growth is in emerging markets.

3.6 Closing remarks

The tradition of brewing is thousands of years old and has played an important social and economic role in many cultures. With this historical significance, it is not surprising that brewing has been highly scrutinized and that many of the processes and mechanisms that take place during fermentations are well understood and documented. However, as scientific methods and tools evolve, there are opportunities to re-evaluate and improve our understanding even of topics that are well understood. Often, apparent discrepancies observed between theoretical and observed results can be explained with a greater understanding of the process. As concluded by many researchers,

modelling fermentations is a powerful tool that is easily used by modern brewers. The examples in this chapter shows that, for brewery applications, the common models used in the fermentation industry each have advantages and disadvantages. The additional accuracy resulting from the use of more complex models will be more easily utilized as modern instrumentation becomes more commonplace, allowing brewers to accurately model and predict their fermentations.

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Advances in metabolic engineering of yeasts

4

C.A. Boulton
University of Nottingham, Loughborough, Leicestershire, UK

4.1 Introduction

The realisation that, in nature, a reservoir existed of strains of brewing yeast with differing properties coincided with the isolation of the first pure cultures by Hansen in 1883. Following the widespread adoption of these techniques, brewing companies rapidly purified and jealously guarded their own proprietary strains. A logical extension of this was that it should be possible to apply the principles of breeding programmes used elsewhere – not only to choose strains from those already existing, but also to actively create new ones possessing even more desirable properties and eliminating disadvantageous traits.

Early strain-improvement programmes were hampered in several ways. There was insufficient knowledge of yeast genetics, especially of brewing strains, and a lack of tools for manipulating the genome with the necessary degree of precision for the creation of new strains with desired new traits and no undesirable changes. Linked to this was a paucity of detailed knowledge of the relationships between the yeast genome and the results of its expression. In consequence, there was a lack of predictability as to how the phenotype of engineered yeast strains would be expressed in the conditions of commercial brewing.

The huge growth in knowledge of the yeast genome acquired over the past 20 years or so, coupled with the development of methods allowing precise genetic manipulation, have superseded the much more difficult approaches of classical breeding or random mutagenesis. These have combined to provide excellent methods for the construction of novel strains. For example, The *Saccharomyces* Genome Database (www.yeastgenome.org) is an excellent online resource providing up-to-date information on this topic.

As these techniques are good, they can be used profitably only if they are underpinned with a precise knowledge of how the make-up and regulation of the yeast genome affects the phenotype. This aspect of yeast behaviour has lagged behind the development of techniques for genetic manipulation. The regulation of metabolism at the level of biochemical pathways is complex, but it is essential to understand whether the benefits of genetic engineering are to be gainfully exploited.

This need is addressed by the concept of metabolic engineering, which can be defined as the application of genetic techniques for the manipulation of metabolic pathways to bring about desirable changes in the activities of an organism. This chapter provides a discussion of metabolic engineering and the tools that are used to apply it.

Brewing Microbiology. http://dx.doi.org/10.1016/B978-1-78242-331-7.00004-6

Developments are described in modern brewing practice, and how these are providing the impetus for developing new brewing yeast strains.

4.2 Metabolic engineering

The concept of metabolic engineering was introduced by Bailey in the early 1990s (Bailey, 1991; Bailey, Birnbaum, Galazzo, Khosla, & Shanks, 1990). Several other terms have been used to describe the similar and allied concepts; these include metabolic pathway engineering (Tong, Liao, & Cameron, 1991), cellular engineering (Nerem, 1991) and in vitro evolution (Timmis, Rojo, & Ramos, 1988). In each case, as the names suggest, the emphasis is on the adoption of a systematic engineering approach to the characterisation of cellular activities so that these can be manipulated to achieve an altered and desired outcome. In other words, rather than adopting the random approach of selecting desirable strains from a pool of natural or induced variants, the likelihood of success is increased, provided there is sufficient prior knowledge of the physiological basis of the trait that is under consideration. Implicit in this is the realisation that although the phenotype is driven by the genome, there are several additional layers of control operating at the pathway level and involving regulation of enzyme activity by effector molecules and the concentrations of substrates and end-products. If these are not quantified and understood, then strain-improvement programmes are unlikely to succeed.

The original definition of Bailey (1991) defined metabolic engineering as 'the improvement of cellular activities by manipulation of enzymatic, transport and regulatory functions of the cell with the use of recombinant DNA technology'. In two later excellent reviews, Stephanopoulis (1999) and Ostergaard, Olsson, and Nielsen (2000) refined the definition to 'the directed improvement of product formation or cellular properties through the modification of specific biochemical reaction(s) or the introduction of new one(s) with the use of recombinant DNA technology'. Key to the approach is the use of the word 'directed'. Unlike earlier attempts at strain improvement, the genetic techniques that are used allow precise changes to be made without the introduction of unwanted and potentially undesirable additional changes.

Metabolic engineering of a desired trait is a two-stage process. First, the cellular basis of the trait that is to be modified is analysed to identify specific targets; second, these target sites are then subjected to genetic modification in a highly focused manner to make the necessary changes. Several operations are possible. These include amplification, inhibition, deletion or deregulation of native genes; in addition, heterologous genes may be introduced. The genetics of brewing yeasts are discussed in Section 4.5. The target traits that have been identified with respect to brewing yeast are described in Section 4.6.

Metabolic engineering seeks to close the gap in understanding between a basic knowledge of biochemical pathways and the overarching mechanisms that regulate cellular functions. Information such as the sequences of chemical steps that make up biochemical pathways, the enzymes that catalyse the individual steps and the genes responsible for their synthesis are essential, but provide relatively little information

as to how the individual pathways are regulated and how the control mechanisms are integrated with other pathways at any given time. Without this higher-level information, it is unlikely that attempts to steer changes to provide an altered outcome will be entirely successful.

With regard to brewing, this can be illustrated by considering the flow of metabolites through pyruvate and acetyl-CoA, as shown in Figure 4.1. Clearly, this is not a complete picture, and many steps in the pathways shown have been omitted; nevertheless, it can be seen that there are major branch points linking the uptake of major wort nutrients such as sugars, free amino nitrogen and oxygen and the formation of key products including ethanol, organic acids, higher alcohols, vicinal diketones, esters, fatty acids and sterols. Many of these compounds will be important participants in potential strain-improvement programmes, and therefore the pathways of which they form a part will be targets for genetic manipulation. Not shown in the figure are the proportions of carbon metabolised via the glycolytic and pentose phosphate pathways; the roles of anaplerotic and gluconeogenic pathways or the issues surrounding cell compartmentalisation. It is obvious that unless the bigger picture is considered, changes made in one pathway could affect carbon flow through others, with unexpected and perhaps undesirable results.

It is helpful to consider cellular activities in terms of a hierarchy that collectively provides a framework within which the various levels of function can be viewed as an ordered whole. These are the genome, the transcriptome, the metabolome and the fluxome (Figure 4.2).

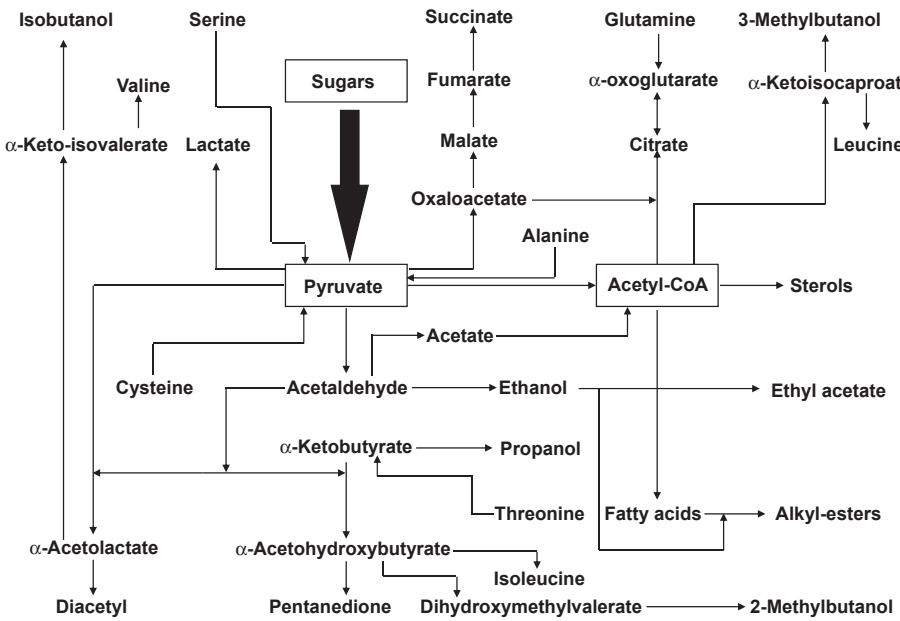

Figure 4.1 Overview of some of the metabolic pathways flowing to and from pyruvate and acetyl-CoA in yeast.

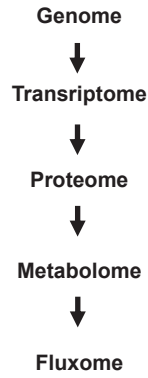

Figure 4.2 Hierarchy of cellular functions (see text for discussion).

The genome delineates the potential capabilities of the cell, and strain-improvement programmes may seek to delete or amplify part of it or augment it with heterologous genes. As discussed later in this chapter (Section 4.5), the genomes of many brewing strains are by no means simple, and these complexities make them not always easily manipulated. By definition, the transcriptome is that part of the genome that at any given instant has been transcribed and is therefore represented by molecules of RNA. An analysis of the transcriptome gives a snapshot of gene activity at any given instant. The products of translation collectively constitute the proteome, and this provides a picture of the immediate results of transcription. Many of these will be enzymes; the proteome describes the types and quantities present but provides no indication of activity. Some components of the proteome interact directly with the genome by acting as transcriptional factors. The longevity of enzymes in the proteome is of interest because mechanisms for regulating protein turnover will have an obvious impact on the results of genetic manipulations, particularly where heterologous genes are involved.

The metabolome describes the concentrations of the entire complement of small molecules present in the cell at any given time (Zamboni & Sauer, 2009). Advances in the power of analytical techniques, as discussed in the next section, now allow this detailed scrutiny to be made such that it is possible to monitor the whole of the metabolome. The compounds involved are direct participants in metabolic pathways, and they can also function as modulators of enzyme activity. They can be used to infer the presence of heretofore unknown pathways, and they provide information as to how nutrients become distributed throughout the cell and how and where excreted products arise.

Metabolomic information is obviously very helpful, but it does not provide any information regarding flow rates of metabolites through individual pathways. For this, it is necessary to measure changes in the concentrations of components of the metabolome as a function of time. Collectively these changes are termed the fluxome (Stephanopoulis, 1999; Wiechart, 2001; Zamboni, 2011). Studies that seek to investigate changes in the metabolome are referred to as metabolic flux analysis (Christiansen & Nielsen, 2000; Zamboni, 2011). Thus, Stephanopoulis (1999) defines a biochemical pathway as a ‘sequence of feasible and observable biochemical steps connecting a specified set of input and output metabolites’, whereas the

metabolic flux is 'the rate at which material is processed through a metabolic pathway'. The flux is considered with measurement of the concentrations of metabolites; collectively, these are considered to be the minimum information required to define the physiology of a cell under a given set of environmental conditions. The study of metabolic fluxes provides the necessary level of detail for a proper appreciation of the effect of changes in physiological condition on cellular function. With reference to the genome and transcriptome, the effect of genetic engineering can be assessed.

The totality of cellular functions needs to be considered. This includes the basic pathways and also global regulatory functions. In the context of brewing, the growth of yeast on wort, usually with an initial aerobic phase, followed by a transition to anaerobiosis, requires the cells to have mechanisms for dealing with the complex mixture of nutrients available and the constantly changing conditions. Apart from short-term regulation of metabolic pathways via interactions between the metabolome and enzyme activity, global effects on the genome must also be considered. These signal transduction pathways regulate the cell cycle (Nishida & Gotsh, 1993; Posas, Takekawa, & Saito, 1998), responses to applied stresses, for example, the osmo-sensing (HOG) pathway (Brewster, de Valoir, Dwyer, Winter, & Gustin, 1993), and responses to mixtures of nutrients as embodied by carbon (Gancedo, 1998; Schüller, 2003) and nitrogen catabolite repression (Boczo et al., 2005; Wiame, Grenson, & Arst, 1985). Of particular note in brewing, where serial fermentation is the norm, is the transition of cells between the G_0 phase of the cell cycle, which occurs at the end of fermentation and persists through storage and back into G_1 at the end of the lag phase when pitched into the next fermentation (Gibson, Lawrence, Leclaire, Powell, & Smart, 2005; Wei, Nurse, & Broek, 2008).

Initiation of these pathways requires communication between the cell and the external environment, and therefore, the role of receptor sites and uptake mechanisms must be considered. The onset of flocculation would be another response that requires responses with cells and the environment and with each other. The appearance of metabolic end-products in the medium requires mechanisms for intracellular transport and excretion. Within the cell, trafficking of metabolites and enzymes is important, particularly in the case of eukaryotic cells, where the impact of compartmentalisation has to be considered in strategies for strain improvement.

4.3 Tools for metabolic engineering

Successful metabolic engineering of microbial cells requires a stringent set of criteria to be satisfied. A high degree of knowledge of the organisation and function of the target cell is required. It is preferable for the genome to be sufficiently characterised, to allow annotation of gene to cellular function. Knowledge of the underlying metabolic pathways are needed, together with information as to how these are linked together and regulated.

It is unlikely that it will be possible to make some measurements in batch fermentations, especially commercial large-scale fermentations featuring the growth of yeast on a relatively uncharacterised medium such as wort. Batch cultures are inherently

difficult to work with because, by definition, conditions are in a state of continuous change. The corollary is that physiological condition is also in a state of continuous change in response to the altered environment. The conditions that yeast is exposed to in commercial brewing fermentations are very different from those in which the same strains are used in laboratory studies. This poses a dilemma, as it is clearly difficult to impose the appropriate degree of experimental stringency in production scale trials in which the results of metabolic engineering must ultimately be assessed. However, when carrying out the initial studies of cellular function and subsequent genetic manipulation, it is essential to have a properly controlled experimental system. Where batch fermentations are used at laboratory scale, the apparatus should be capable of both controlling and monitoring all basic parameters such as temperature, gas analysis, pH and agitation. For more controlled experimentation, it is desirable to use a chemostat or related cultivation system (Pirt, 1975). Chemostats form the basis of continuous cultivation and therefore allow establishment of steady states in which growth rate is proportional to the rate of supply of nutrients and physiological state is constant. Many of the techniques used in flux analysis require that steady-state conditions be used. The ability to perturb growth conditions in chemostats by allowing transitions between different steady states is a particularly powerful tool for elucidating metabolic fluxes (Daran-Lapujade et al., 2003; Kuyper et al., 2005).

Many metabolic engineering initiatives in which the cell may be viewed as a factory have some choice regarding the host organism. It is best that this is stable and capable of growing on a simple and, preferably, reasonably well-characterised medium. In the case of brewing yeast, these niceties are of course irrelevant, and it is certainly true that the genome of lager yeasts in particular poses some problems (see Section 4.5). Nevertheless, a range of tools are available to probe and modify the different levels of cellular activities that are summarised in Figure 4.2.

The yeast genome can now be sequenced and manipulated with relative ease, and there is a great deal of literature describing the techniques that can be used and the information obtained (e.g. Fromont-Racin, Rain, & Legrain, 1991; Oliver, Winson, Kell, & Bayang, 1998). The complete sequencing of several genomes, including that of yeast (Goffeau et al., 1996) has allowed comparisons of coding regions of genes with known function, such that a large proportion of the component parts of genomes can be associated with specific cellular functions (Liti & Louis, 2012; Schilling, Schuster, Palsson, & Heinrich, 1999).

Individual genes can be amplified or deleted and heterologous genes can be inserted using recombination or with suitable vectors. Vectors must be stable, form the desired product and be present in all transformed cells with a consistent copy number (Nevoight, 2008). Single or multi-copy vectors can be used to regulate copy number. In the case of brewing yeast, the most appropriate tool is to increase copy number by integrating several gene copies into chromosomes. Successful attempts have made use of the delta regions that occur in several locations in yeast genomes and thereby provide a means of inserting multiple gene copies (Kudla & Nicolas, 1992; Lee & da Silva, 1997).

The transcriptome can be monitored using a variety of techniques that allow the quantification of the degree of expression of entire genomes. Techniques such as serial analysis of gene expression (SAGE) and array analysis have been applied to yeast

(Gibson et al., 2008; Oleson et al., 2002; Velculescu et al., 1997), in the latter two papers, to lager yeasts during the course of commercial large-capacity fermentations.

Control of the proteome is obviously of primary importance, since control at the cellular level is exerted via the activities of enzymes either via catalysis of individual steps in pathways, performing transport functions, or acting as regulatory proteins. The proteome is amenable to analysis via extractive techniques followed by chromatographic or electrophoretic separation and analysis, usually by mass spectrometry (MS) (Gavin et al., 2002; Picotti et al., 2013; Washburn, Worters, & Yates, 2001). These methods have been particularly useful for gene annotation.

Transcription of individual genes can be modified by the addition of a suitable promoter (Mumberg, Muller, & Funk, 1995). It is possible to regulate transcription efficiency by choosing alternative yeast promoters with the appropriate strength. Verstrepen and Thievelein (2004) successfully applied this technique to control the transcription of homologous genes in *Saccharomyces cerevisiae*. However, this may have to be used in conjunction with methods for manipulating gene copy number. Another approach is to delete or modify the function of transcription factors by targeting the encoding genes. This has been applied to the alleviation of glucose repression, allowing constitutive maltose uptake (Nielsen, 2001) and modulation of ethanol formation by manipulation of the activity of pyruvate decarboxylase (Nevoight & Stahl, 1996). Omura, Fujita, Miyajima, and Fukui (2005) introduced efficient proline assimilation into a lager yeast strain using an alternative strategy by inhibiting ubiquination of an amino acid permease and thereby rendering the protein more long lasting.

Metabolomic and flux analysis are the most challenging. The components of the metabolome can be characterised by separation via liquid or gas chromatography followed by analysis using mass spectrometry (MS) or nuclear MS (NMS). Typically, several methods of separation and detection are combined to allow quantification of several classes of compounds (Büscher, Czernik, Ewald, Sauer, & Zamboni, 2009; Dettmer, Aronov, & Hammock, 2007; Ohashi et al., 2008; Van der Werf, Overkamp, Muilwijk, Coulier, & Hankemeier, 2007). These approaches have been used to characterise nearly 400 individual compounds, albeit only in bacteria. Sampling remains a problem because, after removal from the growth vessel, all cellular activities must be rapidly quenched to avoid artefacts. In the case of bacteria, the process disrupts the membrane, and corrections must be made for leakage into the medium. In the case of eukaryotes, such as yeast cells, quenching will disrupt intracellular membranes, making probing of compartmentalisation difficult.

The dynamic nature of flux analysis introduces much more complexity. Studies are dependent on measurement of the relevant components of the metabolome followed by the application of mathematical modelling and statistical analysis to unravel the huge quantities of data generated. The usual approach is to use a combination of tracer studies using isotopes of carbon, mainly ^{13}C together with Gas-liquid chromatography-mass spectrometry (GC-MS) or nuclear magnetic resonance (NMR) (Whitmann, 2007). The ^{13}C-labelled substrate is fed into the system, and the label becomes distributed into the intermediates and products of subsequent metabolism. The identity of the compounds formed and the position of the label in the molecule are of significance. These combinations of isotopes and isomers are referred to as isotopomers (Wiechart, 2001). The use of

isotopomers dramatically increases the value of the information supplied. A compound containing n carbon atoms, each of which may or may not be labelled, can form 2n different isotopomers. From the position of the label in a product, it may be possible to identify the pathway by which it was formed or the presence of unknown pathways. Where several isotopomers of the same compound are detected, the presence of multiple pathways can be inferred. At steady states, the concentrations of each labelled intermediate provides flux information.

Zamboni (2011) describes flux analysis via two approaches, termed stationary or non-stationary. In both cases, the basic requirements are a model metabolic network, a closed carbon balance measured in terms of rates of substrate uptake and product excretion, and information regarding the patterns of ^{13}C labelling in product and intermediates. The stationary strategy relies on measurement of the proportions of isotopomers of a common compound formed at steady state from which converging alternate pathways can be resolved. The non-stationary method monitors the spread of the ^{13}C label with time. Together with analyses of the concentrations of relevant components of the metabolome, the latter approach is capable of quantifying fluxes. It is much more demanding in terms of data handling.

Undoubtedly, flux analysis offers great promise as a tool to be used in metabolic engineering; however, there remain significant challenges, and these are very pertinent to applications in brewing. The problems of compartmentalisation in eukaryotic cells are problematic (Niklas, Schneider, & Heinzle, 2010). With regard to brewing, the enzymes associated with branched chain amino acid metabolism, such as valine, which are presumed to be implicated in diacetyl formation and reduction in beer, are located in mitochondria, and by inference two transport steps must be involved between this compartment and the cell exterior. To understand properly, and to manipulate metabolic fluxes and how these relate to events such as FAN uptake and vicinal diketone formation, it seems probable that compartmentalisation will be pertinent. Nevertheless, as a result of metabolic engineering, the subcellular localisation of enzymes has been successfully moved from the mitochondria to the cytosol (Moreira dos Santos, Raghevendran, Kotter, Olsson, & Nielsen, 2004).

Much work remains to be done to begin to understand the control of cellular functions in non-steady states during growth on complex media. The great promise of metabolic engineering and the analytical methods on which strategies for genetic manipulation are based is that it allows a much more integrated view of cellular function from metabolic pathways up to the genome. An output from these studies has been the appreciation of the role of regulation networks: in particular, the importance of non-transcriptional events such as phosphorylation of proteins (Heinemann & Sauer, 2010).

4.4 Strategies for metabolic engineering

The complete appreciation of cellular functions continues to lag behind the ability to analyse and manipulate the genome. As a result of this, strategies for metabolic engineering still remain largely reliant on successive rounds of genetic manipulation and analysis to hone the acquisition of the desired phenotype. Metabolic engineering

is based on what has been defined as rational metabolic engineering in which genetic manipulation is based on prior knowledge. The results are often disappointing because of the appearance of unpredicted side-effects and the fact that the advances made in laboratory studies fail to translate to commercial fermentations (Nevoight, 2008).

For these reasons, other strategies have been devised. Evolutionary engineering (Sauer, 2001) relies on a random approach in which the genome is altered via mutagenesis or DNA shuffling, followed by screening for variants with a desired phenotype. The obvious problem is the random nature of the process and the need for precise screening methods. The latter can be a particular problem with some traits. Bayer and Smolke (2005) describe a work-around in which the activation of riboregulators by a target ligand is connected to the expression of a gene that generates a fluorescent dye. In this way, any phenotype should be amenable to analysis.

The major drawback of evolutionary metabolic engineering is that is does not produce an understanding of the metabolic networks involved, nor does it relate this to the relevant genomic information. Global transcription machinery engineering (gTME) targets random mutations involving protein transcription factors. Mutants showing phenotypic improvements are selected and the gene mutations are identified. The phenotype modifications can be transferred to other cells and, via analysis of the transcriptome, the associated changes in gene expression can be identified. An advantage of this strategy is that it can be applied to polygenic traits such as stress responses (Alper, Moxley, Nevoight, Fink, & Stephanopoulos, 2006). In another strand, the concept of reverse metabolic engineering has been introduced (Bailey et al., 2002). This requires selection of systems in which the desired phenotype is expressed differently. These might be different but related strains or the same strain in which the trait is expressed differently under varying environmental conditions. The genetic basis of the observed differences is then resolved, and hopefully this provides the necessary information required to reproduce the desired phenotype in another host. Confirmation that the genetic basis of the phenotype has been correctly identified is made via deletion or amplification of the target portion of the genome.

The strengths and weaknesses of this approach are embodied by the foregoing discussion regarding the different levels of cellular function (Figure 4.2). Assessing the phenotype based on genome analysis is unlikely to reveal the precise source of phenotypic variations. The metabolome and fluxome provided the greatest level of detail but are the most difficult to relate to the genome. Nevoight (2008) claims several advantages of inverse metabolic engineering: it is not necessary to have prior knowledge of the relevant pathways or their regulation; industrial strains can be used under production conditions; heterologous genes are not involved, and the transformants can be considered self-cloned; it may lead to the chance discovery of novel genetic targets.

4.5 Brewing yeast genetics

Brewing yeasts are differentiated into ale and lager types. The former are considered older in evolutionary terms and are classified as *S. cerevisiae*. They express considerable genetic variability (Pedersen, 1985, 1986a, 1986b, 1994). Lager strains are

currently classified as *S. pastorianus* (Vaughan-Martini and Martini, 1987) and form a less diverse group of strains compared with ale types. The genome of lager strains differs significantly from ale types. Ale strains are polyploid whereas lager types are allotetraploids (Smart, 2007). Studies have shown that the lager genome is hybrid in nature in which chromosomes may show homology with those of *S. cerevisiae*, with no homology with *S. cerevisiae* or mosaics of the two. Individual genes may be *S. cerevisiae* (Sc-) type or non-cerevisiae, termed *S. pastorianus* (Sp-), lager (lg-) or *S. carlsbergensis* (CA-) type. Lager strains apparently arose as a result of one or more hybridisation events between two closely related *Saccharomyces* strains. One parental type was *S. cerevisiae*; the identity of the other remains as a subject for debate, but is currently considered most likely to be *Saccharomyces bayanus* (Smart, 2007). The mitochondrial genome is circular and in lager yeast strains shows most homology with that of *S. bayanus* (Smart, 2007).

The hybrid nature of lager strains introduces much complexity. Kodama, Omura, Miyajima, and Ashikari (2001) investigated the uptake of branched chain amino acids in a lager strain. Two permeases were present, coded for by *BAP2* genes. Of the two genes, one (*cer-BAP1*) was identical to that of *S. cerevisiae*, whereas the second (*lg-BAP2*) was identical with that from *S. bayanus*. The genes showed 88% homology with each other but were regulated differently. Generally, two copies of each gene were present.

Nakao et al. (2009) sequenced the entire genome of a lager strain and found that a 25-Mb genome could be divided into two distinct nuclear subgenomes with homologies with *S. cerevisiae* and *S. bayanus*. The size was roughly double that of ale strains. Some 36 different chromosomes could be distinguished, of which 8 were mosaic types where the breakpoints were within open reading frames.

The tetraploid nature of the lager yeast genome appears to confer inherent genetic instability. The applications of applied stresses of the types now commonplace in commercial brewing, such as the use of fermentations at elevated temperatures with very–high-gravity worts, are causes of genetic changes (James, Usher, Campbell, & Bond, 2008).

4.6 Targets for engineering of brewing yeast

Traits chosen for manipulation in brewing strains fall into the following categories:

1. Process improvement
2. Altered spectrum of substrate use
3. Improved control of beer flavour and stability
4. Production of novel beers by fermentation

Many examples of the development of engineered brewing strains with traits falling into the categories given above have been published. Space does not permit a complete listing of these, but some examples are given in Table 4.1. From the references it may be seen that many of these works are now comparatively old; this possibly reflects the fact that the lack of commercial take-up has made many researchers switch to more receptive alternative industries such as bioenergy.

Table 4.1 Targets for metabolic engineering of brewing yeast

Trait	Driver	Trait	Target	References
Process improvement	Increased yield	Increased ethanol yield	Overexpression of *GLT1*	Cao et al. (2007)
		Improved ethanol tolerance	Altered transcription via mutation of Spt15p transcription factor	Alper et al. (2006)
		Improved osmotolerance	NAD^+-dependent glycerol 3-phosphate dehydrogenases	Ansell, Granath, Hohmann, Thevelein, and Adler (1997) and Siderius, Van Wuytswinkel, Reijenga, Kelders, and Mager (2000)
		Improved performance at high gravity	Selection of variants from UV-treated brewing yeast	Blieck et al. (2007)
		Dextrin utilisation	Insertion of glucoamylase and α-amylase from *Lipomyces kononenkoae*	Eksteen, Van Rensburg, Cordero Otero, and Pretorius (2003)
			Addition of *DEX* gene from *Saccharomyces diastaticus*	Perry and Meaden (1988)
		Improved sugar utilisation	Constitutive expression of *MAL* genes	Kodama et al. (1995)
	Reduced fermentation cycle time	Increased thermo-tolerance	Selection and isolation of variants in distillery yeasts	Abdel-Fattah, Fadil, Nigam, and Banat (2000)
		Increased or altered diacetyl metabolism	Disruption of *ILV2* via self-cloning	Zhang, Wang, He, Liu, and Zhang (2008) and Wang, He, Liu, and Zhang (2008)
			Disruption of *ILV* genes	Gjermansen et al. (1988)
			Introduction of α-acetolactate decarboxylase	Fujii et al. (1990)
	Enhanced substrate utilisation	Dextrin utilisation	As above	Eksteen et al. (2003) and Perry and Meaden (1988)
		Pentose utilisation	Incorporation of xylose-utilising pathway, manipulation of redox control	Jeffries and Jin (2004)

Continued

Table 4.1 Continued

Trait	Driver	Trait	Target	References
	Better beer filterability	β-glucan utilsation	Incorporation of β-glucanase from *Trichoderma reesei*	Penttilä, Suihko, Lehtinen, Nikkola, and Knowles (1988)
	Resistance to contamination	Acquisition of killer phenotype	Transfer of killer factor via rare mating	Young (1981)
	Cropping behaviour	Altered flocculation	Manipulation of expression of *FLO1*	Verstrepen et al. (2001)
Beer quality	Altered flavour	Altered volatile spectrum	Alcohol acetyltransferase	Fujii et al. (1994)
			Manipulation of *BAP2* to modulate higher alcohols	Kodama et al. (2001)
		Reduced hydrogen sulphide	Increased copy number of MET25 gene	Omura, Shibano, Fukui, and Nakatani (1995)
		Reduced dimethyl sulphide	Removal of dimethyl sulphide oxidase by deletion of *MXR1*	Hansen, Bruun, Bech, and Gjermansen (2002)
	Enhanced flavour stability	Elevated sulphur dioxide	Over-expression of *MET14* and *SSU1*	Donalies and Stahl (2002)
Novel beers	Low or zero alcohol beers	Increased glycerol production	Manipulation of *GDP1*	Nevoight et al. (2002)
	Low carbohydrate beers	Dextrin utilisation	As above	Eksteen et al. (2003) and Perry and Meaden (1988)

4.7 Future perspective

The global brewing industry continues to be dominated by a relatively small number of large companies. They are internationals operating in some markets that are flat or declining and others where volume growth is very high. Competition is very fierce and growth via acquisition is likely to continue. Pale pilsner-style lager beers vastly out-sell all other beer styles. Against this backdrop there is a burgeoning craft brewing sector that has a relatively small but growing volume. Although small, in many countries, the craft segment has a loud voice and they have done much to polarise arguments over what is and what is not 'real beer'. Undoubtedly, this will colour the views of many consumers regarding the probity of using genetically engineered yeast strains. Hammond (1995) summarised the then current situation regarding the use of genetically modified yeast for brewing. Several strains had been developed successfully and as a test case one had been granted approval for commercial use in the UK. To date, no brewers have chosen to use modified strains and this situation does not show signs of changing. Interestingly, there is less resistance to using commercial exogenous enzymes, some of which have been derived from transgenic organisms.

The major brewers face several challenges. They must minimise costs to maintain a competitive edge. Sustainability is a big driver, and in many markets minimising water usage is important. The move from draught to small-pack continues and coupled with the need to export there is a much focus on extending beer shelf life and maximising beer flavour stability. Large international brands and multi-site brewing must be backed up by excellent control of beer flavour and quality. In the case of developments in selection of brewing yeast, many of these needs are reflected in the strategies for metabolic engineering summarised in Table 4.1.

A common wish is to brew at very high gravity (>20° Plato) using high fermentation temperatures for short vessel residence times and with very large batch sizes. If this is to be combined with serial re-pitching, as would be the norm, many currently used brewing yeast strains are barely fit-for-purpose, and there is a very real danger that the stresses imposed on yeast will lead to greater than normal losses in viability and consequent problems with beer quality. The usual response, namely, to reduce the number of acceptable serial fermentations before introduction of a new culture, is not a particularly tenable strategy, since many propagation facilities are unable to cope with the additional demands. It may be argued therefore that there is a real need to develop brewing strains that are able to tolerate these conditions. These may not occur in nature, and therefore they need to be constructed. Implicit in this is that, for the first time, it many not be possible to isolate suitable new strains from the nature.

Public acceptance of genetically engineered strains is not forthcoming in many countries. The brewing industry has a difficult task in this regard, since many of the goals of yeast genetic engineering are good for brewers' profits but perhaps less obviously of direct benefit to the consumer. The trend towards an increased need for ingredient labelling may become problematic in many markets. The development of engineered strains via self-cloning techniques in which the introduced material is not viewed as 'foreign' is likely to have importance.

Although the brewing industry will exercise its usual caution with regard to the adoption of engineered strains, it is essential that the development work continues in which the principles of metabolic engineering are exploited. Many previous attempts to engineer yeasts have failed, or at least have achieved only partial success, because there was insufficient knowledge of the physiological basis of the traits under investigation. The use of metabolomics and metabolic flux control studies will greatly assist in filling this gap. This basic knowledge is bound to provide benefits, whether or not the information is ultimately used for yeast strain modification.

4.8 Additional sources of further information

For more information on brewing and the role of yeast in fermentation and beer quality, see Boulton and Quain (2006). Metabolic engineering and the tools available for its application are described comprehensively by Wittmann and Lee (2012). The Journal of Metabolic Engineering (www.journals.elsevier.com/metablic-engineering) is an excellent resource for up-to-date information regarding all aspects of metabolic engineering.

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Yeast identification and characterization

5

M. Hutzler, J. Koob, R. Riedl, H. Schneiderbanger, K. Mueller-Auffermann, F. Jacob
Research Center Weihenstephan for Brewing and Food Quality, TU München, Munich, Germany

5.1 Biodiversity and characterization of yeast species and strains from a brewing environment

Approximately 1500 yeast species are currently known (Kurtzman & Fell, 2006). Estimations indicate that an additional 669,000 extant yeast species have not yet been described (Verstrepen, Chambers, & Pretorius, 2006). The most important yeast species for fermentation technology belong to the genus *Saccharomyces* and are taxonomically grouped in the *Saccharomyces sensu stricto* complex (Rainieri, Zambonelli, & Kaneko, 2003; Vaughan-Martini & Martini, 2011). The *Saccharomyces sensu stricto* complex consists of the following: *Saccharomyces cerevisiae,* the yeast used for the production of top-fermented beers (often referred to as "ale"), wine, distillers' mash, sake, and many other alcoholic beverages; *Saccharomyces bayanus*, applied in wine, cider, and apple wine production; *Saccharomyces pastorianus,* the starter culture for bottom-fermented beer (lager) and apple wine production; as well as six additional species (*Saccharomyces cariocanus*, *S. kudriavzevii*, *S. mikatae*, *S. paradoxus, S. arboricolus*, and *S. eubayanus*) that are not used industrially (Bamforth, 2005; Libkind et al., 2011; Rainieri et al., 2003, 2006; Wang & Bai, 2008). Libkind et al. reported that the bottom-fermenting (BF) strains of the species *Saccharomyces pastorianus* used in the lager beer production are genetic hybrids of *Saccharomyces cerevisiae* and the Patagonian wild yeast *S. eubayanus* (Libkind et al., 2011). Dunn and Sherlock postulated that at least two hybridization events took place and that all *Saccharomyces pastorianus* lager strains consist of at least two types (Dunn & Sherlock, 2008). The *S. pastorianus* strains that they studied were divided into the groups: Saaz and Frohberg. Some industrial strains exhibiting strong fermentation performance belong to the Frohberg group. Rapid species identification within the *Saccharomyces sensu stricto* group is of great importance for verifying the purity of a species in a beer starter culture and for detecting cross-contaminations. In addition, there are some non-*Saccharomyces* yeast species that are used as starter cultures for special beer styles. *Schizosaccharomyces pombe* is found in some traditional African beers and *Dekkera bruxellensis* in Belgian beers and in German Berliner Weiße. *Saccharomycodes ludwigii* is used for the production of low- and nonalcoholic beer styles, where *Torulaspora delbrueckii* can be used in the production of top-fermented wheat beer

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as a supplemental yeast strain to generate a distinct fruity aroma. In spontaneous beer fermentations, other non-*Saccharomyces* species can be involved, such as *Debaryomyces* spp., *Meyerozyma guilliermondii*, *Pichia membranefaciens*, *Candida friedrichii*, *Naumovia castellii*, *Dekkera anomala*, *Priceomyces* spp. in lambic beer (Spitaels et al., 2014) and *Cryptococcus keutzingii*, *Rhodotorula mucilaginosa*, *Candida krusei*, *Pichia fermentans*, *Pichia opuntiae* in American coolship ale (Bokulich, Bamforth, & Mills, 2012). Table 5.1 provides an overview of the yeast species common in beer production.

Breweries either maintain individual brewing strains or they order yeast strains from yeast strain providers or culture collections. Yeasts are available as pure cultures or in dried form. For dry yeast, a rehydration process in a tank or a reactor is necessary before the yeast can be pitched. Pure culture yeast must be cultivated in a laboratory until the required volume is reached. Afterward, the yeast can be transferred from the Carlsberg flask to the propagator. In the propagation system, once the target cell concentration for yeast growth under ideal conditions has been achieved, the yeast is grown to the appropriate volume and cell concentration for pitching in wort for beer production. It must be said that handling pure culture yeast is more demanding than working with dry yeast, but it is generally safer from a microbiological point of view. The greater the number of yeast strains maintained in one brewery, the greater

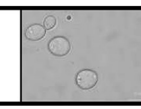
Table 5.1 Brewing strains of yeast species used for different beer types

	Fermentation/flocculation characteristic		Beer type	Genus/species
Brewing yeast strains	Bottom-fermenting	Strong flocculation Low flocculation	Lager, pilsener, export, bottom-fermented special beers, bottom-fermented low alcohol beer, etc.	*S. pastorianus* (spp. *carlsbergensis*)
	Top-fermenting	Low flocculation	German wheat beer, ale, stout, koelsch, alt, Belgian special beer styles (Witbeer, Trapist beer), African indigenous beer styles, etc.	*S. cerevisiae*
	-	-	Non- and low-alcohol beer styles	*Saccharomycodes ludwigii*
	-	-	Berliner Weiße, Belgian special beer styles (e.g., Lambic)	*Dekkera bruxellensis*
	-	-	African indigenous beer styles	*Schizosaccharomyces pombe*
	-	-	Special beer styles with fruity character	*Torulaspora delbrueckii*

the danger for cross-contamination. A brewing yeast strain should be taxonomically classified by means of molecular biological methods at species and strain levels. In addition, its propagation and fermentation performance, as well as its aroma profile, should be characterized. Classification with molecular biological methods is described in Sections 5.1 and 5.2. Figure 5.1 shows a brief description of the BF lager yeast strain TUM 34/70 according to parameters relevant for fermentation, beer quality, and aroma.

Strain TUM 34/70 is one of the most abundant lager yeast strains in the brewing industry, and is a strain to which all others are compared regarding fermentation performance and the pure flavor of lager beers produced with it. The genome of TUM 34/70 was also the first of the BF strains to be sequenced and published (Nakao et al., 2009). A recent study conducted by Mueller-Auffermann used the characteristics of TUM 34/70 as a reference for developing a method to rapidly compare the performance of lager yeast strains (Mueller-Auffermann, 2014b). Data comparing six BF lager yeast strains are found in Figure 5.2, which shows 9 of 17 properties of yeast analyzed and characterized using this method. Strain TUM 66/70 does not flocculate

Description of **yeast strain TUM 34/70**
Saccharomyces pastorianus ssp. carlsbergensis,
bottom-fermenting, flocculent yeast



This yeast strain is excellently suited for the production of bottom-fermented beers of all types. The resulting beer possesses an extremely pure flavor, a fine, subtle aroma, and a mild overall impression. Fermentation is rapid with optimal yeast flocculation. The length of time that the yeast remains in suspension is dependent on conditions present in the brewery. The degree to which the color lightens during fermentation is sufficient.

Beer analysis after 6 days of primary fermentation (original gravity: 11.6 %)

Beer analysis	
Apparent degree of attenuation (%)	73
Cells in suspension (millions/ml)	12.5
pH value	4.6
Difference between final and apparent degree of attenuation (%)	0
Diacetyl (mg/L)in green beer	0.7
Diacetyl (mg/L)in matured beer	0.1
Acetaldehyde (mg/L)	6
Higher (aliphatic) alcohols (mg/L)	58
Esters (mg/L)	18.5
Foam, Ross & Clark method (s)	132

TUM 34/70 overview of attributes

Fermentation rate	High
pH reduction	Normal
Flocculation	Optimal
Diacetyl reduction	Very good
Foam	Very good
Difference between final and apparent degree of attenuation	Very low
Acetaldehyde	Normal
Higher alcohols	Very low
Esters	Pronounced

Figure 5.1 Description of *Saccharomyces pastorianus* ssp. *carlsbergensis* TUM 34/70, a bottom-fermenting lager yeast strain, in terms of fermentation parameters, beer-quality parameters, and aroma.
Hutzler et al. (2014).

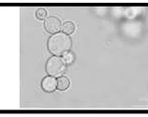
	Saccharomyces pastorianus ssp. carlsbergensis strains					
Fermentation Characteristics	**TUM 34/78**	**TUM 193**	**TUM 194**	**TUM 66/70**	**TUM 44**	**TUM 69**
Physical characteristics of yeast cells						
Low flocculation strength (powdery character)	↑	-	↓	↑↑	-	↑
Sedimentation	-	-	-	-	↑	-
Turbidity	↑↑	-	↑↑↑	-	-	↑
Fermentation by-products						
Diacetyl	↓	-	-	↓↓	↓	↓
SO_2	↓↓	↑↑↑	↓↓	↑	-	-
Acetaldehyde	↓	↑	↓↓	-	-	↑
Esters	↓	↑	↓	↓	-	↓
Fusel alcohols	↓	↑	↓	↓	↑	-
Other quality parameters						
Foam	↑↑	-	↓↓	-	↓↓	↓

Figure 5.2 Characterization of six lager stains in comparison to the reference TUM 34/70 by a new pilot-scale approach.
Mueller-Auffermann (2014b).

well and possesses a "powdery" character, whereas the other five strains in Figure 5.2 are flocculent.

The pilot plant used to conduct these trials consists of 27 2-L small-scale fermentation tanks. In each trial, two strains (2×9 = 18 tanks) were compared to the reference strain TUM 34/70 (in nine tanks). The values measured during fermentation (Figure 5.2) for the two strains characterized in the trials were compared to the values for strain TUM 34/70. An arrow in Figure 5.2 represents a shift of 5% in the value for a specific parameter relative to the same one for strain TUM 34/70, whereas two arrows represent a shift of 10% (Mueller-Auffermann, 2014b). TUM 193 produces more SO_2 than TUM 34/70 as well as more acetaldehyde, esters, and fusel alcohols. Fermentation performance is similar to that of TUM 34/70M; however, the pH drops more slowly at the beginning of fermentation (data not shown). TUM 193 is advantageous for improving the flavor stability of beers (SO_2) and also produces a slight estery, fruity note (esters, acetaldehyde, fusel alcohols) relative to TUM 34/70. These

Description of **yeast strain TUM 68**
Saccharomyces cerevisiae, top-fermenting,
Bavarian wheat beer yeast, POF positive



Color:	light brown, naturally cloudy
Foam (visual):	good
Aroma:	pure, pleasant top-fermented aroma, pleasant clove aroma, trace of banana
Flavor:	pure, pleasant top-fermented flavor, pleasant clove aroma, trace of banana, full-bodied, mild, well-balanced aftertaste

Analysis (fermentation and beer parameters)	
Degree of attenuation	0.83
ΔpH (pH reduction)	1.0
Cells in suspension — value at maximum	39.67 million/mL
ΔFAN	131.1 mg FAN/L
Sugar spectrum, total	79.54 g/L → 0.77 g/L
Isoamyl acetate after 96 h	3.16 mg/L
Ethyl acetate after 96 h	30.16 mg/L
4-Vinylguaiacol after 96 h	2.71 mg/L
Isoamyl alcohol after 96 h	63.91 mg/L
Diacetyl after 96 h	0.51 mg/L
Total score according to the DLG rating scheme	4.45

Abbreviations:
POF = phenolic off flavor
FAN = free amino nitrogen
DLG = Deutsche-Landwirtschafts-Gesellschaft (German agricultural society)

Figure 5.3 Description of *Saccharomyces cerevisiae* TUM 68, a top-fermenting Bavarian wheat beer strain, in terms of fermentation parameters, beer-quality parameters, and aroma. Hutzler et al. (2014).

kinds of trials are very useful for breweries wishing to replace their yeast strain or introduce a second one to develop specialty beers with particular properties or to modify or improve existing lager beer styles. The booming North American craft beer scene is now conquering Europe, and a lot of specialty beers with distinctive flavors are appearing on the market, especially beers fermented with top-fermenting (TF) *Saccharomyces cerevisiae* strains. These strains produce intense flavors and they are in the focus of many craft- and microbreweries. These include Bavarian wheat beer, ales, and Belgian specialty beers. The wide biodiversity and availability of different strains of *Saccharomyces cerevisiae* offer brewers enormous possibilities to create beers with unique attributes and flavor profiles. Therefore, descriptions of these TF specialty yeast strains are of great importance in selecting suitable strains for developing special products. Figure 5.3 provides a description of the most widely used Bavarian wheat beer strain TUM 68.

TUM 68 is a phenolic off-flavor (POF)–positive Bavarian wheat beer strain. Depending on the production process, Bavarian wheat beers can exhibit very strong fruity, clove-like, estery flavors or a more neutral, yeasty, top-fermented character with a decent fruity note or they can fall somewhere in between the two. In addition to the process parameters, the strain and how it is handled play a prominent role in determining the aroma of the finished beer. Schneiderbanger recently described the impact of the different wheat beer yeast strains on fermentation performance and their respective aroma profiles (Schneiderbanger, 2014). These authors found that the Bavarian wheat beer strain TUM 127 used to ferment the first batch does not ferment maltotriose, which results in a differing mouthfeel and aroma compared to wheat beer strains without this maltotriose gap (Schneiderbanger, Strauß, Hutzler, & Jacob, 2013). Describing both existing and new brewing yeast strains will aid in our understanding of their characteristics and will open doors to experimentation for innovative brewers around the world to create novel products for the beer market. The potential for increasing the biodiversity among brewing yeast strains is more or less infinite.

5.2 Microbiological, physiological, identification, and typing methods

5.2.1 Differences in top- and bottom-fermenting brewing yeast strains

Saccharomyces pastorianus ssp. *carlsbergensis* strains are BF lager yeast strains, whereas *Saccharomyces cerevisiae* brewing yeast strains are TF. Both differ significantly from one another with regard to numerous characteristics. The former strains are able to ferment at lower temperatures, able to flocculate well during primary fermentation, and are harvested from the bottom of a fermentation tank, for example, a cylindroconical tank (CCT). The latter strains cease to function or they are only able to ferment very slowly at low temperatures (6–10 °C). They ferment most readily at temperatures between 15 °C and 25 °C, depending on the strain and type of wort. Cell growth is more vigorous among TF brewing yeast strains, and the cells do not flocculate out as rapidly compared to BF yeast strains. A large portion of the yeast population remains suspended in the liquid phase for a long time and is even buoyed to the top of the fermentation vessel by bubbles of CO_2. If it is possible to crop the yeast from the top, a positive selection of the most vital yeast can be carried out. In this way, many Bavarian wheat beer brewers select their yeast over numerous generations and can continue to repitch it virtually a countless number of times. In ale and kölsch beer production, most yeast strains have been selected by means of bottom cropping from CCTs and have therefore lost their vigorous TF character to some extent. A further distinguishing feature of bottom- and TF yeast becomes apparent through microscopic analysis. Bottom-fermenting yeast strains occur as single cells and perform unilateral budding. Most TF yeast

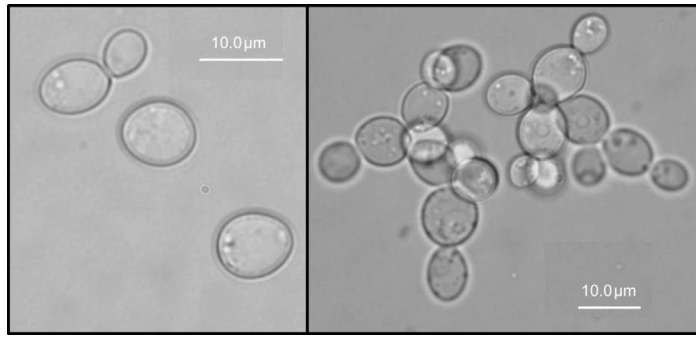


Figure 5.4 Microscopic picture of *Saccharomyces pastorianus* ssp. *carlsbergensis* TUM 34/70 and *Saccharomyces cerevisiae* TUM 127.

strains occur in groups consisting of more than two cells and perform multilateral budding. The BF lager strain *Saccharomyces pastorianus* ssp. *carlsbergensis* TUM 34/70 is depicted on the left in Figure 5.4, both as a single cell and as a cell having undergone unilateral budding. The Bavarian wheat beer strain *Saccharomyces cerevisiae* TUM 127 (right) forms two three-dimensional stellar patterns through multilateral budding.

Large cell formations or large star-like clusters are not formed by all *Saccharomyces cerevisiae* strains, however. Most *Saccharomyces cerevisiae* strains used to produce altbier and kölsch as well as certain ale strains form only very small cell clusters consisting of one to four cells. These are often very difficult to differentiate from *Saccharomyces pastorianus* lager strains, especially when they appear close together. Additional characteristics differentiating BF and TF yeast are as follows:

- Respiration rate (higher in TF)
- Metabolism rate (higher in TF)
- Optimum growth temperature (higher in TF)
- Bands of cytochrome spectrum (TF four bands, BF two bands)
- Optimum catalase pH (TF 6.2–6.4, BF 6.5–6.8)
- Complete use of melibiose and raffinose (BF positive)
- Yeast crop cell mass (higher in TF if cropped from above)
- Sulfite production (BF positive)
- Flocculation (stronger in BF)
- Fructose transport via active transporter (BF positive)
- Growth at 37 °C (TF positive)
- Ascospore formation on acetate agar according to a defined procedure (TF positive)
- Glucose effect, carbon catabolite repression (BF positive)
- Re-pitching cycles (higher in TF)
- Growth on pantothenate agar, a culture medium containing no pantothenic acid (BF positive)

Detailed descriptions of these differences and the related methods are described in several publications and overviews (Back, 1994; Dufour, Verstrepen, & Derdelinckx, 2003; Hutzler, 2009; Priest & Campell, 2003; Röcken & Marg, 1983).

5.2.2 Overview of identification methods

Over recent decades, the classical standard methods for microbiology and physiology have been modified and, in part, replaced by sophisticated molecular microbiological, physical–chemical methods, such as polymerase chain reaction (PCR)–based DNA techniques, or chemotaxonomic, spectrometric methods. Methods commonly used in the brewing industry for identifying and differentiating yeast species can be found in Table 5.2, including the degree of differentiation possible with each method.

Some promising new methods are not provided in this table; however, they are discussed in Section 5.2.4 below. The variety of identification and differentiation methods is extensive; only a few are implemented in the routine analysis conducted in commercial laboratories, and even fewer in the microbiology laboratories of breweries. A list of some of the established and reliable routine methods for rapid yeast identification is found in Section 5.2.3. Which method is applied in brewery laboratories or combined with the established routine methods depends on the practicability and the degree of acceptance for new techniques, which require special training and knowledge of yeast handling.

5.2.3 Selection of successful standard and recently introduced methods

For the detection of contamination by wild yeast, culture media are still the state-of-the-art in brewery laboratories. Many, however, do not even test for wild yeast, only for beer spoilage bacteria. More than one medium is necessary to detect a broad range of these yeasts. Differentiation of strains in the *Saccharomyces sensu stricto* complex and top- and BF brewing yeasts using culture media is particularly challenging. In the differentiation of *Saccharomyces cerevisiae* (TF) and *Saccharomyces pastorianus* ssp. *carlsbergensis* (BF) strains, media such as WLN agar, X-α-GAL, YM agar or wort agar at 37 °C, melibiose/bromocresol purple agar, or pantothenate agar may be used.

WLN agar is based on a color reaction of bromocresol green. *S. cerevisiae* (TF) strains are not able to reduce bromocresol green and therefore form dark green colonies. Lager strains (BF) as well as *Saccharomyces* and non-*Saccharomyces* wild yeasts reduce bromocresol green, forming pale green, bluish, or white colonies (Jespersen & Jakobsen, 1996). For this reason, WLN agar is very useful in analyzing *S. cerevisiae* (TF) starter cultures for contamination with lager yeast and varieties of wild yeast. The method is described in Analytica-Microbiologica EBC 3.3.2.2 (Analytica-EBC, 2014b).

The X-α-GAL medium exploits the fact that BF yeast strains secrete α-galactosidase (melibiase) and TF strains do not. Whereas TF brewing yeast colonies remain white, BF brewing yeast colonies turn blue-green. A description of this method is given in the ASBC Methods of Analysis, Microbiology, Yeast-10 A (ASBC, 2014). BF colonies grow on melibiose/bromocresol purple agar. This agar, described by Back, turns yellow when BF colonies are cultured on it, whereas TF colonies exhibit no growth or develop only micro-colonies (Back, 1994). However, a study has shown that the

Table 5.2 Overview of methods for yeast identification and differentiation that can be applied for yeast species from a brewing environment

Methods	Degree of differentiation	Reference source
Physiological, morphological methods		
Standard methods	Genus, species	
Miniature commercial systems (e.g., API 20C AUX, rapid IDyeast plus)	Genus, species	
Chemotaxonomic methods		
Total fatty acids analysis (FAME = determination of fatty acid methyl ester compounds)	Species	Timke, Wang-Lieu, Altendorf, and Lipski (2008)
Protein fingerprinting (e.g., 2D protein map)	Species, strain	Abdel-Aty (1991), Kobi, Zugmeyer, Potier, and Jaquet-Gutfreund (2004)
Mass spectrometry methods (e.g., MALDI-TOF MS, Py-MS, DIMS, GC-TOF MS)	Species, strain	Blattel, Petri, Rabenstein, Kuever, and Konig (2013), Pope et al. (2007), Schuhegger, Skala, Maier, and Busch (2008), Timmins et al. (1998), Usbeck et al. (2013), Usbeck, Wilde, Bertrand, Behr, and Vogel (2014)
Fourier transform infrared spectroscopy (FT-IR)	Species, strain	Buchl, Wenning, Seiler, Mietke-Hofmann, and Scherer (2008), Buchl et al. (2010), Timmins et al. (1998), Wenning et al. (2002)
Immunological methods		
Technique based on monoclonal antibodies (e.g., enzyme-linked immunosorbent assay)	Species, strain	Abdel-Aty (1991), Kuniyuki, Rous, and Sanderson (1984)

Continued

Table 5.2 Continued

Methods	Degree of differentiation	Reference source
Molecular genetics methods		
Sequencing	Species	Arias, Burns, Friedrich, Goodrich, and Parish (2002), Hutzler (2009), Laitila et al. (2006), Timke et al. (2008), Van Der Aa Kuhle, Jesperen, Glover, Diawara, and Jakobsen (2001)
Karyotyping	Species	Antunovics, Irinyi, and Sipiczki (2005), Demuyter, Lollier, Legras, and Le Jeune (2004), Esteve-Zarzoso, Peris-Toran, Garcia-Maiquez, Uruburu, and Querol (2001), Guerra et al. (2001), Naumov, Masneuf, Naumova, Aigle, and Dubourdieu (2000), Naumov, Naumova, Antunovics, and Sipiczki (2002), Naumov et al. (2001), Martinez, Gac, Lavin, and Ganga (2004), Rodriguez et al. (2004)
Restriction fragment length polymorphism (RFLP) mt DNA	Species	Beltran et al. (2002), Cappello, Bleve, Grieco, Dellaglio, and Zacheo (2004), Comi, Maifreni, Manzano, Lagazio, and Cocolin (2000), Esteve-Zarzoso, Fernandez-Espinar, and Querol (2004), Esteve-Zarzoso et al. (2001), Fernandez-Gonzalez, Espinosa, Ubeda, and Briones (2001), Granchi, Ganucci, Viti, Giovannetti, and Vincenzini (2003), Lopes, Van Broock, Querol, and Caballero (2002), Martinez et al. (2004), Pramateftaki, Lanaridis, and Typas (2000), Rodriguez et al. (2004), Torija, Rozes, Poblet, Guillamon, and Mas (2001), Torija et al. (2003)
Fluorescence/chemoluminescence in-situ hybridization (FisH/CisH)	Genus, species	Roder, Konig, and Frohlich (2007), Stender et al. (2001), Xufre, Albergaria, Inacio, Spencer-Martins, and Girio (2006)
Polymerase chain reaction (PCR)-based methods		
PCR (specific primers)	Species	Muir, Harrison, and Wheals (2011)
PCR-RFLP of the 5.8s ITS rDNA region	Species	Arias et al. (2002), Beltran et al. (2002), Esteve-Zarzoso et al. (2001), Ganga and Martinez (2004), Las Heras-Vazquez, Mingorance-Cazorla, Clemente-Jimenez, and Rodriguez-Vico (2003), Morrissey, Davenport, Querol, and Dobson (2004), Pramateftaki et al. (2000), Rodriguez et al. (2004), Torija et al. (2001), Van Der Aa Kuhle et al. (2001)

PCR-DGGE, PCR TGGE	Species, strain	Cocolin, Bisson, and Mills (2000), Prakitchaiwattana, Fleet, and Heard (2004)
PCR-DHPLC	Species, strain	Buchl et al. (2010), Hutzler (2009), Hutzler et al. (2010)
Real-time PCR	Species, subspecies	Bleve, Rizzotti, Dellaglio, and Torriani (2003), Brandl (2006), Brandl et al. (2005), Casey and Dobson (2004), Delaherche, Claisse, and Lonvaud-Funel (2004), Dörries (2006), Hutzler (2009), Phister and Mills (2003)
RAPD-PCR	Strain	Gomes et al. (2002), Guerra et al. (2001), Scherer (2002)
SAPD-PCR	Species, strain	Blattel et al. (2013)
Microsatellite PCR	Strain	Howell, Bartowsky, Fleet, and Henschke (2004), Scherer (2002)
AFLP-PCR	Strain	Schöneborn (2001)
δ-Sequence PCR	Strain	Ciani, Mannazzu, Marinangeli, Clementi, and Martini (2004), Cappello et al. (2004), Demuyter et al. (2004), Legras and Karst (2003), Lopes et al. (2002), Pramateftaki et al. (2000), Scherer (2002), Tristezza, Gerardi, Logrieco, and Grieco (2009)

discriminative power of the method, that is, the capability of differentiating real colonies and micro-colonies, is insufficient (Anonymous, 1994).

As stated above in Section 5.2.1, pantothenate agar does not contain pantothenic acid. Top-fermenting *Saccharomyces cerevisiae* strains do not grow on pantothenate agar, unlike BF strains, which flourish on it. Röcken and Marg found that some *S. cerevisiae* strains could also be cultivated on the pantothenate agar. This agar nevertheless provides a valuable tool for breweries to test for the presence of TF strain *S. cerevisiae*, which in contrast to a broad variety of wild yeasts, does not grow on this medium (Röcken & Marg, 1983). Before performing this test, the yeast sample must be thoroughly washed to avoid transferring of pantothenic acid to the pantothenate agar (Röcken & Marg, 1983; Back, 1994).

The 37 °C method has been approved by the ASBC and is found in its Methods of Analysis, Microbiology, Yeast-10 B (ASBC, 2014), and Analytica-Microbiologica EBC 4.2.5.2 (Analytica-EBC, 2014b). Hutzler tested the YM medium at an incubation temperature of 37 °C for differentiation of BF and TF brewing yeasts and for detection of wild yeast. In addition, he compared YM medium with three other media (YM + $CUSO_4$, CLEN, and XMACS) developed for the detection of a wide range of wild yeasts (Hutzler, 2009). The results are shown in Table 5.3.

The results in Table 5.3 provide proof of the differentiation potential of this agar for TF and BF brewing yeast strains. YM agar is also an adequate medium for detecting wild yeast cells in BF lager yeast at 37 °C, with the exception of some *Saccharomyces bayanus/pastorianus* strains that do not grow on YM agar at 37 °C. In Table 5.3, wort agar was used as a positive control.

YM + 195 ppm $CuSO_4$ can serve as a good culture medium for the detection of non-*Saccharomyces* and *Saccharomyces cerevisiae* wild yeast cells among BF and TF brewing yeast strains. It has also been successfully used in the form of YM + $CuSO_4$ broth for real-time PCR pre-enrichment of *S. cerevisiae* var. *diastaticus*, to directly detect these super-attenuating strains in beer samples containing brewing yeast (Brandl, Hutzler, & Geiger, 2005).

Use of CLEN is an improved method over simple lysine agar for the detection of non-*Saccharomyces* wild yeasts (Anonymous, 1997, 1998). Most non-*Saccharomyces* yeast species grow on this medium. The disadvantages inherent to this medium are that it necessitates washing the sample and that brewing yeasts are able to grow in micro-colonies on the medium, making it difficult to distinguish them from other yeasts.

The XMACS medium exhibits similar disadvantages. De Angelo and Siebert proposed the XMACS with five carbon sources for the detection of *Saccharomyces* and non-*Saccharomyces* wild yeast strains (De Angelo & Siebert, 1987). They also demonstrated that more *Saccharomyces* wild yeast strains were more readily detectable with this medium than with YM + $CuSO_4$ and YM agar at 37 °C (De Angelo & Siebert, 1987). This was also tested by Hutzler, whose results are shown in Table 5.3 (Hutzler, 2009). *Saccharomyces bayanus* wild yeasts were able to grow on XMACS medium, and a number of brewing strains grew to form larger micro-colonies with diameters of approximately 2 mm (strains TUM 34/78 and strain TUM 175 in Table 5.3). Therefore, the detection of wild yeasts among these brewing strains is difficult.

Table 5.3 Growth spectra of brewing and wild yeast strains on five different cultivation media

Yeast strain/media	Wort agar	YM+ 195 ppm $CuSO_4$	YM + 37 °C	CLEN	XMACS
***S. pastorianus* bottom-fermenting brewing strains**					
S. pastorianus TUM 34/70	+/1/<6	-/7/-	-/7/-	+/3/<1	+/3/<1
S. pastorianus TUM 34/78	+/1/<5	-/7/-	-/7/-	+/3/<1	+/2/<2
S. pastorianus TUM 44	+/1/<6	-/7/-	-/7/-	+/3/<1	+/3/<1
S. pastorianus TUM 66	+/1/<6	-/7/-	-/7/-	+/3/<1	+/2/<1
***S. cerevisiae* top-fermenting brewing strains**					
S. cerevisiae TUM 68	+/1/<6	-/7/-	+/2/<5	-/7/-	+/3/<1
S. cerevisiae TUM148	+/1/<6	-/7/-	+/2/<5	+/3/<1	+/2/<1
S. cerevisiae TUM 175	+/1/<11	-/7/-	+/2/<8	-/7/-	+/3/<2
S. cerevisiae TUM 184	+/1/<6	-/7/-	+/2/<5	+/3/<1	+/3/<1
***S. cerevisiae* wild yeasts**					
S. cerevisiae DSM 70451	+/1/<3	+/5/<2	+/4/<2	+/6/<1	-/7/-
S. c. var. *diastaticus* TUM K 3-D-2	+/1/<4	+/5/<2	+/1/<5	+/2/<1	+/2/<1
S. c. var. *diastaticus* TUM K 1-H-7	+/1/<4	+/1/<2	+/1/<2	+/2/<1	+/2/<3
S. c. var. *diastaticus* TUM K 1-B-8	+/1/<5	-/7/-	+/1/<3	+/2/<1	+/2/<3
***S. bayanus/pastorianus* wild yeasts**					
S. bayanus DSM 70411	+/1/<4	-/7/-	-/7/-	+/2/<1	+/2/<2
S. bayanus DSM 70412T	+/2/<9	-/7/-	+/1/<1	+/2/<1	+/2/<4
S. bayanus DSM 70508	+/1/<6	-/7/-	-/7/-	+/5/<1	-/7/-
S. bayanus DSM 70547	+/1/<6	-/7/-	-/7/-	+/2/<1	+/3/<4
S. bayanus TUM K 1-C-3	+/1/<5	-/7/-	-/7/-	+/2/<1	+/3/<3
S. pastorianus DSM 6580NT	+/3/<4	-/7/-	-/7/-	-/7/-	-/7/-

Continued

Table 5.3 Continued

Yeast strain/media	Wort agar	YM+ 195 ppm $CuSO_4$	YM + 37 °C	CLEN	XMACS
Non-*Saccharomyces* wild yeasts					
C. sake TUM K 1-B-3	+/1/<3	+/1/<3	+/3/<1	+/1/<2	+/2/<4
C. tropicalis TUM K 1-A-3	+/1/<11	+/1/<6	+/3/<1	+/1/<5	+/1/<7
D. bruxellensis CBS 2797	+/3/<5	+/4/<2	+/3/<1	+/5/<2	–/7/–
L. kluyveri CBS 3082T	+/1/<8	+/1/<6	+/1/<6	+/2/<1	+/2/<4
N. castellii TUM K 3-I-1	+/1/<2	+/1/<2	+/4/<2	+/2/<1	+/3/<1
P. membranifaciens CBS 107	+/1/<3	+/1/<3	+/3/<1	+/2/<2	+/2/<2
Sch. pombe CBS 356	+/1/<5	+/1/<2	+/3/<1	+/1/<4	+/1/<3
Z. bailii CBS 1097	+/1/<7	+/3/<2	+/3/<1	+/1/<3	+/2/<4
Growth [+/–]/incubation time until positive result [days]/colony diameter after 7 days [mm]					

In selecting the most suitable medium for wild yeast, it quickly becomes apparent that one must first know which brewing strains a brewery uses and the number of different media that a brewery laboratory is inclined to keep on hand. YM agar at 37 °C is often used for analysis procedures involving BF yeast strains, whereas YM + 195 ppm $CuSO_4$ is typically used for TF yeast strains. WLN agar, X-α-GAL medium, pantothenate agar, and XMACS agar can be effective in conjunction with additional media, depending on which microbes are to be targeted. YM agar + bromophenol blue + coumaric acid at a pH of 6.0 offer an effective means for detecting some *Saccharomyces* and non-*Saccharomyces* wild yeasts present in brewing yeast, and can be used to detect phenolic off-flavor–positive yeast strains (Hutzler, 2009). They can be distinguished based on the different colors produced by the colonies.

The real-time PCR is well established internationally as a method in brewing microbiology, primarily in relation to the detection of beer spoilage bacteria (Hutzler, Schuster, & Stettner, 2008). Large brewing companies, the central laboratories of brewing groups, and commercial service laboratories use a real-time PCRs for the detection and identification of beer spoilage bacteria and, to some extent, also for wild yeast as well as brewing yeast. It is very common among breweries and laboratories of this size to use commercially available PCR kits; however, in small-to-mid-sized breweries, real-time PCR is rarely used. It provides a rapid and reliable means for identifying and differentiating *Saccharomyces* and non-*Saccharomyces* brewing species. Real-time PCR can be used to identify single colonies of an unknown yeast strain at the species level, and can also serve as a tool for finding trace contaminations in mixed populations at concentrations of one contaminating cell in 1000 culture yeast cells (e.g., one cell of *Saccharomyces cerevisiae* in 1000 cells of *Saccharomyces pastorianus* ssp. *carlsbergensis*)(Hutzler, 2009; Hutzler, Schoenenberg, Koetke, Geiger, & Rainieri, 2008). Identifying the correct species to which a brewing yeast strain belongs can rapidly be carried out. Schönling et al., Brandl, Hutzler, and Dörries have published information pertaining to PCR systems for the detection of wild and beer spoilage yeast (Brandl, 2006; Brandl et al., 2005; Dörries, 2006; Hutzler, 2009; Schönling, Koetke, Wenning, & Hutzler, 2009). Table 5.4 lists the primer sets and target specificities of real-time PCR systems for the detection and identification of the *Saccharomyces sensu stricto* and non-*Saccharomyces* species that are used in brewing.

The real-time PCR probes belonging to the systems and primers listed in Table 5.4 can be found in Table 5.5.

All real-time PCR systems in Tables 5.4 and 5.5 are compatible, operate using the same temperature protocol (95 °C 10 min; 40 × 95 °C 10 s, 60 °C 55 s; 20 °C ∞), and detect the products in the FAM channel, which is available in most real-time PCR cyclers. The composition of the PCR mix as well as the DNA sequences and the application of the internal positive/amplification control (IPC or IAC) in the HEX channel are described by Brandl and Hutzler (Brandl, 2006; Hutzler, 2009). Qualitative results for certain strains of the *Saccharomyces sensu stricto* complex analyzed with the real-time PCR systems in Tables 5.4 and 5.5 are shown in Table 5.6.

Saccharomyces cariocanus, paradoxus, kudriavzevii, and *mikatae* can be identified using the specific real-time PCR systems presented in Tables 5.4 and 5.5. No specific real-time PCR system has been developed for *S. arboricolus*. Up to now,

Table 5.4 Primer sequences of real-time polymerase chain reaction (PCR) systems to differentiate *Saccharomyces sensu stricto* and other brewing culture species

Target-specificity	Primer	Probe	System name	Primer sequence (5′→3′)	Reference
D. bruxellensis	Db-f Db-r	Y58	Dbr	TGCAGACACGTGGATAAGCAAG CACATTAAGTATCGCAATTCGCTG	Brandl (2006)
S. bayanus, *S. pastorianus*	Sbp-f Sbp-r1 Sbp-r2	Y58	Sbp	CTTGCTATTCCAAACAGTGAGACT TTGTTACCTCTGGGCGTCGAGTTTGT-TACCTCTGGGCTCG	Josepa, Guillamon, and Cano (2000), Brandl (2006)
S. cariocanus	Sca-f Sca-r	Scar	Sca	TTAGACTTACGTTTGCTCCTCTCATG TGCAAATGACAAATGGATGGTTAT	Hutzler (2009)
S. cerevisiae, *S. pastorianus* ssp. *carlsbergensis*, *S. paradoxus*, *S. cariocanus*	Sc-f Sc-r	Scer	Sce	CAAACGGTGAGAGATTTCTGTGC GATAAAATTGTTTGTGTTTGTTACCTCTG	Josepa et al. (2000), Brandl (2006)
S. cerevisiae *S. pastorianus* ssp. *carlsbergensis*	Sc-GRC-f Sc-GRC-r	Sc-GRC	Sc-GRC3	CACATCACTACGAGATGCATATGCA GCCAGTATTTTGAATGTTCTCAGTTG	Hutzler (2009)
S. cerevisiae	TF-f TF-r	TF-MGB	TF-COXII	TTCGTTGTAACAGCTGCTGATGT ACCAGGAGTAGCATCAACTTTAATACC	Hutzler (2009)
S. cerevisiae	SCF1 SCR1	SCTM	SC	GGACTCTGGACATGCAAGAT ATACCCTTCTTAACACCTGGC	Salinas, Garrido, Ganga, Veliz, and Martinez (2009)
S. cerevisiae var. *diastaticus*	Sd-f Sd-r	Sdia	Sdi	TTCCAACTGCACTAGTTCCTAGAGG GAGCTGAATGGAGTTGAAGATGG	Scherer (2002), Brandl (2006)
S. kudriavzevii	Sk-f Sk-r	Skud	Sku	TCCTTACCTTATTCATCATATTCTCCAC CGATATTTGGTAAGGGGAGGTAGA	Hutzler (2009)

S. mikatae	Sm-f Sm-r	Smik	Smi	ACAACCGCCTCCCCAATT AAATGACAAGTAGTGGGTTGGAAGT	Hutzler (2009)
S. paradoxus	Sp-f Sp-r	Spar	Spa	CATACTATCAATACTGCCGCCAAA GGCGGATGTGGGTGGTAA	Hutzler (2009)
S. pastorianus, S. *bayanus* (partially) Main target: Bottom-fermenting culture yeast	BF300E BF300M	BF	BF300	CTCCTTGGCTTGTCGAA GGTTGTTGCTGAAGTTGAGA	Brandl (2006)
S. pastorianus, S. *bayanus* (partially) Main target: Bottom-fermenting culture yeast	UG-LRE-f UG-LRE-r	UG-LRE	UG-LRE1	ACTCGACATTCAACTACAAGAGTA-AAATTT TCTCCGGCATATCCTTCATCA	Hutzler (2009)
Saccharomycodes ludwigii	Sl-f Sl-r	Y58	Slu	GACGAGCAATTGTTCAAGGGTC ACTTATCGCAATTCGCTACGTTC	Brandl (2006)
T. delbrueckii	Td-f Td-r	Y58	Tde	AGATACGTCTTGTGCGTGCTTC GCATTTCGCTGCGTTCTT	Hutzler (2009)

Table 5.5 **Probe sequences of real-time PCR systems to differentiate *Saccharomyces sensu stricto* and other brewing culture species**

Probe name	Reporter	Quencher	Sequence 5´→3	Reference
TF-MGB	FAM	BHQ1	ATGATTTTGCTATCCCAAGTT	Hutzler (2009)
SC	FAM	BHQ1	CCCTTCAGAGCGTTTTCTCTAAATTGATAC	Salinas et al. (2009)
Sbp	FAM	BHQ1	ACTTTTGCAACTTTTTCTTTGGGTTTCGAGCA	Brandl (2006)
Scar	FAM	BHQ1	TCACCAAAACTGCACCATACGTACAAAATACC	Hutzler (2009)
Scer	FAM	BHQ1	ACACTGTGGAATTTTCATATCTTTGCAACTT	Brandl (2006)
Sc-GRC	FAM	BHQ1	TCCAGCCCATAGTCTGAACCACACCTTATCT	Hutzler (2009)
Sdia	FAM	BHQ1	CCTCCTCTAGCAACATCACTTCCTCCG	Brandl (2006)
Skud	FAM	BHQ1	TGCTATTACTTTTGCTTTTTCACTCACCACACCCT	Hutzler (2009)
Smik	FAM	BHQ1	AACATCCATCATCTATGTGCTCTAAATCCT-CACTTTATCA	Hutzler (2009)
Spar	FAM	BHQ1	CTGCACCATACGTACAAAATCTCCCTCCTTC	Hutzler (2009)
BF	FAM	BHQ1	TGCTCCACATTTGATCAGCGCCA	Brandl (2006)
BF-LRE	FAM	BHQ1	ATCTCTACCGTTTTCGGTCACCGGC	Hutzler (2009)
Y58	FAM	BHQ1	AACGGATCTCTTGGTTCTCGCATCGAT	Brandl (2006)

Table 5.6 Comparison of qualitative results of different strains analyzed with Real-Time PCR systems for differentiation of *Saccharomyces sensu stricto* species with focus on the differentiation of brewing species

Species	Strain examples	PCR system							
		Sc-GRC3	Sce	SC	TF-COX II	Sbp	BF-LRE1	BF-300	Sdia
S. bayanus	DSM 70412T, 70547, TUM K 1-C-3 (type B)	–	–	–	–	+	–	–	–
	DSM 70411, 70508 (type A)	–	–	–	–	+	+	+	–
S. bayanus/ pastorianus	CBS 2440, 6017	–	–	–	–	+	+	+	–
S. eubayanus	CBS 12537	–	–	–	–	+	+	+	–
S. pastorianus	CBS 1503, 1513 DSM 6580NT, 6581	–	–	–	–	+	+	+	–
S. pastorianus (bottom-fermenting brewing yeast)	TUM 34/70, 34/78, 69, 128, 168,194 (Flocculating yeasts)	+	+	–	–	+	+	+	–
	TUM 71, 144 (non-flocculating yeasts)								
	CBS 1484, 5832, CBS 6903, NBRC 2003, TUM B-I-4, B-J-4, B-J-5								
	TUM 120 (flocculating yeast)	+	+	–	–	+/–	+	+	–
	TUM 66/70, 204 (non-flocculating yeasts), CBS 5832, CBS 6903								
S. cerevisiae	DSM 70424, 70449T, 70451, CBS 1464, 8803, TUM K 3-A-1, 3-C-3	+	+	+	+	–	–	–	–
S. cerevisiae (Top-fermenting brewing yeast)	TUM 68, 127, 149, 175, 205, TUM K 5-A-8 (wheat beer yeasts)	+	+	+	+	–	–	–	–
	TUM 148, 184, 208 (Alt beer yeasts)	+	+	+	+	–	–	–	–
	TUM 165, 177 (Koelsch beer yeasts)	+	+	+	+	–	–	–	–
	TUM 210, 211, 213 (ale yeasts)	+	+	+	+	–	–	–	–

Continued

Table 5.6 Continued

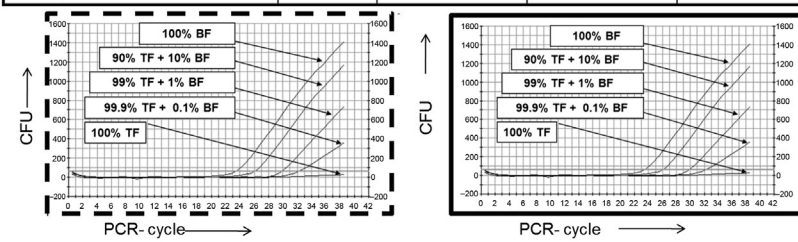
Species	Strain examples	PCR system							
		Sc-GRC3	Sce	SC	TF-COX II	Sbp	BF-LRE1	BF-300	Sdia
S. cerevisiae (top-fermenting yeast from other fermentation industries)	TUM V Bingen, V Bordeaux, V Eperney, V Wädensvil, V Laureiro (wine yeast)	+	+	+	+	–	–	–	–
	TUM D4 (distillery yeast)	+	+	+	+	–	–	–	–
	TUM S2 (sparkling wine yeast)	+	+	+	+	–	–	–	–
S. cerevisiae var. *diastaticus*	CBS 1782, DSM 70487, TUM 1-B-8, 1-H-7, 2-A-7, K 2-F-1, 3-D-2, 3-H-2	+	+	+	+	–	–	–	+
S. cariocanus	CBS 7995, 8841	–	+	–	–	–	–	–	–
	CBS 5313	+	+	+	+	–	–	–	–
S. kudriavzevii	CBS 8840	–	–	–	–	–	–	–	–
S. mikatae	CBS 8839	–	–	–	–	–	–	–	–
S. paradoxus	CBS 406, 432, 2908, 5829, 7400, 8436	–	+	–	–	–	–	–	–

Culture collection abbreviations.TUM, Technische Universität München, Forschungszentrum Weihenstephan BLQ, Freising, Germany; CBS, Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; DSMZ, Deutsche Stammsammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany; NBRC, Biological Resource Center, Tokyo, Japan.
Source: Hutzler (2009).

S. arboricolus has not been observed in a brewing environment. The only strain that produced a negative result with this specific system was *S. cariocanus* CBS 5313, but it produced a positive result with all *Saccharomyces cerevisiae*-specific systems and was subsequently sequenced on both IGS rDNA regions as *S. cerevisiae* (data not shown). *S. cerevisiae* can be distinguished from other species with TF-COXII and SC real-time PCR systems. *Saccharomyces pastorianus* ssp. *carlsbergensis* strains (BF, lager type) can be discerned using two different real-time PCR systems. The hybrid character of lager strains can be differentiated by using, for example, a combination of Sc-GRC3 and BF-LRE1 or Sce and BF-300. *S. bayanus/eubayanus/pastorianus* strains produce negative results with Sc-GRC3 or Sce systems and therefore can be differentiated (in single colonies). *S. bayanus* consists of two types (varying BF-300 and BF-LRE1 results). *S. cerevisiae* brewing strains cannot be distinguished from *S. cerevisiae* wild yeast strains using these methods. Contaminations by all relevant *Saccharomyces sensu stricto* species can be detected directly in beer or starter cultures containing *S. cerevisiae* brewing yeast through use of the systems listed in Tables 5.4–5.6 An analysis scheme for contaminations in mixed populations is shown in Figure 5.5.

Contamination by all relevant *Saccharomyces sensu stricto* species can be detected directly in *Saccharomyces pastorianus* ssp. *carlsbergensis* brewing yeast containing beer or starter cultures with the exception of *Saccharomyces bayanus/eubayanus/pastorianus* contaminations. For this specific problem, an additional quantitative approach, which is shown in Figure 5.6, can be implemented.

	Real-time PCR assay			
	Sc GRC3	**BF-300**	**TF-COXII**	**Sbp**
***S. cerevisiae* (TF)**	+	-	+	-
***S. cerevisiae* (wild yeast)**	+	-	+	-
***S. pastorianus* (BF)**	+	+	-	+
***S. pastorianus* (wild yeast)**	-	+	-	+
S. bayanus type A	-	+	-	+
S. bayanus type B	-	-	-	+



TF= top fermenting brewing yeast, BF= bottom fermenting brewing yeast

Figure 5.5 Real-time polymerase chain reaction detection scheme and results for contaminations of *S. cerevisiae* brewing yeast in *S. pastorianus* ssp. *carlsbergensis* brewing yeast and vice versa. Hutzler (2009).

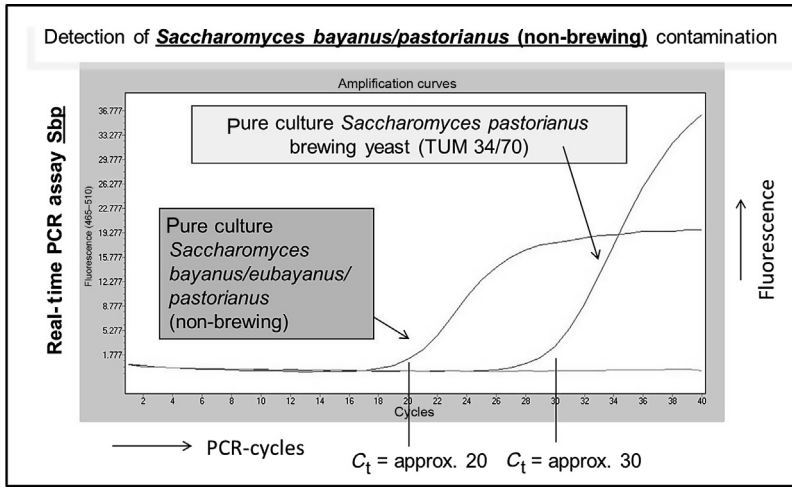


Figure 5.6 Real-time polymerase chain reaction basis for the detection of *S. bayanus/eubayanus/pastorianus* contaminations in *S. pastorianus* ssp. *carlsbergensis* brewing yeast using the C_t shift of the Sbp system.
Hutzler, Mueller-Auffermann, and Jacob (2012).

The PCR signal of the Sbp system for *Saccharomyces pastorianus* ssp. *carlsbergensis* brewing yeast is weak, and therefore the C_t value (cycle number with positive fluorescence signal) is high (~30), as can be seen in Figure 5.6. This means that only a small number of target DNA copies of this gene were in the sample. A DNA isolate consisting of approximately 50 million cells/mL from a sample of fermented beer was analyzed. Pure cultures of *Saccharomyces bayanus, eubayanus,* or *pastorianus* produce C_t values of approximately 20 using the Sbp system. This indicates that a large number of target DNA copies of this gene were present in the sample. Hence, the C_t value of an *S. pastorianus* ssp. *carlsbergensis* brewing yeast sample contaminated with *S. bayanus/eubayanus/pastorianus* should be between 20 and 30. The Sbp C_t value is strain dependent, and is ≥30 for the strain *S. pastorianus* ssp. *carlsbergensis* TUM 34/70, given that DNA is extracted from cell concentrations of ≤100 million cells/mL. To obtain a reliable result for contamination, the C_t shift should be at least 3 to 4 C_t values. For example, if yeast TUM 34/70 is to become contaminated, the Sbp C_t value should be ≤26 to 27. At the moment, this is the only rapid method that can directly detect contamination by *S. bayanus/eubayanus/pastorianus* in *S. pastorianus* ssp. *carlsbergensis* brewing yeast.

Identifiying the species of brewing yeast as well as the strain must be possible in order to maintain reproducible beer quality. Therefore, frequently monitoring strain identity and purity is of great consequence. Table 5.2 in Section 5.2.2 lists a number of methods applicable for differentiating yeasts on the strain level. Karyotyping and other PCR typing or PCR fingerprint techniques are standard for brewing strains, and karyotyping can be regarded as the reference method or gold standard. For the most part, *Saccharomyces cerevisiae* strains are more heterogeneous than lager strains, and one suitable typing method is sufficient to differentiate between two strains of this

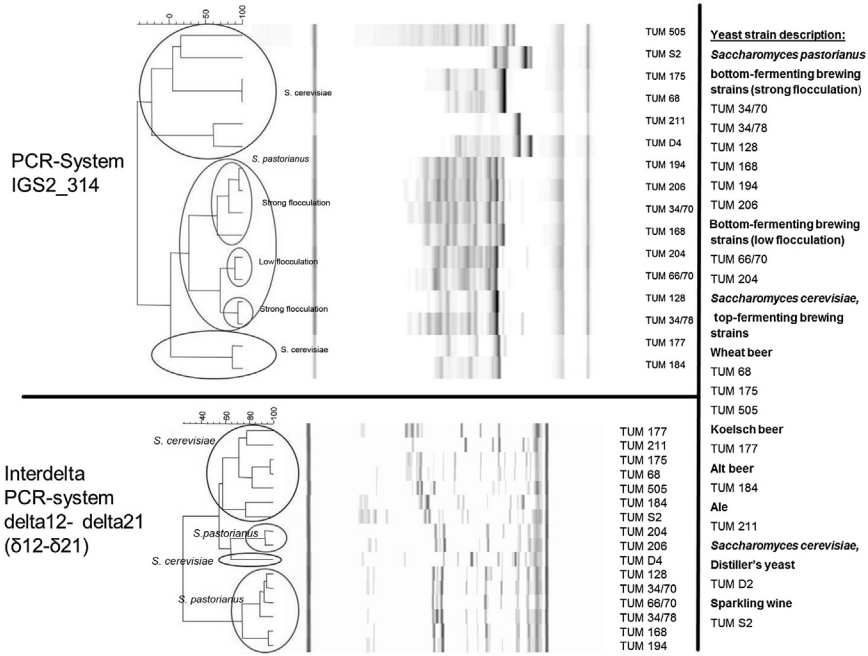


Figure 5.7 Differentiation on the strain level of *S. pastorianus* ssp. *carlsbergensis* and *S. cerevisiae* brewing yeasts and *S. cerevisiae* strains from other beverage fermentations using polymerase chain reaction systems IGS2_314 and Interdelta δ12-δ21. Hutzler et al. (2014).

species. If they are very closely related, two typing methods have to be combined (e.g., when one strain is the ancestor of the other strain and was used for a long period of time under different conditions). *S. pastorianus* ssp. *carlsbergensis* lager strains are very homogenous genetically, and a set of lager strains can generally be distinguished using merely two or more typing techniques (Van Zandycke, Bertrand, & Powell, 2007). Figure 5.7 shows the two PCR typing methods IGS2_314 and Interdelta PCR system delta12-delta21 (δ12-δ21), both of which are combined with capillary electrophoresis (lab on a chip, Bioanalyzer, Agilent, Santa Clara, CA, USA) and subsequent data analysis with Bionumerics (Applied Maths, Ghent, Belgium). The IGS2_314 system was originally developed for adjacent gel electrophoresis and was subsequently combined with DHPLC (Buchl, Hutzler, Mietke-Hofmann, Wenning, & Scherer, 2010; Hutzler, 2009; Hutzler & Goldenberg, 2007; Hutzler, Geiger, & Jacob, 2010). The system can differentiate most lager strains. The PCR system δ12-δ21 was developed by Legras and Karst, and then combined with capillary electrophoresis and subsequent data analysis with Bionumerics by Hutzler et al. (Hutzler, Stretz, Schneiderbanger, Mueller-Auffermann, & Riedl, 2014; Legras & Karst, 2003).

Figure 5.7 shows the homogeneity of *Saccharomyces pastorianus* brewing strains and the heterogeneity of *Saccharomyces cerevisiae* brewing strains, as well as the discriminatory power of combining the two methods. Further combinations of typing methods are also possible and can be evaluated by using Bionumerics, for example.

Aside from rapid identification and differentiation of brewing yeast strains and common wild yeast species, a reliable and easy-to-handle identification technique for "unknown" yeast species from a brewing environment would also be advantageous. The reference method entails sequencing the 26S rDNA D1/D2 domain (Kurtzman & Robnett, 1998). Other useful regions for species identification are, for example, the ITS1-ITS4 rDNA and the IGS2 rDNA (Fernandez-Espinar, Martorell, De Llanos, & Querol, 2006; Hutzler, 2009).

5.2.4 Promising new methods

Yeast databases for both brewing yeast and wild yeast were established for the methods matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and Fourier transform infrared spectroscopy (Hutzler & Wenning, 2009; Timmins, Quain, & Goodacre, 1998; Usbeck, Kern, Vogel, & Behr, 2012, 2013; Wenning, Seiler, & Scherer, 2002). Strains stored in databases can easily be rechecked with the relevant methods, and databases can be rapidly expanded to allow mapping of culture collections or microhabitats. Identification on the basis of large database analyses is inexpensive compared to the use of molecular biological methods. Multilocus sequencing and whole-genome/next-generation sequencing will be of immense importance in describing lineages of brewing yeasts and for exploring the potential of as-yet unproven brewing yeast strains from other industries or from the environment for applications in brewing. Metagenomics, microbiome analysis, and droplet PCR can play important roles in the clarification of mixed species populations, such as those found in spontaneously fermented beer.

5.3 Brewing yeast cell count/viability/vitality methods

5.3.1 Cell-counting methods

Yeast cell concentration is an important parameter in beer fermentation technology. It is usually expressed in millions of cells per milliliter and should be able to be determined rapidly to ensure that, for example, pitching yeast volumes or harvest yeast dilutions can be calculated and adjusted expeditiously. The most common method used in brewery laboratories for determining yeast concentration is to count the cells under a microscope in a counting chamber, for example, with a Thoma cell-counting chamber. Figure 5.8 shows *Saccharomyces pastorianus* ssp. *carlsbergensis* TUM 34/70 cells on the grid of a Thoma cell-counting chamber square, which consists of 16 small squares.

There are 16 large grid squares in a Thoma cell-counting chamber, and each one of these is divided into 16 smaller squares, for a total of 256 small squares. The Thoma cell-counting chamber is designed to hold a defined volume so that the cell count can be converted to a cell concentration using a formula provided

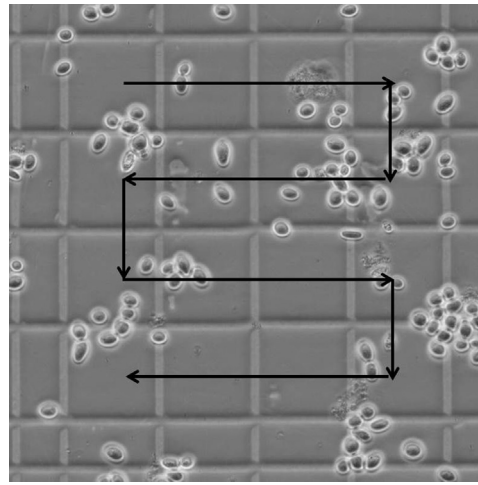


Figure 5.8 *Saccharomyces* pastorianus ssp. carlsbergensis TUM 34/70 in a Thoma cell-counting chamber, which is counted following the arrows over 16 small grid squares.

with each specific type of chamber. A Thoma cell-counting chamber suitable for counting yeast cells is 0.1 mm deep with an area of 0.00,025 mm^2. Concentrations are calculated with the following formula:

$$\text{Yeast cell number/mL} = (\text{average yeast cell number per single grid square}) \times 4 \times 10^6$$

Manual cell-counting methods are described in MEBAK III 10.4.3.1/10.11.4.4 (Pfenniger, 1996), Analytica-Microbiologica EBC 3.1.1.1 (Analytica-EBC, 2014b) and in ASBC Methods of Analysis, Microbiology, Yeast-4 (ASBC, 2014). Currently, there are automated cell chambers (e.g., Cellometer®, Nexcelom, Lawrence, KS, USA) available that are very practical for rapidly counting a large number of yeast samples. Manual counting in such cases can be very time consuming. Automated cell chambers can also be combined with fluorescence staining to simultaneously measure viability (see 6.3.2). Other non–microscope-based automated yeast counting systems such as Coulter counter® (Beckman Coulter, Brea, CA, USA) or the flow cell-based system Nucleocounter® (IUL Instruments, Königswinter, Germany) are also used in some breweries. The system Nucleocounter® can also be used to measure viability. An exemplary electronic automated yeast counting technique is described in MEBAK III 10.11.4.5 (Pfenniger, 1996) and in Analytica-Microbiologica EBC 3.1.1.2 (Analytica-EBC, 2014b). Photometric determination of the yeast cell concentration is of less practical relevance and is outlined in MEBAK III 10.4.3.2 (Pfenniger, 1996) and in Analytica-Microbiologica EBC 3.1.1.3 (Analytica-EBC, 2014b). The correct yeast cell concentration is of great importance for reproducibility in fermentation processes. Automated methods should be optimally calibrated before introducing them into a brewery laboratory. They should also be thoroughly compared with existing manual methods. Top-fermenting *Saccharomyces*

cerevisiae brewing strains (e.g., Bavarian wheat beer strains TUM 68 and 127), which form larger cell clusters, can cause cell concentrations to vary in automated systems. In such cases, the introduction of a correction factor can be necessary. Other microbiological methods (e.g., pour plate technique, surface spread-plate technique) can also be used to determine the number of colony-forming units (CFU) per milliliter. These methods are described in MEBAK III 10.11.4.1/10.11.4.2 (Pfenniger, 1996), Analytica-Microbiologica EBC 2.3.3.1 (Analytica-EBC, 2014b), and ASBC Methods of Analysis, Microbiology, Microbiological Control-2 (ASBC, 2014). The microbiological method for determining the CFU per defined volume is far too slow for the breweries to measure yeast concentration, because results are needed quickly so that adjustments to brewing process parameters can be made. However, for many microbiological analyses, the CFU per defined volume is highly practical. Inline systems for the analysis of the yeast cell concentrations are described in Section 5.4.2.

5.3.2 Viability methods

Yeast viability describes the percentage of living cells in a yeast population. For a population of brewing yeast, this value should be at least >95% living cells. One of the objectives of yeast management and yeast handling in a brewery should be to achieve a yeast viability of 99–100%. Viability is the chief analysis criterion, which must be determined in order to rapidly evaluate the condition and quality of the yeast. The standard method for measuring yeast viability is methylene blue or methylene violet staining, described in MEBAK III 10.4.4.1/10.11.3.3 (Pfenniger, 1996), Analytica-Microbiologica EBC 3.2.1.1 (Analytica-EBC, 2014b) and in ASBC Methods of Analysis, Microbiology, Yeast-3 A (ASBC, 2014). Additionally, various fluorophores are used for staining living or dead cells to facilitate counting them under a fluorescence microscope. A standard stain for living cells is fluorescein diacetate (FDA). Dead cells can be stained with propidium iodide (PI) and 1,8-ANS (1-anilinonaphthalene-8-sulfonic acid). The viability method in Analytica-Microbiologica EBC 3.2.1.1 describes dead yeast cell staining with 1,8-ANS. The substance 4′,6-diamidino-2-phenylindole (DAPI) is able to cross the cell membrane and thus can be used for staining both living and dead cells. This technique can be applied as counterstaining against PI. Figure 5.9 shows a microscopic image of *Saccharomyces pastorianus* ssp. *carlsbergensis* TUM 34/70 cells stained with DAPI (blue, living cells) and PI (red to purple, dead cells).

Another combination that is routinely used is FDA (green, living cells) and PI (red, dead cells). Other fluorescent dyes that can be used for this purpose include DiBAC4(3), berberine, acridine orange, primuline, Sytox Orange, cFDA, trypan blue, or Hoechst stains. A study by Van Zandycke et al. found that fluorophore staining was perceived to be less subjective by individuals conducting the analysis than bright field dye staining because of the lack of intermediate color variations (Van Zandycke, Simal, Gualdoni, & Smart, 2003). If a fluorescence-based viability method has been established in a brewing microbiology laboratory, then a comparison with the standard method methylene blue staining is recommended. Back described the difference in viability using FDA/PI fluorescence staining and methylene blue staining

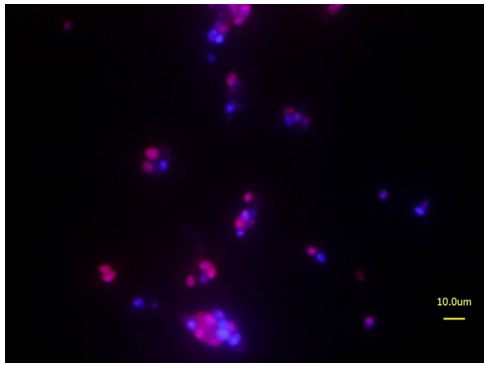


Figure 5.9 Microscopic picture of *Saccharomyces pastorianus* ssp. *carlsbergensis* TUM 34/70 cells stained with DAPI (blue, living cells) and propidium iodide (red to purple, dead cells).

(Back, 1994). Automated yeast counting systems (outlined in Section 5.3.1), which offer a fluorescence measurement option, also find applications in automated yeast viability measurements. Flow cytometry can be used to measure viability as well. A standard fluorescent stain for this application is PI.

5.3.3 Vitality methods

Yeast vitality expresses the activity of the yeast metabolism or in simple words the "fitness" of a yeast population. Vital brewing yeast should exhibit an excellent growth rate and a strong fermentation performance. Different characteristics or parameters depend on or influence the vitality of the yeast. Table 5.7 presents various methods to quantify yeast vitality, which are based on the measurement of metabolic activity, cellular components, or fermentation capacity.

Only a few of the methods in Table 5.7 are actually used in brewing laboratories. Most breweries do not measure yeast vitality at all. A few use time-consuming small fermentation trials yielding delayed results, or have in-house methods to evaluate the fermentation capacity of brewing yeast. It is exceedingly rare for service laboratories and for breweries around the world to use the intracellular pH (ICP) method. Weigert et al. modified the ICP method to be based on a flow cytometry platform and developed a rapid technique to determine yeast vitality. Flow cytometry can also be used to determine the life cycle of yeast cells and to measure specific cell components, which provide an indication of the yeast vitality. Various flow cytometry protocols and applications for these methods with brewing yeast have been described by Boyd et al., Hutter, Kobayashi et al., Novak et al. and Schönenberg (Hutter, 2001; Boyd et al., 2003; Kobayashi, Shimizu, & Shioya, 2007; Novak, Basarova, Teixeira, & Vicente, 2007; Schönenberg, 2011). Methods that have little practical relevance are not discussed further in this chapter but can be reviewed in the publication by Heggart et al. (Heggart et al., 2000). Recently, Mueller-Auffermann developed two methods based on measurement of the CO_2 volume produced by a defined yeast cell concentration within a specified time period (Mueller-Auffermann, 2014a; Mueller-Auffermann, Schneiderbanger, Hutzler, & Jacob, 2011). One method is

Table 5.7 Classification and evaluation of yeast vitality methods according to Heggart et al., Thiele, and Mueller-Auffermann

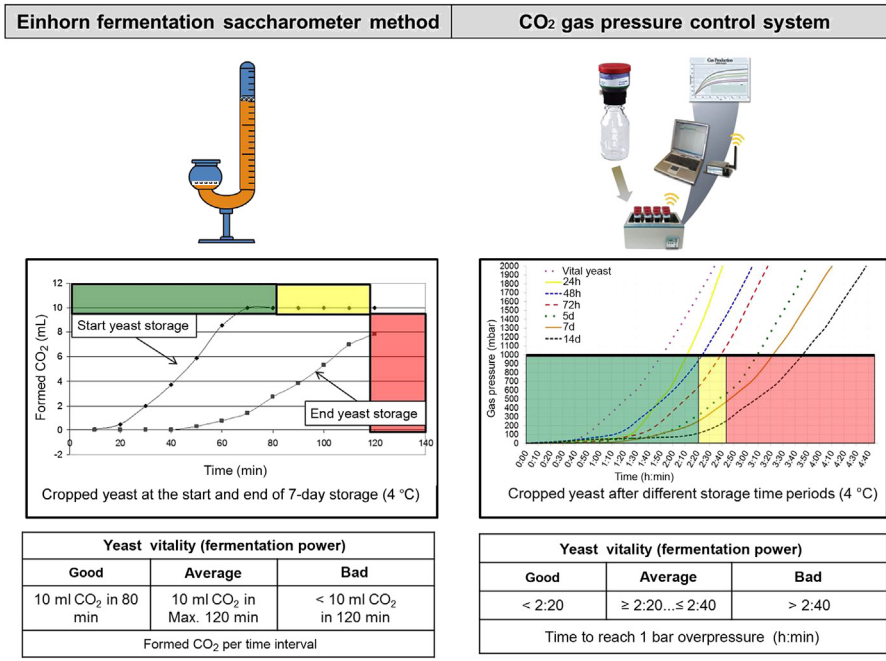
Method based on	Examples	Direct	Practical relevance
Metabolic activity	Vitality staining	-----	----
	Microcalorimetry	-----	----
	Reduction of vicinal diketones (VDK)	----	----
	Protease activity of yeast	----	----
	Magnesium ion release test (MRT)	----	----
	Specific oxygen uptake	----	----
	Acidification power test	----	----
	Intracellular pH value (ICP)	----	**X**
Measurement of cellular components	Adenosin triphosphate (ATP)	----	----
	Adenylate energy charge (AEC)	----	----
	NADH (fluorometry)	----	----
	Glycogen and trehalose	----	----
	Sterols und unsaturated fatty acids	----	----
Fermentation capacity or glycolytic flow rate	Glycolytic flow rate	**X**	----
	CO_2 measurement	**X**	**X**
	Rapid fermentation trials	**X**	**X**

Source: Mueller-Auffermann et al. (2011), Heggart et al. (2000), Thiele (2006).

based on the volumetric measurement of CO_2 in an Einhorn saccharometer, whereas the other system is a CO_2 gas pressure control system. Both methods are shown in Figure 5.10.

Both methods can easily be transferred to brewery laboratories because the equipment is small and compact and can be placed in incubators. For the Einhorn fermentation saccharometer measurement, the protocol was optimized and is subject to the following conditions: maltose substrate consisting of a 10% ([m/v]) solution; centrifugation of the yeast sample at 750 g for 5 min; adjustment of the yeast cell concentration to 200 million cells/mL with water; mixing according to a ratio of 6 mL yeast suspension (200 million cells/mL) + 14 mL maltose solution; mixing in Einhorn saccharometer; equilibration for 60 min in an incubator at 28 °C; after 120 min of fermentation, readings are taken for the produced CO_2 volume every 10 minutes. Conditions of the CO_2 gas pressure control system have been described by Mueller-Auffermann (Mueller-Auffermann, 2014a).

Empirically obtained, sound values for yeast vitality using the two methods are given in Figure 5.10. ICP, which serves as a kind of reference method, provides the following range of values in assessments of yeast quality: good vitality ≥5.8; average vitality 5.8–5.4; poor vitality ≤5.4.



Yeast vitality (fermentation power)		
Good	Average	Bad
10 ml CO_2 in 80 min	10 ml CO_2 in Max. 120 min	< 10 ml CO_2 in 120 min
Formed CO_2 per time interval		

Yeast vitality (fermentation power)		
Good	Average	Bad
< 2:20	≥ 2:20...≤ 2:40	> 2:40
Time to reach 1 bar overpressure (h:min)		

Figure 5.10 Yeast vitality measurement using Einhorn fermentation saccharometer and the CO_2 gas pressure control system.
Mueller-Auffermann (2014a), Mueller-Auffermann et al. (2011).

5.4 Monitoring yeast and fermentation

5.4.1 Wort and yeast specifications

A rapid start by the yeast and a "nicely progressing" fermentation necessitate that the wort composition and the condition of the yeast meet certain requirements. Wort and yeast specifications important for favorable propagation and good fermentation performance are presented in Table 5.8.

High-gravity worts with extract concentrations of ≥15° Plato are not generally recommended for yeast propagation processes, due to the osmotic stress on the yeast. High-gravity worts blended with de-aerated water to extract levels of approximately 11–12° Plato must be carefully checked according to the wort specifications in Table 5.8. Here, the parameters FAN and zinc are the most essential. Under normal conditions, the wort specifications in Table 5.8 guarantee that enough nitrogen and carbon sources for growth and fermentation are available for the yeast. Table 5.8 also contains microbiological specifications for wort and yeast. Brewing yeast should be free of other microorganisms that can negatively influence propagation and fermentation processes negatively. In propagation systems, aerobic or facultative anaerobic microorganisms such as Gram-negative acetic acid bacteria or aerobic and facultative anaerobic wild yeasts may cause problems. During the initial phase of fermentation, until the brewing yeast has reduced the pH to below 4.8 and the ethanol concentration rises above 1.5–2%,

Table 5.8 Yeast and wort specifications for sufficient yeast growth and fermentation performance

Wort specifications			
Total nitrogen	900–1100		mg/L
FAN free amino nitrogen cast wort	200–250		mg/L
pH value (depending on additional acidification)	5.0–5.6		
Zinc	0.1–0.3		mg/L
Attenuation limit of wort (apparent)	≥78		%
Photometric iodine method (MEBAK)	<0.45		
Wort-associated microbial background flora (wort agar)	≤1		per mL
Beer spoilage bacteria (NBB)	Negative		In 1 mL
Yeast specifications			
Target cell concentration in propagation end sample	80–100		Million cells/mL
Target cell concentration in fermenter full sample	≥10 (BF)	≥3–10 (TF)	Million cells/mL
Viability (methylene blue)	≥95		%
Vitality (Einhorn saccharometer, 80 min runtime)	≥10		mL
Vitality (CO_2 pressure system, 1 bar over pressure)	≤2:20		h:min
pH of yeast supernatant	≤5.8		
Bacterial flora in fermentation systems (WLD)	≤1		per mL
Beer spoilage bacteria (NBB)	Negative		In 1 mL
Wort-associated bacterial background flora (Wort agar + actidione)	≤1		per mL
Acetic acid bacteria	Negative		In 0.1 mL
Wild yeast ($CuSO_4$ broth)	Negative		In 0.01 mL
Wild yeast (37 °C method)	Negative		In 0.01 mL
Microscopic pre-check	No spoilage organisms, No or few non-yeast particles		

BF, bottom fermenting; TF, top fermenting.

the pitched wort is exceedingly susceptible to microbial spoilage. Background flora associated with wort, mostly Gram-negative bacteria such as *Enterobacter* sp., *Rahnella* sp., *Citrobacter* sp., *Klebsiella* sp., *Pantoea* sp., and *Serratia* sp., are particularly apt to cause spoilage and generate off-flavors (Hutzler, Mueller-Auffermann, Koob, Riedl, & Jacob, 2013). Therefore, yeast should be monitored using wort agar and Wallerstein differential agar (WLD agar) and wort on wort agar. A test for *Enterobacteriaceae* may also be performed on VRBD agar. Wild yeasts may also compromise the quality of the fermenting wort by competing with brewing yeasts in the initial stage of fermentation. For this reason, wort should be tested for wild yeast growth using wort agar or any other universal nutrient medium for yeast. Brewing yeast should be monitored for the presence of wild yeast, for example, with the 37 °C test (for BF) or YM + 200 ppm $CuSO_4$ (for BF and TF). There are also other media for the detection of wild yeasts. Furthermore, wort and yeast should also be checked for beer spoilage microorganisms. The total colony count is a good comparative value that enables CFU/mL comparisons with other foods, beverages, water, or intermediate products. Methods for determining and assigning values to yeast viability and vitality are described in Sections 5.3.2 and 5.3.3. If the pH of the supernatant of a centrifuged yeast suspension is above 5.8, the yeast population most likely contains residues of autolyzed yeast cells, which increase the alkalinity of the suspension. Preliminary examination of the yeast with a microscope provides quick information on the amount of nonyeast particulates present. Especially bottom-cropped brewing yeast that has been re-pitched many times may contain higher percentages of nonyeast particulates, for example, oxalate.

5.4.2 Monitoring of fermentation and maturation parameters and their application with yeast

Measurement of the fermentation and maturation parameters allows the fermentation performance, as well as any problems or any other developments that may arise, to be monitored and rectified if necessary. Table 5.9 shows a list of analysis methods for monitoring the principal fermentation parameters and also for selected fermentation by-products approved by the ASBC, MEBAK, and EBC.

The principal fermentation parameters are useful for creating fermentation diagrams and therefore for comparing different fermentation processes with each other. The same is true for propagation and maturation processes. The process can be tailored to fit specific parameters, according to an individual brewery's needs. Hence, a fruity beer character can be augmented by increasing ester production, or the flavor stability can be improved by enhancing SO_2 production. In Figure 5.11, the values for various fermentation by-products from two different beers are discussed with regard to the errors in the brewing process that may have produced them.

The following errors in the brewing process listed in Table 5.11 can result in extreme values for the parameters measured: high fermentation/maturation temperature, insufficient aeration, long maturation time (yeast excretion), very strong yeast growth/propagation, FAN deficiency, low yeast cell concentration, and short maturation.

In-process or in-line measurements throughout the entire brewing process, and for yeast and fermentation parameters in particular, are growing in significance and have

Table 5.9 Approved analysis methods for fermentation main parameters and selected fermentation by-products of the analysis commissions of ASBC, MEBAK, and EBC

	Methods		
Fermentation main parameters	**ASBC**	**MEBAK**	**EBC**
Extract	Beer 2, 3, 5	2.9	8.3, 9.43
Ethanol	Beer 4	2.9	9.2-9.4
CO_2	Beer 13	2.26	9.28
Final attenuation of beer	Beer 16	2.8	9.7
pH	Beer 9	2.13	9.35
Selected fermentation by-products	**ASBC**	**MEBAK**	**EBC**
Volatile fermentation by-products (headspace)	Beer 48	2.21.1	-
Fermentation by-products (other methods)	-	2.21.2	-
Fatty acids	-	2.21.4	-
Aromatic alcohols and phenolic acids	-	2.21.3	-
Vicinale diketones	Beer 25	2.21.5	9.24
Acetoin	-	2.21.5.4	-
Higher alcohols and esters	-	2.21.6	-
Organic acids	-	2.21.7	9.32, 9.34
SO_2	Beer 21	2.21.8	9.25
Glycerol	-	-	9.33

Source: ASBC (2014), Analytica-EBC (2014a), Jacob (2012).

already become well established in some areas. Boulton published, in a review article, a list of in-line analyses for all stages of the brewing process. In this article, he described the types of sensors necessary for monitoring the corresponding processes, including all those involving yeast (Boulton, 2012). Boulton also discussed the equipment required for determining the concentration or cell mass of the yeast, turbidity measurements (Optek-Danulat, Germany), and capacitance measurement/dielectric spectroscopy (Aber Instruments, UK/Hamilton Messtechnik, Germany/Fogale Nanotech, France). Capacitance measurement/dielectric spectroscopy can also be used to measure the amount of viable yeast cells. Many breweries, especially small- and mid-sized breweries, adjust their yeast cell concentrations, pitching rates, and quantities of yeast cropped by means of volumetric measurements, sight glass inspection, and manual process control without knowing the exact yeast concentration and the percentage of viable cells in the pitching yeast population. Essentially there is a room for improvement in the in-line yeast process control at most breweries. Even in breweries where in-line measurement and control systems for yeast are already in place, optimal adjustment of the finer points of these processes according to

		Beer 1	Beer 2	
Diacetyl		0.05	0.13 **E**	mg/L
Pentandione-2,3	7	0.02	0.08 **E**	mg/L
Acetoin		2.8 **A**	7.9 **A**	mg/L
Ethyl acetate		12.4 **D**	25.1 **F**	mg/L
Isobutyl acetate		0.01 **D**	0.05 **F**	mg/L
Isoamyl acetate		0.6 **D**	1.8	mg/L
2-Phenylethanol		31.5 **A**	15.5	mg/L
Decanoic acid		1.9 **D**	1.0	mg/L
Ethyl decanoate		0.11 **D**	0.05	mg/L
Ethyl hexanoate (ethyl caproate)		0.16 **B**	0.23 **B**	mg/L

Possible fermentation/process errors causing out-of-range values:
A High fermentation/ maturation temperature
B Insufficient aeration
C Long maturation time (yeast excretion)
D Strong yeast growth/propagation
E Deficient FAN, low yeast concentration, short maturation
F Low yeast growth/propagation

Figure 5.11 Interpretation of selected fermentation by-products of two beers in terms of process errors.
Jacob (2014).

the parameters of each individual process is necessary and may require some time for sufficient optimization. Selected publications by Tibayrenc et al., Sandhar, Austin et al., Mas et al., and Krause et al., discuss the principles, applications, and scientific approaches pertaining to the topic of in-line yeast measurement (Kiviharju, Salonen, Moilanen, & Eerikainen, 2008; Krause, Birle, Hussein, & Becker, 2011; Mas, Ossard, & Ghommidh, 2001; Sandhar, 2014; Tibayrenc, Preziosi-Belloy, & Ghommidh, 2011). Aside from yeast concentration, ethanol, CO_2, and specific gravity/extract are considered to be principal fermentation parameters and thus are of great importance. The relevant methods for in-line measurement of these parameters were also included by Boulton review (Boulton, 2012). Boulton and Nordkvist, and Biering and Bockisch cited different methods for in-tank measurement of fermentation parameters that are used to observe certain conditions present in fermentation tanks (e.g., layer effects, homogeneous distribution, etc.) (Biering & Bockisch, 2014; Boulten & Nordkvist, 2014). Grassi et al. demonstrated that FT-NIR spectroscopy, when combined with locally weighted regression, is a perfectly suitable quantitative method for measuring pH, biomass, and °Brix (extract), and can be readily implemented in the beer production process (Grassi, Amigo, Lyndgaard, Foschino, & Casiraghi, 2014a,b). This method offers a potential in-line measurement tool for recording the values of principal fermentation parameters.

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Part Two

Spoilage bacteria and other contaminants

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Toxigenic fungi and mycotoxins in the barley-to-beer chain

6

A. Laitila
VTT Technical Research Centre of Finland, Espoo, Finland

6.1 Introduction

Contamination by toxigenic fungi of cereals used as raw materials in brewing is a great concern. Harmful fungal metabolites can cause failures during both malting and the brewing process. Furthermore, toxic metabolites may have severe adverse effects on human and animal health. Mycotoxins are toxic, low-molecular-weight natural compounds produced as secondary metabolites by various different filamentous fungi (Bennett & Klich, 2003). Mycotoxins are considered as climate-dependent plant-, storage- and process-associated problems (Paterson & Lima, 2010). The growth of toxigenic fungi and subsequent toxin production vary considerably from year to year and place to place, depending especially on climatic conditions.

Figure 6.1 shows the transmission of toxigenic fungi and their metabolites in the barley-to-beer chain. Mycotoxins can enter human and animal food chains through direct or indirect contamination (Edite Bezerra da Rocha, da Chagas Oliveira, Erlan Feitosa Maia, Florindo Guedes, & Rondina, 2014). Products can be directly contaminated with toxigenic fungi with the concomitant toxin production. More often mycotoxins enter the food and feed chain indirectly. Raw materials used in the barley-to-beer chain can be contaminated with toxigenic fungi, and even though the fungi have been eliminated in the process, the mycotoxins survive and remain in the final product. Mycotoxin present in grain dust can also be transmitted via air. Mycotoxins can be transmitted to by-products used as animal feed. Barley rootlets and brewers' spent grains are important by-products used as animal feed. When contaminated grains or by-products are fed to livestock, mycotoxins can be transferred into milk, eggs and meat and then back to human consumption.

Health hazards associated with mycotoxins in humans are rarely seen in western countries, but mycotoxins are recognized as a serious food safety issue, especially in developing countries, due to a combination of subsistence farming, poor postharvest handling and storage and unregulated local markets (Chakraborty & Newton, 2011). The public health risks related to mycotoxins in beer are generally regarded as low for moderate consumers. This is mainly because malting and brewing raw materials are carefully selected and inspected for quality prior their use. However, clear risk has been identified for by-products from malting and brewing processes as well as for rejected barley batches used as animal feed. In addition to mycotoxin production, the activity of barley-associated toxigenic fungi may lead to serious quality problems

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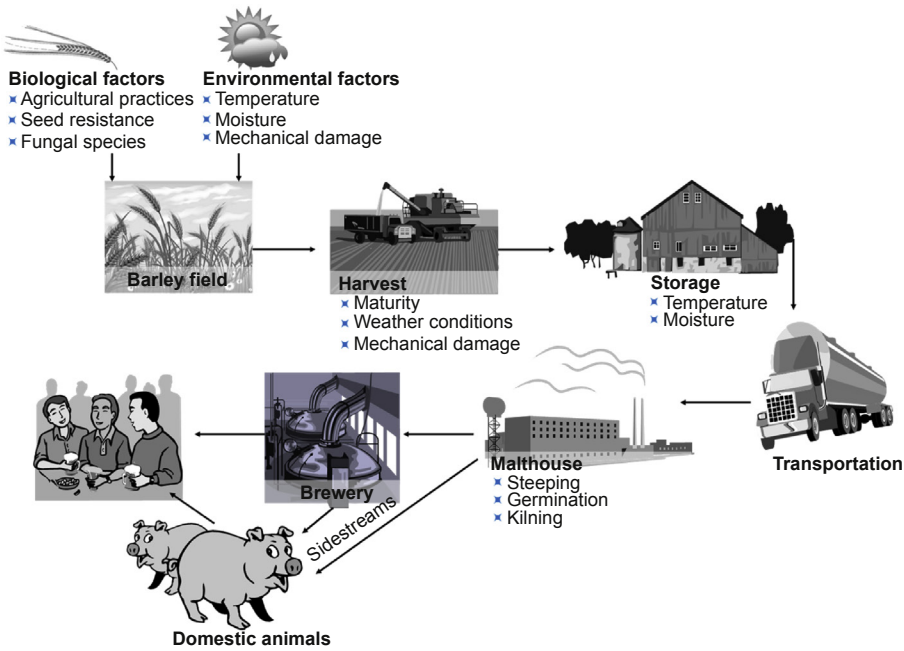

Figure 6.1 Transport of toxigenic fungi and their metabolites in the barley-to-beer chain.

(discussed in Section 6.4). Occurrence of mycotoxins in the food and feed chain is expected to increase due to climate change (Miraglia et al., 2009). Thus, toxigenic fungi in cereal processing will be a great concern in the future.

This chapter begins by discussing the evolution and impacts of fungi in the barley-to-beer chain. It then gives an overview of the current knowledge on toxigenic fungi and mycotoxins. Regulation and emerging mycotoxin issues, such as modified mycotoxins, will also be discussed. Finally, this chapter gives a review on preventive actions.

6.2 Barley malt: a key raw material in brewing

Barley (*Hordeum vulgare* L.) is one of the most important cereals worldwide. Barley can be cultivated in highly diverse geographical regions from subarctic to subtropical. In 2012, global barley production was over 132 million tonnes (http://faostat.fao.org/site/567/default.aspx). The largest value-added use for barley is the production of malt (Schwarz & Li, 2011). Malted barley provides the basis of most beers in the world. Approximately 13% of the barley produced worldwide is processed into malt. The rest is used as animal feed or for other human consumption.

Barley malt is produced by germinating grains under controlled moisture and temperature conditions. Currently, the global malting capacity is around 23 million tonnes, with 42% located in EU countries (www.euromalt.be). Malting is a natural, biological process involving a wide range of biochemical and physiological reactions. Malting

traditionally involves three stages: steeping, germination and kilning. The main goal is to produce various enzymes capable of degrading the grain macromolecules into soluble compounds. The outward appearance of the final malt resembles that of the unmalted barley, but the physical, biochemical and microbiological composition is changed.

In addition to germinating grain, the malting process includes another metabolically active component: a diverse microbial community that naturally colonizes the barley grains (Laitila, 2007; Raulio, Wilhelmson, Salkinoja-Salonen, & Laitila, 2009). The indigenous microbial community of barley harbours a wide range of microbes, including numerous species of bacteria, yeasts and filamentous fungi (moulds). Malting can be considered as a complex ecosystem involving two metabolically active groups: the barley grains and the diverse microbial community. The grain ecosystem is greatly influenced by the whole history experienced by the grain during the growth period, harvesting and storage. Furthermore, the behaviour of both barley and microbes during the malting process is influenced by multiple interactive factors, such as moisture, temperature, gaseous atmosphere and time.

In the brewery, malt is milled and mashed with water. In the mashing stage, malt enzymes break down the grain components into fermentable sugars and other yeast nutrients. The liquid fraction (wort) is then separated from the grain insoluble parts (spent grains). The spent grains are most often used as animal feed. After boiling with hops, cooling and aeration wort is ready for beer fermentation. In addition to barley malt, many other cereals, such as maize, millet, oats, rice, rye, sorghum and wheat, can be applied to bring additional sources of carbohydrates and proteins into wort (Goode & Arent, 2006).

6.3 Evolution of fungi in the barley–malt ecosystem

The microbial community characteristics of barley products develop in the field, under storage and during processing. Many intrinsic and extrinsic factors, including plant variety, climate, soil type, agricultural practices, storage and transport, influence the diversity and structure of the microbial community present in the barley grains (Flannigan, 2003; Noots, Delcour, & Michiels, 1999; Petters, Flannigan, & Austin, 1988). Of these, climate plays a particularly important role. Therefore, barleys cultivated in different geographic locations have different microbial communities. The composition of the microbial community on barley grains changes dramatically as a result of postharvest operations. Some of the grain-associated microbes and their metabolites are removed during the processing of grains, whereas every process step in the barley–malt–beer chain can be a source of additional populations.

Fungi that contaminate barley grains may come from air, dust, soil, water, insects, rodents, birds, animals, humans, storage and transport containers and handling and processing equipment. More than 150 fungal species can be found on grains as surface contaminants or as internal invaders (Sauer, Meronuck, & Christensen, 1992). The number of identified species is expected to increase due to the new detection methods based on the genetic diversity. It has been estimated that only 7% of the world's fungi have been so far described (Hawksworth, 2004).

The filamentous fungi have traditionally been divided into two rather distinct ecological groups: field fungi and storage fungi. Field fungi invade the kernels while the plant is growing or during the harvest. Among the most common and widespread field fungi are species of *Alternaria*, *Cladosporium*, *Epicoccum*, *Fusarium* and *Cochliobolus*, *Drechslera* and *Pyrenophora*. The latter three are formerly known as the *Helminthosporium* group (Ackermann, 1998; Flannigan, 2003; Noots et al., 1999). These fungi require relatively high water availability for growth (a_w >0.85, moisture content >18%). Thus, their growth is restricted during storage by appropriate drying of barley. However, dormant spores can survive in normal storage conditions for years.

After harvest, barley grains are stored for about 2 months to 1 year to allow the breakup of the normal dormancy before malting. Microbes are not usually active and their number generally decreases during storage under appropriate conditions. Microbial growth and spoilage of stored barley are determined especially by water activity and temperature (Frisvad, Andersen, & Samson, 2007). Xerophilic *Aspergillus*, *Eurotium* and *Penicillium* are the most characteristic fungi found in the storage environment (Samson, Hoekstra, Frisvad, & Filtenborg, 2000). Storage fungi are able to grow on kernels of moisture content as low as 13–15% (a_w ~0.70). Storage fungi are habitually present in the dust and air of the storage environment and can also be found in different farm and malting equipment such as harvesters and elevators (Sauer, Meronuck, & Christensen, 1992). Thus, it is almost impossible to avoid contamination of cereals by these fungi. The best way of controlling fungal activity in stored barley is to ensure that conditions do not allow their growth. It is well known that temperature and moisture content together determine the length of safe storage.

It must be noted that this differentiation into field and storage fungi is applicable only in temperate climates, since in warmer regions some species normally considered as storage fungi may be found already in the developing barley (Medina et al., 2006; Noots et al., 1999). *Aspergillus* species capable of producing mycotoxins have been detected in malting barley in the Mediterranean countries where the temperatures during ripening can be high (Medina et al., 2006).

The microbial ecology of barley changes again during malting. Before entering the malting process, barley is cleaned and graded in order to remove foreign material, dust and small and broken kernels. Cleaning procedures also diminish the microbial load. However, malting conditions are favourable for microbial growth in terms of available nutrients, temperature, moisture content and gaseous atmosphere. Steeping of barley leads to leakage of nutrients into steeping water and rapidly activates the dormant microbes present in barley grains (Laitila, 2007). Although some of the microbes and soluble nutrients are washed away along with steep water draining, the viable microbial numbers increase markedly during the steeping period (Flannigan, 2003; O'Sullivan, Walsh, O'Mahony, Fitzgerald, and van Sinderen et al., 1999). Steeping is generally regarded as the most critical step in malting with respect to microbiological safety (Laitila, 2007; Noots et al., 1999).

Microbial activity remains high throughout the germination period. Furthermore, microbial growth is accelerated during the first hours of kilning (Wilhelmson et al., 2003). The kilning regime has been identified as a significant factor in controlling microbial communities. Although high temperatures effectively restrict the growth and activity of microbes, kilning appears to have little effect on the viable counts of

bacteria and fungi. The viable counts of microbes are generally higher in the finished malt than in the native barley (Noots et al., 1999). The barley bed dries progressively from the bottom to the top of the grain bed. Temperature and moisture gradients are formed in the grain bed. The conditions that prevail during the first hours of kilning before the temperature breakthrough, especially in the top layers of the grain bed, are rather favourable for microbial growth and activity (Laitila et al., 2006; Wilhelmson et al., 2003).

The microbial community is also significantly influenced by the malthouse operations, and it has been shown that a specific microbial community develops in each malting plant (O'Sullivan et al., 1999). Great variation in fungal communities has been observed due to the differences in malting practices in different geographic locations (Ackermann, 1998; Flannigan, 2003). The microbial community of final malt reaching the brewery is naturally influenced by the handling and storage operations after the malting process as well as during the transport of malt. In addition to barley malt, adjuncts used in breweries are potential sources for fungi and their metabolites, thus they may contribute to the mycotoxin levels in the final product.

6.4 Impacts of barley-associated fungi on malt quality

It is evident that fungi as well as other microbes associated with barley actively interact with the grain and thus greatly influence barley, malt and beer quality and safety (Table 6.1). Deterioration or damage caused by intensive fungal proliferation during storage or processing include decrease of germination, postharvest dormancy, discoloration, off-flavors and off-odours, dry matter losses, changes in chemical and nutritional composition of grains, heating and caking of cereal lots during storage, production of gushing factors and formation of toxic metabolites (Laitila, 2007; Noots et al., 1999; Sarlin et al., 2005a, 2005b; Sauer et al., 1992; Schwarz, Beattie, & Casper, 1996; Schwarz, Casper, & Beattie, 1995; Wolf-Hall, 2007).

Fusarium fungi are considered as perhaps the most important group of filamentous fungi with respect to malt and beer quality. Many barley-associated Fusaria are plant

Table 6.1 Overview of reported negative impacts of filamentous fungi associated with barley and malt during storage and processing

Quality reduction	Process failures	Health hazards
Plant diseases	Spontaneous heating of grain batch in silos	Allergens
Qualitative and quantitative changes in grain carbohydrates, proteins, lipids	Reduced grain germination	Mycotoxins
Off-odours and off-flavours	Factors inducing premature yeast flocculation (PYF)	
Discolouration of kernels	Production of gushing inducers	

pathogens causing devastating infections and thus lead to quality and yield reduction. Small cereal grains, such as barley, are greatly influenced by the plant disease *Fusarium* head blight (FHB), also known as scab (McMullen, Jones, & Gallenberg, 1997; Parry, Jenkinson, & McLeod, 1995; Paulitz & Steffenson, 2011). FHB is a disease complex in which several *Fusarium* and *Microdochium* species are involved. *Fusarium graminearum* is the most common causal agent of FHB, especially in the temperate and warmer regions of the USA, China and the southern hemisphere. *Fusarium culmorum* is more frequently found in cooler regions such as the UK, Northern Europe and Canada. A number of other species are also reported with this disease complex, especially in Europe, including *Fusarium avenaceum, Fusarium poae*, *Fusarium sporotrichioides* and *M. nivale* (Bottalico & Perrone, 2002; Osborne & Stein, 2007).

Fusarium damaged barley cannot be processed in the malting plant. They produce a wide range of enzymes and a diverse array of secondary metabolites with a range of biological activities, including pigmentation, plant growth regulation and toxicity to other microbes, humans and animals (Brown & Proctor, 2013). The main problem associated with using *Fusarium*-infected barley malt in brewing is in the alteration of wort-soluble nitrogen compounds. This will have an impact on colour, flavour, texture and foaming properties of the beer (Sarlin et al., 2005a; Schwarz, Horsley, Steffenson, Salas, & Barr, 2006; Wolf-Hall, 2007). Yeast fermentation failures may also be due to fungal activity in barley malt. Premature yeast flocculation (PYF) has been associated with fungal activity in barley (Van Nierop, Rautenbach, Axcell, & Cantrell, 2006). PYF is a phenomenon in which the brewing yeast prematurely settles at the bottom of the fermentation tank leading to an incomplete fermentation and undesirable beer flavour (Blechova, Havlova, & Havel, 2005; Van Nierop et al., 2006). Heavy contamination of the barley crop by fusaria or other filamentous fungi may increase the gushing (beer overfoaming) propensity of beer (Sarlin et al., 2005b). Fungi may produce gushing factors during the cultivation period in the field or during the malting process. It has been shown that small secreted fungal proteins called hydrophobins act as gushing factors (Sarlin et al., 2005b).

Fungi present in barley and malt or in grain dust are also potent sources of allergens to the workers in malthouses and breweries. Diseases such as farmer's or maltworker's lung and brewer's asthma are results of allergic responses to high concentrations of inhaled spores (Flannigan, 1986; Heaney, McCrea, Buick, & MacMahon, 1997; Rylander, 1986). Mycotoxins can also be concentrated on grain dust (Norby et al., 2004).

Most plant-pathogenic or spoilage fungi can produce a wide range of toxic metabolites, mycotoxins, that are toxic to other microbes, plants, animals and humans. The most important fungal genera producing mycotoxins include *Aspergillus, Fusarium* and *Penicillium*.

6.5 *Aspergillus*, *Penicillium* and *Fusarium* mycotoxins

The problems associated with moulds and concomitant mycotoxin production are worldwide. The Food and Agricultural Organization (FAO, 2008) of the United Nations has estimated that 25% of the world's crops are contaminated by mycotoxins

each year. Mycotoxins are fungal metabolites that cause sickness or death in people and other animals when ingested, inhaled and/or absorbed (Paterson & Lima, 2010). Mycotoxins include a very large, heterogeneous group of substances, and toxigenic species can be found in all major taxonomic groups. Thousands of mycotoxins exist, but only a few present significant food safety challenges (Murphy, Hendrich, Landgren, & Bryant, 2006). The relevant mycotoxins related to foods and beverages are mainly produced by species in the genera *Aspergillus*, *Penicillium* and *Fusarium* and include aflatoxins (AFs), ochratoxin A (OTA) and *Fusarium* toxins such as trichothecenes, zearalenone and fumonisins. When present in high levels, mycotoxins can have toxic effects ranging from acute (for example kidney or liver damage) to chronic symptoms (increased cancer risk and suppressed immune system).

Production of a particular mycotoxin is a species- or strain-specific property, and usually a toxigenic fungus can produce several toxins. Furthermore, several different toxins are often present in the contaminated raw materials, and they have poorly understood synergistic effects.

In addition to health hazards, several mycotoxins have phytotoxic impacts on host plants and may cause loss of viability and reduced quality of plant seed (Nishiuchi, 2013). Several mycotoxins have antimicrobial activity and thus may also influence the behaviour of other microbes present in the same surrounding. Mycotoxins may have adverse effects on animal health if they are transmitted to sidestreams used as animal feed. Consumption of contaminated batches at farms can lead to reduced livestock productivity and to serious illness or even death (Murphy et al., 2006). Some of the most common mycotoxins associated with foods and beverages are presented in Table 6.2.

6.5.1 Aflatoxins

AFs are the most important mycotoxins worldwide (Bennett & Klich, 2003; Murphy et al., 2006). They are typical toxins in tropical and subtropical regions. They are mainly produced by *Aspergillus* species, particularly *Aspergillus flavus* and *Aspergillus paraciticus*. AFs occur in several chemical forms and four compounds are commonly produced in foods: aflatoxins B_1, B_2, G_1 and G_2. Letters B and G refer to the blue or green fluorescence observed under ultraviolet light. Furthermore, aflatoxin M1, which is a metabolite of aflatoxin B1, can be found in milk and milk products. When cows consume aflatoxin-contaminated feed, they metabolize aflatoxin B_1 into a hydroxylated form called M_1. Aflatoxins are considered to be the most toxic natural compounds and are classified as proven human carcinogens (Edite Bezerra da Rocha et al., 2014). The International Agency for Research on Cancer (IARC) has classified aflatoxins as human carcinogens Class 1. They have been detected as natural contaminants in brewing materials and may pass from contaminated raw materials or adjuncts even into final beer (Mably et al., 2005; Scott, 1996). However, malting barley and malt are not the major source for these toxins if barley is properly dried and secondary contamination is restricted during storage (Benesova, Belakova, Mikulikova, & Svoboda, 2012).

Table 6.2 Some mycotoxins most commonly associated with particular fungi

Mycotoxin	Major producer fungi	Common food and beverage source
Aflatoxins B_1 (AFB_1), AFB_2 AFB_1, AFB_2 AFG_1, AFG_2	*Aspergillus flavus, Aspergillus paracitus*	Cereals, nuts, seeds, dried fruits, spices
Ochratoxin A (OTA)	*Aspergillus carbonarius, Penicillium verrucosum*	Dried fruits, cereals, grape juice, wine, coffee
A-type trichothecenes T-2 and HT-2, diacetoxyscirpenol (DAS)	*Fusarium acuminatum, Fusarium langsethiae, Fusarium poae, Fusarium sambucinum, Fusarium sporotrichoides*	Cereals and cereal products
B-type trichothecenes[a] deoxynivalenol (DON), nivalenol (NIV)	*Fusarium cerealis, Fusarium culmorum, Fusarium graminearum, Fusarium poae*	Cereals and cereal products
Zearalenone (ZEA)	*Fusarium crookwellense, Fusarium culmorum, Fusarium equiseti, Fusarium graminearum, Fusarium semitectum*	Cereals and cereal products, other food commodities
Fumonisin B_1 (FB_1)	*Fusarium verticillioides, Fusarium proliferatum, Fusarium nygamai*	Corn, corn meal, grits

[a]Over 200 compounds are included in the trichothecenes. In addition to *Fusarium* fungi, species belonging to the genera *Myrothecium*, *Phomopsis*, *Stachybotrys*, *Trichoderma* and *Trichothecium* can also produce trichothecenes.
Source: Bennett and Klich (2003), Frisvad, Andersen, and Samson (2007), Murphy et al. (2006), Paterson and Lima (2010).

6.5.2 Ochratoxin A

OTA can be found in a large variety of products since it is produced by several fungal strains of *Aspergillus* and *Penicillium*. OTA is a derivative of isocumarin linked via peptide bond with L-phenylalanine. It is considered to be nephrotoxic, teratogenic, immunotoxic and carcinogenic (Creppy, 1999). Based on the IARC classification, OTA is considered as a possible human carcinogen (Group 2B). Cereals are the major source of human OTA exposure. Cereals contribute 50–80% of the OTA intake among European consumers. OTA has been found in barley, oats, rye, wheat, coffee beans and other plant products, with barley having a particularly high likelihood of contamination (Anli & Mert Alkis, 2010; Bennett & Klich, 2003). OTA is considered as a postharvest problem and not produced in the field in Europe. Toxin production is often related to improper storage conditions. It is mainly produced by *Penicillium verrucosum* in cool and temperate zones and by *Aspergillus ochraceus* in warmer regions. OTA occurrence in malting barley has been associated with *P. verrucosum* (Mateo, Medina, Mateo, Mateo, & Jimenez, 2007). OTA producers require rather high water activity for growth. Rapid growth occurs at a_w 0.98–0.99 (≥27–30% moisture content) over

the temperature range 10–25 °C. Growth and toxin production is almost completely inhibited at about 0.80–0.83 (=17.5–18% m.c.) (Anli & Mert Alkis, 2010; Magan & Aldred, 2005). OTA can also be present in other adjuncts used in breweries.

6.5.3 *Fusarium* toxins

Production of mycotoxins is probably the most negative consequence associated with heavy contamination of barley and malt by *Fusarium* fungi.

Trichothecenes are tricyclic sesquiterpenes, and they can be classified into four major types (A-D) based on their chemical structure. More than 200 trichothecenes have been identified (Nishiuchi, 2013). Although a high number of molecules have been characterized, only a few of them have been characterized from barley. Types A and B are frequent contaminants in cereal grains and cereal-based products. Type A includes T-2 toxin, neosolaniol (NEO) and diacetoxyscirpenol (DAS). Type B includes fusarenon-x, nivalenol (NIV) and deoxynivalenol (DON).

Trichothecenes bind to eukaryotic ribosomes and inhibit protein synthesis (Pestka, Zhou, Moon, & Chung, 2004). Different trichothecenes interfere with initiation, elongation and termination stages of protein synthesis. They are also immunosuppressive. Acute trichothecene mycotoxicosis are rare, but when ingested in high doses by farm animals they cause nausea, vomiting and diarrhea. DON is also called a vomitoxin or food refusal factor (Bennett & Klich, 2003). Trichothecenes are not classifiable as to their carcinogenicity to humans (Class 3) (IARC).

Deoxynivalenol (DON) is the most important trichothecene worldwide and is often detected in small cereal grains such as barley, oats and wheat. Due to relatively good thermal stability DON can be transmitted from contaminated barley into the final product (Schwarz et al., 1995). DON is frequently detected in barley and in commercial beer (discussed in Section 6.6.2). The occurrence of DON is largely dependent on weather conditions in the particular location and year. DON is predominantly produced by *F. culmorum* and *F. graminearum* species.

The mycotoxin T-2 and its deacetylated form HT-2 toxins are Type A trichothecenes and perhaps the second most important *Fusarium* toxins. They are often treated as a pair when considering incidence and regulatory aspects as these closely related mycotoxins have equivalent toxicity. The major producers of T-2 and HT-2 are *F. sporotrichioides* and *F. langsethiae*. T-2 is a potent inhibitor of protein synthesis and it is considered to be significantly more toxic than DON. The LD50 value (mg/kg for mice) for DON is 70 and for T-2 it is only 5.2. The data available shows that T-2 and HT-2 have been an increasing problem, especially in oats (Edwards, 2009a; van der Fels-Klerx & Stratakou, 2010). Since 2004, the occurrence of T-2 and HT-2 producers and toxin incidences appear to also be increasing in barley (van der Fels-Klerx & Stratakou, 2010; Malchova et al., 2010; Strub, Pocaznoi, Lebrihi, Fournier, & Mathieu, 2010). Studies have indicated that T-2 and HT-2 production in malting barley is rather unpredictable (Euromalt 2013). Modelling based on the weather data can be used for prediction of DON. Currently, forecasting systems for T-2 and HT-2 production are not available. More knowledge is required for the understanding of the biological role and induction of the T-2 and HT-2 toxin synthesis.

Zearalenone (ZEA) is a nonsteroidal estrogenic mycotoxin produced by several *Fusarium* species, including *F. graminearum, F. equiseti, F. culmorum, F. tricinctum* and *F. crookwellense* (Zinedine, Soriano, Molto, & Manes, 2007). Chemically it is a phenolic resorcyclic acid lactone. The detrimental effects caused by consumption of zearalenone-contaminated grains include impaired reproduction and altered sexual development in farm animals (Muphy et al., 2006). ZEA often coexist with DON, as *F. graminearum* and *F. culmorum* may produce both compounds (Richard, 2007). Most often this toxin is found in maize but also in other important cereal crops, including barley. ZEA was evaluated by the IARC in 1993. Based on inadequate evidence in humans and limited evidence in experimental animals it was categorized in Group 3 (not classifiable as to its carcinogenicity to humans) together with trichothecenes.

Fumonisins are produced by a number of *Fusarium* species, notably *Fusarium verticillioides* (formerly known as *Fusarium moniliforme*), *Fusarium proliferatum, Fusarium nygamai* and also *Alternaria alternata*. Fumonisins in brewing processes are seldom related to barley but rather maize adjuncts (Pietri, Bertuzzi, Agosti, & Donadini, 2010). *F. verticillioides* has economic importance, since it is present in almost all maize samples (Bennett & Klich, 2003). However, not all strains are toxigenic, so the presence of this fungus does not necessarily mean that toxin is formed. The presence of fumonisins in maize has been associated with oesophageal cancer in regions of Africa, China and Italy (Edite Bezerra da Rocha et al., 2014). IARC has categorized fumonisins as Class 2B (possible human carcinogen).

6.6 Fate of mycotoxins in the barley-to-beer chain

Toxigenic fungi and their metabolites are a natural part of the barley–malt ecosystem. Table 6.3 compiles the fate of mycotoxins in the malting and brewing process. The majority of fungal metabolites may be produced during the following steps:

1. during crop cultivation in the field,
2. while cereal awaits drying after harvest,
3. if cereal is inadequately dried or becomes damp during storage or transport, and
4. during the malting process.

The various processing steps along the malting and brewing chain such as sorting, cleaning and grading, malting, roasting, milling, mashing and fermentation will influence the final mycotoxin levels (Bullerman & Bianchini, 2007). Generally mycotoxin concentrations significantly decrease in the brewing process but are not completely eliminated.

6.6.1 Mycotoxins in barley and malting

Barley naturally contains *Fusarium* mycotoxins at harvest, whereas *Aspergillus* and *Penicillium* toxins are seldom detected in good quality malting barley (Baxter, Slaiding, & Kelly, 2001; Benesova et al., 2012; Parry et al., 1995). Mycotoxin production in the field is a complex biological process and is influenced by several different interrelated

Table 6.3 Mycotoxins in the malting and brewing process

Process phase	Potential change in mycotoxins	Cause
Barley cleaning and grading	Reduction	Removal of infected grains, fungal spores and dust
Steeping	Reduction	Removal of water-soluble toxins
Germination	No effect/increase/ decrease	Growth of toxigenic fungi suppressed Fungal growth and concomitant mycotoxin production Liberation of conjugated forms/ deconjugation
Kilning	Increase/no effect	Fungi capable of mycotoxin production during early hours
Mashing	Increase	Enzymatic release of toxins from protein conjugates/mycotoxins transmitted via contaminated adjuncts
Wort boiling	No effect/decrease	Mycotoxins stable/removal with trub
Fermentation	Increase/decrease	Increase due to deconjugation/decrease due to absorption to yeast cell or bioconversion
Final beer	No effect/dilution	
Side-streams from malting and brewing	Increase	Accumulation

factors such as fungus type, crop resistance, cultivation practices and climatic conditions. Surveys on the presence of *Fusarium* toxins in barley have been carried out in several countries. During recent years, the amounts of *Fusarium* toxins in malting barley have been below the EU acceptance limits (Belakova, Benesova, Caslavsky, Svoboda, & Mikulikova, 2014; Benesova et al., 2012; Dohnal et al., 2010; Edwards, 2009b; Euromalt, 2013; van der Fels-Klerx & Stratakou, 2010; Ibanez-Vea, Lizarraga, Gonzalez-Penas, & Lopez de Cerain, 2012). However, some single samples occasionally may exceed the legal limits. DON is the prevalent toxin in malting barley. Type-A trichothecenes such as T-2 and HT-2 are detected less frequently and at lower concentrations than DON.

There has been increasing consumer interest in organic products. Bernhoft, Clasen, Kristoffersen, and Torp (2010) and Ibanez-Vea et al. (2012) found less *Fusarium* infestation and mycotoxin production in organically grown barley compared to conventional farming, obviously due to better crop rotation and soil management. Based on the studies by Edwards (2009a and 2009c), organic cultured wheat and oats appear to have lower mycotoxin contamination compared to conventional cultivation, but there were no significant differences in mycotoxin levels of organic and conventional barley samples in the UK (Edwards, 2009b). Due to contradictory results more systematic studies are needed on the farming practices and mycotoxin production.

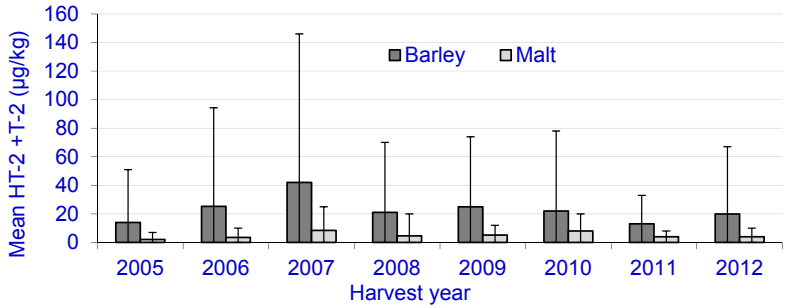

Figure 6.2 Mean and 95th percentile for T2 and HT2 toxins in barley and malt 2005–2012. Euromalt (2013); EU Commission's Mycotoxins Forum September 5, 2013.

Harvesting causes a major change in the balance in fungal ecology. The availability of water decreases and new fungal populations can spread by the machinery, field dust and crop residues (Noots et al., 1999). If the grain has been dried before storage, the risk of enhancing fungal activity is low. Baxter et al. (2001) reported that in normal conditions, OTA was undetectable in malting barley. However, slight changes in temperature and moisture parameters can lead to rapid deterioration of the barley. Fungi are unevenly distributed in silos. If the conditions at one spot allow the proliferation of xerophilic fungi their activity changes the microenvironment so that it becomes more favourable for toxigenic fungi (Noot et al., 1999; Magan & Aldred, 2005). If *P. verrucosum* spores are present at hot spots, they will proliferate and produce OTA (Anli & Mert Alkis, 2010).

The malting process has a significant cleaning function. The majority of the water-soluble toxins are washed out during the steeping step. Euromalt, the trade association representing the malting industry in the European Union, is carefully following the occurrence of mycotoxins in malting barley. They have been performing annual surveys of barley and malt since 2002. Samples (100–200) have been collected from all EU Member States with significant malt production (Figures 6.2 and 6.3).

Recent studies revealed that the levels of Type A trichothecenes decreased during malting (Euromalt, 2013; Malachova et al., 2010). Approximately 60–80% reduction in the sum of T-2 and HT-2 toxins was detected during the malting process (Euromalt, 2013). In all years, a mean value of the sum of T-2 and HT-2 toxin was below 50 µg/kg in barley and below 10 µg/kg in malt (Figure 6.2). Surveys also revealed that the levels of T-2 and HT-2 in winter grown barleys were lower than in spring grown varieties (Euromalt, 2013; van der Fels-Klerx & Stratakou, 2010). However, the initial differences in T-2 and HT-2 contamination of winter and spring varieties disappeared during malting, resulting in comparable levels in the final malt (Euromalt, 2013).

The impact of malting on type B trichothecenes is different. Processing barley into malt has generally little effect on the overall DON level. Both a decrease and production of DON can occur during malting. Dohnai et al. (2010) studied DON production in laboratory scale malting with 20 different barley varieties. They observed a decrease of DON levels during malting in 10% of samples and an increase in 20% of samples. A statistically significant impact of malting to DON content was not found. A similar

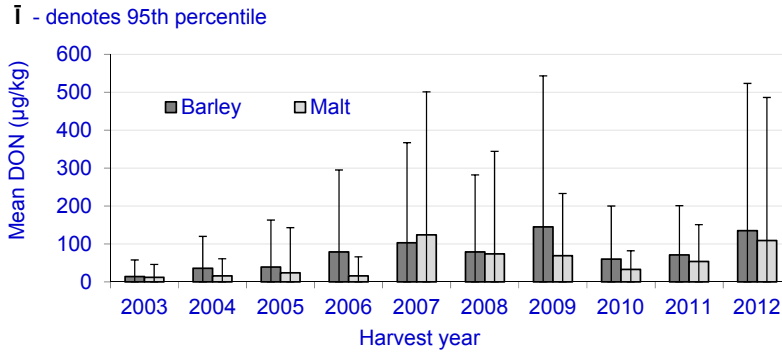

Figure 6.3 Mean and 95th percentile for deoxynivalenol (DON) in barley and malt 2005–2012. Euromalt (2013); EU Commission's Mycotoxins Forum September 5, 2013.

trend was reported by Malachova et al. (2010). In the Euromalt survey, the mean levels of DON in malt were generally lower than in barley (Figure 6.3). However, little correlation between individual barley and malt samples was observed. Schwarz et al. (2006) reported that barley with a DON level <1.0 ppm produced acceptable malt.

It is well known that malting conditions are favourable for fungal growth. Dormant spores present in barley are activated during the steeping. Reformation of toxins may occur with the growth of toxigenic fungi during steeping, germination and early hours of kilning (Schwarz et al., 1995; Sarlin, Laitila, Pekkarinen, & Haikara, 2005a; Wolf-Hall, 2007). Stress conditions for fungi during the kilning phase can trigger the mycotoxin synthesis. It has been shown that the temperature rise can activate mycotoxin production (Schwarz et al., 1995; Wolf-Hall, 2007). Thus, kilning can be regarded as an important step with regard to mycotoxin production and safety. The elevated temperatures at malt kilning are not enough to destroy mycotoxins.

6.6.2 *Mycotoxins in brewing and beer*

Mycotoxins are stable compounds and can therefore survive throughout the brewing process and enter the final product. Other raw materials used in beer production can be additional sources for mycotoxins. Many factors influence the concentration in the final product (raw materials, production steps in the breweries such as the mashing step, fermentation, yeast type). Thus, prediction of transmission is difficult. Several mycotoxins, like trichothecenes, are water soluble and are extracted into wort during mashing. Cantrell (2008) concluded that the majority (65–100%) of T-2 and HT-2 toxins present in malt persist into beer. There was little or no significant losses of these toxins into by-products such as spent grains or brewers' yeast. Furthermore, mashing conditions and enzymatic activity may contribute to the release of mycotoxins from the grain matrix during mashing (Wolf-Hall, 2007). Mashing may increase toxin levels such as DON due to the release of additional DON from conjugates (see Section 6.8.1 for modified mycotoxins). Mycotoxins are relatively stable at temperatures used in mashing and wort boiling. For example, DON is stable at 120 °C (Hazel & Patel, 2004).

Table 6.4 Mycotoxin concentration (ppm = µg/ml) causing significant inhibitory effects on the growth and activity of brewing yeast

Mycotoxin	Lager yeast	Ale yeast
Deoxynivalenol	100	50
Nivalenol	50	50
Fumonisin B1	10	50
T2	10	nd
Zearalenone	50	50
Diacetoxyscirpenol (DAS)	5–10	nd

nd: not determined.
Source: Boeira et al. (1999a, 1999b, 2000), Whitehead and Flannigan (1989).

Mycotoxins may cause process failures in beer production. They have been shown to disturb yeast metabolism during fermentation (Boeira, Bryce, Stewart, and Flannigan, 1999a, 1999b, 2000; Koshinsky, Crosby, & Khachhatourians, 1992; Whitehead & Flannigan, 1989). Mycotoxins may have negative impacts on the viability, biomass growth and metabolic activity of yeasts. AFB_1 and trichothecenes are known to inhibit the alcohol hydrogenase activity resulting in decreased fermentation activity and lower CO_2 liberation (Klosowski, Mikulski, Grajewski, and Blajet-Kosicka, 2010). Mycotoxin contamination of raw materials has also led to significant reduction of ethanol yield (Klosowski et al., 2010). The degree of growth inhibition has been shown to depend on the toxin concentration, yeast type, fermentation conditions and length (Boeira et al., 1999a, 1999b; Boeira, Bryce, Stewart, Flannigan, 2002). Table 6.4 illustrates some mycotoxins and their levels that have shown to cause significant adverse impacts. A combination of various toxins at low concentrations may be one of the reasons for unexplained unfinished fermentations. Synergistic effects of various toxins are still poorly understood.

Although mycotoxins have been shown to influence yeast behaviour, they do not form immediate concern for brewing. Even if highly contaminated grains were used in the brewing process, it is unlikely that any of the toxins would be found at high concentrations during fermentation (Wolf-Hall, 2007). Studies have indicated that DON has little importance in conventional brewing or distilling fermentations (Boeira et al., 2000; Kostelanska et al., 2009; Wolf-Hall, 2007). The inhibitory DON level is 10,000 times higher than that measured in the survey of commercial beers (Kostelanska et al., 2009).

The fermentation process can also decrease the amount of mycotoxins. Part of the toxins can be bound to yeast cells and thus removed from the wort. In addition, brewers' yeast also has the ability to detoxify mycotoxins by bioconversion of mycotoxins to less toxic derivatives (Halady Shetty & Jespersen, 2006; Inoue, Nagatomi, Uyama, & Mochizuki, 2013). Mizutani, Nagatomi, and Mochizuki (2011) showed that the major part of ZEA (89.5%) was converted to β-zearalenol, which has lower estrogenic activity than ZEA.

Although part of the mycotoxins are removed or destroyed during brewing, some toxins can be transmitted into beer. Surveys on mycotoxins in beer are continuously being carried out in different countries. Detectable amounts of mycotoxins have been found in commercial beer samples regardless of the market where they have been collected. In all surveys the mycotoxin levels have been rather low. DON, fumonisin, nivalenol, T-2, HT-2, diacetoxyscirpenol, zearalenone, aflatoxins and OTA have been detected in beers at trace (ppb) levels (Anli & Mert Alki 2010; Cantrell, 2008; Kostelanska et al., 2009; Mably et al., 2005; Mateo et al., 2007; Scott, 1996; Tangni, Ponchaut, Maudoux, Rozenberg, & Larondelle, 2002).

Aflatoxins are rarely found in beers. However, some warmer climates, such as South America and equatorial Asia, can have a higher incidence for AFs in raw materials and thus contamination levels in final products (Mably et al., 2005; Scott, 1996). Mably et al. (2005) carried out a survey of aflatoxins in beer sold in Canada. Both domestic and imported beers from 36 countries were included and 12 samples were positive for AFs. The highest incidence (4/5) and levels (max 230 ng/l) occurred in beers from India. Pietri et al. (2010) studied the transfer of AFB_1 and fumonisin B_1 from naturally contaminated raw materials to beer during a full-scale industrial brewing process. The content of AFB_1 in maize grit ranged from 0.31 to 14.85 µg/kg. AFB_1 was also found in malted barley at levels of 0.20–4.07 µg/kg. Approximately 0.6–2.2% of AFB_1 was recovered from the final beer. Recently, Benesova et al. (2012) analysed AFs from different brewing materials (61 malting barley, 77 malt, 54 hop, 12 brewers' yeast and 12 spent grain) and 117 beers obtained from EU malting plants and breweries. AFs in trace concentration were found in approximately 3% of the material samples and in 5% of beer samples coming from European countries.

OTA present in contaminated grains can be transmitted to beer, although the brewing process decreases the concentration (Baxter et al., 2001; Mateo et al., 2007; Tangni et al., 2002). Baxter et al. (2001) reported that substantial loss of OTA (up to 40%) was observed during mashing. This was obviously due to the proteolytic degradation and conversion of OTA to nontoxic ochratoxin α. OTA structure includes a peptide bond, thus the cleavage is possible. Approximately 16% of OTA was removed along with the spent grain. The final beer contained 13–32% of the toxin present in the contaminated malt sample. OTA has been found in beers all over the world in surveys carried out since 1998. More than 50% of the analysed samples have shown trace amounts of toxins <0.2 ng/ml. Mateo et al. (2007) concluded that beer is not a relevant contributor to OTA exposure in human consumption. However, highly contaminated batches may occur in the beer production chain. Thus, it is important to follow and control OTA in brewery products to maintain OTA intake at the lowest achievable level (Tangni et al., 2002).

Fusarium toxins can be frequently found in beers. For DON, the transfer from malt in finished beer was between 80–93% (Schwarz et al., 1995). European beers were surveyed for the presence of T-2 and HT-2 toxins during the years 2006 (including 195 samples) and 2007, (including 196 samples) from 27 countries (Cantrell, 2008). The majority (65–100%) of T-2 and HT-2 toxins present in malt persists into beer. There were little or no significant losses of these toxins into side-products such as spent grains or brewers' yeast. The mean level was 0.098 µg/l in 2006 (maximum 0.73) and 0.053 µg/l in 2007 (maximum 2.67). Schwarz et al. (1995) studied the fate of ZEA in brewing.

They reported that the majority of ZEA (60%) was detected from the spent grains. ZEA was not detected in the final beer. This might be due to conversion of ZEA to zearalenol by the brewers' yeast

Overall it can be concluded that mycotoxins can be transmitted to beer. However, mycotoxin in commercial beers does not form a significant health risk for moderate consumers (Ibanez-Vea et al., 2012; Varga, Malachova, Schwartz, Krska, & Berthiller, 2013). However, rather high incidences of aflatoxins (AFs), ochratoxin A (OTA) and zearalenone (ZEA) have been found in locally brewed commercial and home-brewed beers (maize- or sorghum-based) in warm climates, particularly in Africa (Mably et al., 2005; Odhav & Naicker, 2002). Increased toxin levels were often due to improper storage of raw materials.

6.6.3 Mycotoxins in by-products

The potential health risks related to beer consumption are low, but mycotoxins present in grain dust or in by-products used as food ingredients or as animal feed are a concern (Nordby et al., 2004; Wolf-Hall, 2007). Mycotoxins present in contaminated rootlets and spent grains have caused serious mycotoxicoses in production animals (Flannigan, 2003). Cavaglieri et al. (2009) reported that potential toxin producers such as *A. flavus* (potential aflatoxin producer) and *F. verticilloides* (potential fumonisin producer) were frequently found in barley rootlets. Poor management of by-products during storage and transportation can lead to fungal growth and mycotoxin production. Several mycotoxins have been shown to accumulate in spent grains and thus form a health risk to animals (Gonzalez Pereyra, Rosa, Dalcero, & Cavaglieri, 2011).

Aspergillus clavatus is considered to be one of the major causes of allergic alveolitis among malt workers (Flannigan, 2003). It can also produce various mycotoxins, including patulin and cytochalasin E. Lopez-Diaz and Flannigan (1997) showed that *A. clavatus* could produce these toxins during laboratory-scale malting. *A. clavatus* and toxins have been associated with mycotoxicoses in animals fed with by-products from malting houses and breweries (Lorretti et al., 2003).

6.7 Regulation of mycotoxins in Europe

Management of mycotoxin contamination in cereals is a global objective for farmers, breeders, manufacturers, regulatory agencies and the research community (Cheli, Battaglia, Gallo, & Dell'Orto, 2014). Mycotoxin regulations have been established in about 100 countries. The European Union Commission Regulation No. 1881/2006 (European Commission, 2006b) has set maximum levels for several mycotoxins in foods and beverages in order to protect consumer health. Beer must conform to legal limits for raw materials. Mycotoxins originating from raw materials are diluted in the brewing process. It has been estimated that the toxin levels in the final beer are decreased by one order of magnitude compared to the levels of raw material (Kostelanska et al., 2009).

Legal limits for the total amount of aflatoxins are 4 and 2 µg/kg for aflatoxin B1 in cereals.

The legal limits for ochratoxin A in cereals in the EU are 5 µg/kg for raw grain and 3 µg/kg for processed grain (which includes malt). The EU Commission Regulation No. 105/2010 (European Commission, 2010) did not set a separate maximum level in beer, since it indicated that the presence of OTA is already controlled in malt.

Maximum limits for *Fusarium* toxins DON and ZEA in cereals have been operated in the EU. For DON these are 1250 µg/kg in raw cereals and 750 µg/kg for processed cereals (which includes malt); for ZEA these are 100 and 75 µg/kg, respectively. Legislative limits have not been set for nivalenol (NIV) as it is considered to be a cocontaminant of DON and as such can be controlled by controlling DON.

The maximum levels for fumonisins (sum of B_1 and B_2) are set for maize and maize products and vary from 200 to 2000 µg/kg.

The maximum levels for T-2 and HT-2 are still under consideration. An indicative level for the sum of T2 and HT-2 was set in 2013 (Commission Recommendation 2013/165/EU, European Commission, 2013). It is 200 µg/kg for barley (including malting barley) and 50 µg/kg for processed cereals. According to this recommendation, member states should also collect more data on the occurrence of T-2 and HT-2 in cereal and cereal products. Furthermore, more information is needed on year-to-year variation, the effects of food processing and agronomic factors on the presence of these toxins. It is also encouraged that samples be simultaneously analysed for the presence of T-2 and HT-2 and other *Fusarium* toxins, since they often co-occur in the samples.

The Panel on Contaminants in the Food Chain of the European Food Safety Authority (EFSA) (http://www.efsa.europa.eu/en/panels/contam.htm) deals with contaminants in the food chain. Scientific opinions on health risks are prepared by this panel. It is anticipated that the number of mycotoxins with regulatory status will increase in the future. Other *Fusarium* mycotoxins with possible regulatory interest in the future are fusarenone-x (an acetylated form of nivalenol), fusarin C, enniatins, beauvericin, diacetoxyscirpenol and moniliformin. More scientific opinions and risk assessments on mycotoxins can be found in the EFSA webpages (http://www.efsa.europa.eu/en/topics/topic/mycotoxins.htm).

The EU Commission has also set a regulation for sampling (EC 401/2006, European Commission, 2006a). Representative sampling of biological samples is always a great challenge due to the sporadic distribution of the target molecules. Differences in the mycotoxin contamination pressure can be seen between different regions and also inside one field or in stored barley batches. Thus, sampling is a key factor in mycotoxin control. Regardless of the mycotoxin detection method chosen, the final results will be only as good as the sample taken.

6.8 Emerging mycotoxin issues

6.8.1 Modified mycotoxins

Food or feed are not necessarily safe due to the absence or low concentrations of well-known mycotoxins, as these toxic compounds can be present in disguise as a result of plant, mammal or fungal metabolism or even food processing (Berthiller, Schuhmacher, Adam, & Krska, 2009; Berthiller et al., 2013). Modified mycotoxins

(also called masked mycotoxins) are mainly conjugation products due to detoxification mechanisms of living organisms (Rychlik et al., 2014). The great challenge is that modified mycotoxins are often undetectable by conventional analytical techniques since their structure has been changed due to conjugation with sugars, amino acids or proteins. They can be either in soluble form (then called masked or preferably modified mycotoxins) or in nonextractable form attached to macromolecules (bound mycotoxins). Bound mycotoxins can be considered to be detoxified compounds as long as they are not released from the cereal matrix during processing or digestion (Berthiller et al., 2013). However, possible hydrolysis of modified mycotoxins back to their toxic forms within the mammalian gastrointestinal tract is a great concern. A recent study by Dall'Erta et al. (2013) demonstrated that modified forms of DON and ZEA could be deconjugated by the human colonic microbiota.

Several *Fusarium* toxins such as DON, fumonisins, fusarenon-x, fusaric acid, nivalenol, T-2, HT-2 and ZEA are prone to masking biotransformations or binding by plants (Berthiller et al., 2013). In addition, other mycotoxins such as OTA and patulin have been found in conjugated forms. The major form of modified DON is deoxynivalenol-3-β-D-glucopyranoside (D3G), which is perhaps the most widely studied cereal-associated modified mycotoxin so far.

Food and beverage processing can alter mycotoxins chemically. For example, microbes used in fermentations may transform mycotoxins to less toxic compounds. So far, only a few studies have been carried out concerning the modified mycotoxins in the barley-to-beer chain. The studies have indicated that bound DON appears to be fairly common in barley (Lancova et al., 2008; Zhou, He, & Schwarz, 2008). Zhou et al. (2008) reported that bound DON in naturally infected barley was detected in almost 40% of samples and represented an additional 6–21% of DON determined by the standard gas chromatography method.

D3G has been found at levels comparable or even higher than DON in malt and beer (Lancova et al., 2008). Both an increase and a decrease of modified or bound mycotoxins can occur during malting and brewing (Kostelanska et al., 2011; Lancova et al., 2008). The formation and detoxification mechanisms still need further studies. However, it has been suggested that barley could metabolize the *Fusarium* toxin produced during the malting process. In addition, bound mycotoxins originally present in the plant cell walls could be liberated due to enzymatic actions during processing. Maul et al. (2012) reported that the germination process has a significant impact on biotransformation of DON. Approximately 50% of DON was transformed to D3G during 5 days of germination. D3G has been detected in all types of malts, including light, caramel, Munich and wheat malt (Kostelanska et al., 2011), except roasted malt used in dark beer production. This was obviously due to thermal degradation under roasting temperatures. Kostelanska et al. (2011) reported that D3G was not detected in brewing intermediates, including spent grains, indicating that extractable mycotoxins were effectively transferred into wort. In summary three critical steps can be identified in the barley-to-beer chain with respect to transformation of modified mycotoxins: (1) barley germination, (2) liberation of bound toxins during mashing and (3) yeast fermentation.

Traces of modified mycotoxins can be found in beers worldwide. Kostelanska et al. (2009) analysed 176 commercial beers for DON and D3G. Samples were collected from European and North American markets in 2007. Almost 74% of the samples contained the modified form of DON exceeding the detection limit (1 µg/l). The highest level of D3G was 37 µg/ml. Recently, Varga, Malachova, Schwartz, Krska, and Berthiller (2013) analysed 374 beer samples from 38 countries for the presence of DON, D3G and 3-acetyl-deoxynivalenol (3ADON). They reported that trace amounts of DON were found in 77% of beers and D3G in 93% of beers. 3ADON was not detected in beer. The majority of the samples contained DON and D3G less than 10 µg/l. The highest concentrations of both toxins (>80 µg/l) were found in the same beer sample. It was shown that stronger beers with higher ethanol concentrations contained higher DON and DON-conjugate levels (Kostelanska et al., 2009; Varga et al., 2013). This was obviously due to the use of larger wort extract volumes in strong beer production. DON and its conjugates have also been detected in nonalcoholic beers (Kostelanska et al., 2011; Varga et al., 2013). However, the nonalcoholic products have shown the lowest contamination levels (Varga et al., 2013).

The extractable conjugated or bound mycotoxins are not currently regulated by legislation (Berthiller et al., 2013). Further studies are required on determination of modified mycotoxins and on their stability, transformation, toxic properties and bioavailability. With respect to regulation, a possible approach in the future could be the definition of the sum of all relevant forms of each mycotoxin, including its relevant derivatives.

6.8.2 Enniatins and beauvericin

Barley-associated fungi are also responsible for the production of bioactive compounds called emerging or 'minor' mycotoxins. This group includes toxins such as enniatins (ENNs) and beauvericin (BEA). Recently, the occurrence and fate of these toxins in the barley-to-beer chain have gained attention (Hu, Gastl, Linkmeyer, Hess, & Rychlik, 2014; Vaclavikova et al., 2013).

ENNs and BEA are cyclic hexadepsipeptides consisting of three D-2-hydroxycarboxylic acid and *N*-methylamino acid moieties (Jestoi, 2008). They are often found in *Fusarium*-infected cereals, including barley. ENNs appear in nature as mixtures of four main variants: enniatin A, A_1, B_1 and B_2 and the minor variants C, D, E and F. BEA is produced by a relatively wide range of *Fusarium* spp., including F. *avenaceum*, *Fusarium oxysporum*, *F. poae*, *F. proliferatum, Fusarium subclutinans* and *Fusarium semitectum* and the relatively closely related fungus *Beauveria bassiana*, which is a natural soil fungus (Jestoi, 2008; Logrieco, Rizzo, Ferracane, & Ritieni, 2002). Jestoi (2008) summarized that 26 *Fusarium* species are known to produce BEA. ENNs are also produced by several different *Fusarium* species (17 reported enniatins producers), including *Fusarium acuminatum*, *F. avenaceum*, *Fusarium sambucinum* and *F. tricinctum*. A review published by Jestoi (2008) gives an overview of the properties of these emerging toxins. They are of interest due to their wide range of biological activity.

The ENNs act as ionophores and thus they can serve as antimicrobial compounds (Uhlig, Ivanova, Petersen, & Kristensen, 2009). They may disrupt the membrane permeability of other microbes. Hiraga, Yamamotoa, Fukuda, Hamanakaba, and

Oda (2005) showed that ENNs can influence functions of *Saccharomyces cerevisiae*. They were identified as inhibitors of major drug efflux pumps. Thus, they could potentially cause failures with metabolism of brewers' yeast. However, the impacts of these emerging mycotoxins on the behaviour and activity of brewers' yeast still need further investigation.

Hu et al. (2014) studied the fate of ENNs and BEA during the malting and brewing process. They showed that only a small part of the preformed toxins were leached out during steeping and the major part of ENNs and BEA remained in barley, obviously due to their low water solubility. Furthermore, toxin production can occur during barley germination. Vaclavikova et al. (2013) reported that ENN levels were decreased during malting by approximately 30% of the initial content in barley. Some losses of ENNs and BEA could be seen due to thermal degradation or biodegradation during kilning. Significant proportions of these toxins were removed along with the rootlets (reduction range 28–59% including the losses during kilning and rootlet removal) (Hu et al., 2014). In the brewing process, these toxins were mostly retained spent grains (53–98%) due to low water solubility (Hu et al., 2014; Vaclavikova et al., 2013). Additionally, some part of the toxins remained in the wort and were discarded along with trub. Hu et al. (2014) reported that less than 0.2% of the ENNs and BEA present in barley were detected in the final beer. Thus, they do not form an immediate health risk for consumers. However, high concentrations of the toxins could be concentrated in rootlets and spent grains used as animal feed and should be taken into consideration. Further studies are needed on their toxicity in mammals.

6.9 Preventive actions

Toxigenic fungi and mycotoxins are present in brewing raw materials and recognized as a risk in the beer production chain. Therefore, it is highly important to set up specific procedures to assure the safety of the products.

Management of fungi and their metabolites in the entire barley–malt–beer chain relies on good agricultural practices (GAP) as well as on good malting and brewing practices. The first line of defence is always at farm level. GAP are general procedures to reduce hazards already at the farm level. The selection of barley cultivars and agricultural practices such as crop rotation, tillage practices and fungicide use will influence fungal dynamics and their mycotoxin production.

EU Regulation (No. 852/2004) requires food business operators to establish, implement and maintain permanent procedures based on Hazard Analysis and Critical Control Points (HACCP) (European Commission, 2004). It has also been implemented in the malting and brewing industry (Rush, 2006; Davies, 2006; Erzetti et al., 2009). HACCP involves identifying all points in the manufacturing process where biological, chemical and physical hazards could occur and then controlling and monitoring those risks. It also covers the cereal coproducts such as malt rootlets and spent grains of the malting and brewing process used as animal feed. Erzetti et al. (2009) reviewed how to develop HACCP programmes for mycotoxins, nitrosoamines and biogenic amines in the brewing production chain. They highlighted that small enterprises

(microbreweries, micropubs, etc.) should also pay attention to safety issues, since they often use nonstandardized barley and malt lots.

Preventive actions are highly important in maintaining the quality of malting barley and in assuring safety throughout the malting and brewing process. These procedures must be implemented for both preharvest and postharvest actions. Generally, the preventive actions can be divided into three categories (Wolf-Hall, 2007):

1. removal and/or separation of infected kernels (for example, cleaning, grading, peeling);
2. treatments intended to prevent mould growth; and
3. decontamination or elimination of mycotoxins present.

Controlling the harmful fungi and their metabolites is extremely challenging because the procedures carried out in the barley-to-beer chain should not have negative impacts on the grain germination performance or on malt and beer properties.

6.9.1 Preharvest management

Management of toxigenic fungi in field conditions requires an integrated approach including proper agricultural practices to minimize the risk for fungal growth, development and use of resistant cultivars, use of fungicides and/or biocontrol to protect the susceptible host, minimizing insect infestation and utilization of weather-based risk assessment for disease forecasting. Previous crops and the amount of crop residue on the soil surface are considered major factors in spreading the pathogen (Leplat, Friberg, Abid, & Steinberg, 2013; Osborne & Stein, 2007). For example, the use of maize as a previous crop for barley, wheat and oats may increase the risk for FHB and increased mycotoxin production. Maize is an important host for a number of *Fusarium* fungi, including *Fusarium graminearum*. The survival of fungi is enhanced with reduced tillage systems. Fungi are present in crop residues and may survive in plant debris over winter, while burial of plant residues speeds decomposition and reduces pathogen survival (Osborne & Stein, 2007). More care must be given to the crop rotation scheme to limit the risk for toxigenic fungi (Leplat et al., 2013).

A lot of attention is paid to finding effective fungicide treatments against FHB. However, contradictory results have been obtained with fungicides. The effects are highly dependent on cultivar resistance, fungicide efficacy, fungicide coverage, timing and the aggressiveness of the pathogen. Based on current knowledge, no or only a small impact has been obtained by the use of fungicides on the contamination of cereals with *Fusarium* toxins (van der Fels-Klerx & Stratakou, 2010; Henriksen & Elen, 2005). There are at present no strategies or common practices for the use of fungicides to prevent *Fusarium* infection in cereal grains. Henriksen and Elen (2005) showed that fungicide treatment even increased the *Fusarium* infection level in spring barley when treatment had been applied to control other fungal diseases. The suppression of competing moulds in the barley ecosystem may lead to increased *Fusarium* growth. Even increased mycotoxin production has been observed when fungicides have been applied. Malachova et al. (2010) reported that a combination of two fungicide preparations led to increased NIV production and in some crop years also enhanced DON production in field trials of 12 malting barley cultivars.

6.9.2 Importance of storage

Storage of cereal lots on the farm level or on manufacturing silos can be regarded as the most important postharvest phase with respect to mycotoxin production. Water is the most important single factor limiting microbial growth. Barley should be dried immediately after harvest at least to a moisture content <14% if it is stored for any period of time and to <12.5% to exlude the fungal growth during storage (Flannigan, 2003). During storage the barley moisture content is in equilibrium with the moisture content of the air. Therefore, grains may be further dried or they can absorb water from the surrounding air during storage. The storage life of stored grains is increased by cooling. Barley and malt should always be stored in a dry and cool environment to avoid the potential risks associated with fungal growth and possible mycotoxin accumulation. Furthermore, empty silos should be cleaned to remove the grain residues and occasionally fumigated in order to eliminate the contaminants. Pest control is highly important since they are vectors for toxigenic fungi. Metabolic activity of insects and mites increases the moisture content and temperature of contaminated grains and thus creates conditions favourable for fungal growth. Sanitation of empty malting vessels and air-conditioning systems is also carried out in malting houses in order to avoid harmful process contaminants.

6.9.3 Cleaning and grading

The best preventive method is to avoid highly contaminated material in malt and beer production. Heavily infected barley lots are then discarded before entering the malting process. Cleaning and grading of grains during the harvest and prior processing are crucial steps and significantly reduce fungal contamination and also mycotoxins. Grain deterioration due to plant-pathogenic fungi often leads to poor kernel size and highly contaminated grains can be removed by sorting. By rejection of the smallest sized kernels (<2.5 mm), a significantly reduced level of *Fusarium*-contaminated grains and mycotoxins can be obtained (Perkowski, 1998). Lancova et al. (2008) reported that cleaning of kernels reduced DON content by 30–50%. However, there are differences between the removal of mycotoxins during cleaning and grading procedures. Although cleaning significantly influences *Fusarium* toxins, only 2–3% reduction of OTA in barley/wheat was obtained by cleaning (Scudamore, 2005).

6.9.4 Heat treatment of barley

It is a well-known fact that several filamentous fungi, especially field fungi such as fusaria, are sensitive to heat. High-temperature treatments have been shown to effectively reduce the viable fungi on cereal grains, although it does not eliminate the preformed mycotoxins (Kristensen, Elmholt, & Thrane, 2005). Olkku, Peltola, Reinikainen, Räsänen, and Tuokkuri (2000) reported an invention in which the mould contamination of barley was effectively reduced by exposing grains to heat (60–100 °C) for 0.5–3 s prior to the malting process. Heat treatment of barley notably decreased the *Fusarium* contamination without influencing grain germination. Moreover, it significantly reduced mycotoxin production during the malting process and alleviated the gushing tendency (Olkku et al., 2000).

Kottapalli, Wolf-Hall, Schwarz, Schwarz, and Gillespie (2003) studied hot water treatments for reducing fusaria in malting barley. They showed that soaking barley with water at 45 °C for 15 min resulted in a significant reduction of *Fusarium* contamination without influencing grain germination. The same effect was obtained by soaking at 50 °C for 1 min. A great advantage of hot water treatments was that some water soluble mycotoxins could be washed out of the grain. Briggs (2004) reported that short exposures to hot water, even at 100 °C for 5 s, were advantageous with respect to microbiological safety and grain germination. Thus, grains would be washed and surface-sterilized prior to steeping.

6.9.5 Electron-beam irradiation of grains

In addition to heat treatments, electron-beam irradiation of barley has been reported to be an effective, nonchemical means for reducing fusaria. For dry *Fusarium*-infected barley, an irradiation dose of >4 kGy was required to obtain *Fusarium* reduction (Kottapalli et al., 2003, Kottapalli, Wolf-Hall, & Schwarz, 2006). Although irradiation had no effect on the preformed mycotoxins, it greatly reduced the mycotoxin production during malting (Kottapalli et al., 2006). The advantage of irradiation was that the grain treatment could be carried out either prior to or after storage. It could also help in the maintenance of quality by eliminating insect infestation. Insects such as beetles are the principal vectors of microbes in plant ecosystems. Grain germination was not significantly influenced up to a dosage of 8 kGy (Kottapalli et al., 2003). Irradiation is considered as a promising means to influence fungal activity prior to malting, but the impact on toxin production and malt quality needs further studies (Wolf-Hall, 2007). Röder et al. (2009) reported on new E-ventus technology which utilizes the biocidal effect of soft, low-energy, accelerated electrons. The main application area so far has been the replacement of chemical dressing in seed treatments in order to eliminate seed-borne pathogens.

6.9.6 Effective rinsing during steeping

The treatments carried out pre- or postharvest on barley should not significantly influence the seed vigour. Various common practices are routinely applied to reduce adverse effects of fungi and other microbes during malting, especially during the steeping phase, such as changing the steeping water in order to remove microbes and leached nutrients, balancing the temperature or modifying aeration. Furthermore, steep water must be warm enough to allow rapid water uptake and germination of the grains but cool enough to avoid extensive microbial growth. Therefore, steeping is normally carried out at 10–20 °C. It is also important to provide sufficient aeration and to pulse the circulation throughout the immersion period in order to keep the grains moving and to avoid anaerobic, hot pockets in the grain bed which would lead to increased microbial activity and poor grain germination (Davies, 2006).

It has been estimated that approximately 70–100% of the toxins present in grains can be removed during steeping (van der Fels-Klerx & Stratakou, 2010; Sarlin et al.,

2005a; Schwarz et al., 1995). The following factors influence the removal of toxins: (1) amount of water, (2) temperature, (3) number of steeps, (4) duration of steeping periods and (5) extent of mixing during steeping (van der Fels-Klerx & Stratakou, 2010).

It has been shown that a variety of chemical agents such as acids, bases and oxidizing reagents could be applied to intensify the washing effect during steeping. Papadopoulou, Wheaton, and Muller (2000) suggested that fungal proliferation could be restricted by adding hop beta-acids in the malting process. Moreover, they demonstrated that the growth of fungi was inhibited by washing barley first with sodium hypochlorite (alkaline wash) followed by an acid wash with hypochloric acid. Lake, Browers, and Yin (2007) reported that soaking of barley in sodium bisulfite (10 g/l) enhanced the removal of DON during steeping without influencing grain germination. Most studies have been carried out in a laboratory or pilot scale, and the feasibility and safety of treatments in large scale remain to be confirmed. Although different additives may effectively improve processing, their use in industrial processes is often limited by legislation. Furthermore, the industry has a strong emphasis towards natural processing without chemicals. Ozonation is one of the new potential technologies to reduce fungal growth and mycotoxins since it would not leave residual chemicals (Kottapalli, Wolf-Hall, & Schwarz, 2005; Tiwari et al., 2010). Application of ozone (O_3) in gaseous or liquid form can be used for insect control and to inactivate the toxigenic fungi. Furthermore, ozone has been reported to be effective in detoxification and degradation of mycotoxins such as aflatoxin, OTA, DON and ZEA (Tiwari et al., 2010). Kottapalli et al. (2005) concluded that gaseous ozone and hydrogen peroxide are potential means for reducing *Fusarium* survival during malting.

6.9.7 Biocontrol

Residues and undesirable reaction products in germinating barley and in the subsequent malt are of concern especially with chemical treatments, since they may have a negative impact on malt properties and yeast fermentation performance (Laitila, 2007). Furthermore, precautions must be taken as some of the antimicrobial treatments in sublethal doses may stimulate the production of harmful metabolites such as gushing factors and mycotoxins. Barley- and malt-derived microbes, especially lactic acid bacteria and certain fungi, offer a potential alternative as natural, food-grade biocontrol agents (Dalie, Deschamps, & Richard-Forget, 2010; Laitila, 2007; Lowe & Arendt, 2004; Rouse & van Sinderen, 2008). Biocontrol candidates isolated from the brewing raw materials will most likely persist in the habitat from which they have been isolated. LAB and fungi with antagonistic properties have been shown to restrict fungal growth and prevent mycotoxin formation (Boivin & Malanda, 1997; Laitila, 2007; Laitila, Alakomi, Raaska, Mattila-Sandholm, and Haikara, 2002; Laitila, Tapani, & Haikara, 1997). Natural biocontrol agents are attractive as they have a better public image, and they could potentially be used as starter cultures in bioprocesses in which the use of chemicals is considered undesirable. Starter technology, in which well-characterized microbes are added to the barley during field conditions or during processing, has been introduced into the malting and brewing industry.

Biodegradation of mycotoxins has become an area of great interest. Biological detoxification involves the enzymatic degradation or transformation of toxins to less toxic compounds and is often a detoxification or resistance mechanism used by microbes or plants for protection from adverse impacts of toxins. It has been shown that *S. cerevisiae* and lactic acid bacteria are potential candidates for mycotoxin decontamination (Halady Shetty & Jespersen, 2006).

Biocontrol combined with other physical and chemical preventive actions along the barley-to-beer chain could result in a successful strategy for controlling toxigenic fungi. Several new potential techniques have been studied in the laboratory or pilot scale and transfer of technologies into the industrial scale requires further studies.

6.10 Future trends

It is clear that contamination of brewing raw materials with toxigenic fungi cannot be completely avoided, especially in crop years when bad weather conditions favour the growth of gushing active and toxigenic species in large barley production areas. Recognizing, understanding and management of toxigenic fungi and mycotoxin production require close cooperation and communication between different sectors along the food, beverage and feed production chain. Control of toxigenic fungi in changing climatic conditions together with changing agricultural practices is a global future challenge. Occurrence of mycotoxins is expected to increase due to climate change. Climate represents the key agro-ecosystem driving force of fungal colonization and mycotoxin production. Climate change will have direct effects on the fungal host interactions. Mycotoxin production is also influenced by noninfectious factors in the field, e.g. plant stress, bioavailability of (micro) nutrients, insect damage and other pest attacks, which are in turn driven by climatic conditions. In addition, indirect effects will be due to the changes in agricultural crop production systems. Changes in farming practices and new crop varieties in different cereal production areas are expected due to climate change. The need for sharing information and practices related to food and feed safety issues has been globally recognized.

Currently, reduction of water and energy consumption in all industrial processes is an economical and environmental challenge. Simultaneously, the environment is becoming more favourable for toxigenic fungi. Food safety issues should be taken into account when changes are made in the malting and brewing industry.

According to Codex Alimentarius (2003) complete elimination of mycotoxins in cereals and cereal-based products may not be achievable, but reduction of toxins in every step along the cereal production chain is essential to ensure consumer and animal safety. Thus, effective means are needed to control the growth of fungi and removal of mycotoxins at the preharvest level as well as during processing. Furthermore, fast and reliable early warning tools are required for prediction of toxin production and for multitoxin detection, since several mycotoxins can co-occur in the cereal production chain. The multitarget control strategies in combination with novel monitoring tools will open up new possibilities for ensuring safety along the barley-to-beer chain.

6.11 Sources of further information and advice

Food and Agriculture Organization of the United Nations provides a lot of information and relevant links related to food safety and quality, including mycotoxins. Available from: http://www.fao.org/food/food-safety-quality/home-page/en/.

EFSA collects and evaluates occurrence data on mycotoxins in food and feed. It provides scientific advice and risk assessments on mycotoxins for EU risk managers to help them assess the need for regulatory measures as regards to the safety of mycotoxin-contaminated food and feed. Further information can be found on the EFSA homepages: http://www.efsa.europa.eu/en/topics/topic/mycotoxins.htm.

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Gram-positive spoilage bacteria in brewing

7

K. Suzuki
Quality Control Center, Asahi Breweries, Ltd., Ibaraki, Japan

7.1 Introduction

Beer has been recognized as a microbiologically stable beverage. This is due to the presence of ethanol (0.5–10% w/w), hop bitter compounds (*ca* 17–55 ppm of iso-α-acids), and high carbon dioxide content (approximately 0.5% w/v), as well as low pH (3.8–4.7) and reduced concentration of oxygen (generally less than 0.3 ppm) (Suzuki, Iijima, Sakamoto, Sami, & Yamashita, 2006). Beer is also a poor medium because nutrients are almost depleted by the fermentative activities of brewing yeast. As a result, most Gram-positive bacteria, such as *Bacillus* and *Staphylococcus*, do not grow or survive in beer (Bunker, 1955). Despite these hostile features, a limited number of Gram-positive species are able to grow in beer. The two genera *Lactobacillus* and *Pediococcus* are predominant beer spoilers in Gram-positive bacteria. These beer-spoilage lactic acid bacteria (LAB) exhibit strong resistance to hop bitter acids, a distinguishing feature that is not observed with nonspoilage Gram-positive counterparts. Hop resistance of beer-spoilage LAB is considered as the interplay of several distinct mechanisms that collectively counteract the toxic effects of hop bitter acids. In this chapter, the first section describes the taxonomy and history of beer-spoilage LAB, in addition to other features concerning these spoilage microorganisms. The second section is specifically devoted to the hop resistance mechanisms in LAB, as well as the identification of hop resistance genes and their significance in the brewing industry. The preservation and subculture methods of beer-spoilage LAB strains are described in the third section, to which attention should be paid to maintaining the original states of beer-spoilage LAB when detected as primary isolates from brewing environments. Other Gram-positive bacteria will be briefly summarized in the final section of this chapter.

7.2 Beer-spoilage LAB

7.2.1 Historical backgrounds and taxonomy

Beer-spoilage LAB were found by Pasteur in 1871 through microscopic examinations of spoiled beer (Pasteur, 1876). Initially beer-spoilage LAB were grouped in rods and cocci. Rod-shaped LAB strains were originally designated *Saccharobacillus pastorianus* by van Laer (1892). This species was named in honor of Pasteur and later redesignated *Lactobacillus pastorianus*. van Laer also reported that *L. pastorianus* did

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not show the culturability on ordinary nutrient media, and therefore used unhopped beer solidified with gelatin for isolation. Due to its extremely low culturability on ordinary culture media, *L. pastorianus* had been poorly characterized, despite the fact that this species exhibits very strong beer-spoilage ability (Suzuki, Iijima, Sakamoto, et al., 2006). However, the recent development of new culture techniques has enabled *L. pastorianus* to be isolated from brewing environments (Suzuki, 2012). Since then, the insights into this species have been accumulated, and it was reported that *L. pastorianus* is a much more common beer spoiler than previously assumed (Iijima, Suzuki, Asano, Kuriyama, & Kitagawa, 2007). *L. pastorianus* is now considered as a synonym of *Lactobacillus paracollinoides*, and *L. paracollinoides* has been accepted as a formal species name (Ehrmann & Vogel, 2005; Suzuki, Asano, Iijima, & Kitamoto, 2008). Through the subsequent development of phylogenetic studies, the taxonomy of the rod-shaped lactobacilli have been changed a lot since the end of the nineteenth century, and beer-spoilage lactobacilli are now divided into *Lactobacillus brevis*, *Lactobacillus lindneri*, *L. paracollinoides*, *Lactobacillus backi*, and several other *Lactobacillus* species (Hutzler, Müller-Auffermann, Koob, Riedl, & Jacob, 2013). On the other hand, coccal strains were originally named *Pediococcus cerevisiae* by Blacke in 1884 (Kitahara, 1974). *Ped. cerevisiae* is now redesignated *Pediococcus damnosus*, a species name proposed by Claussen (1903). *Pediococcus claussenii* has been recently described as a new beer-spoilage LAB species (Dobson et al., 2002). *Pediococcus inopinatus* is also recognized as a potential beer spoiler (Back, 2005a; Iijima et al., 2007).

7.2.2 General features of beer-spoilage LAB

LAB contain a large group of genera and species of Gram-positive bacteria, including *Lactobacillus* and *Pediococcus*. In the period 1980–2002, approximately 60–90% of the microbiological spoilage incidents in Germany were caused by *Lactobacillus* and *Pediococcus* (Table 7.1; Back, 1994a, 1994b, 2003). A similar trend was observed in the studies conducted during the 2010–2013 period, using polymerase chain reaction (PCR) analysis (Table 7.2; Hutzler, Koob, Grammer, Riedl, & Jacob, 2012; Koob et al., 2014). Among these LAB, *L. brevis*, *L. lindneri,* and *Ped. damnosus* are considered as major beer spoilers. *L. brevis* has been reported as the most frequently detected LAB species in spoiled beer products, as well as in fermentation and maturation processes (Back, 2005a), and hence most extensively studied in brewing microbiology. *L. brevis* is widespread in the food industry and natural environments, and is generally known to be physiologically versatile in that this species grows relatively well on many laboratory culture media and in temperature ranges wider than most other beer-spoilage LAB species. However, the beer-spoilage ability of *L. brevis* varies considerably depending on the strain and the source of isolation (Back, 2005a; Suzuki, Iijima, Sakamoto, et al., 2006). Some strains spoil almost all kinds of beer, causing turbidity, sediment, and acidification, but produce no diacetyl off-flavor. In contrast, *L. brevis* strains isolated from sources other than brewing environments generally exhibit no or very weak beer-spoilage ability (Kern, Vogel, & Behr, 2014; Nakagawa, 1978; Suzuki, 2009). For these reasons,

Table 7.1 Percentages of beer-spoilage microorganisms in incident reports in Germany during 1980–2002[a]

Genus/species[b]	1980–1990	1992[c]	1993[c]	1997	1998	1999	2000	2001	2002
L. brevis	40	39	49	38	43	41	51	42	51
L. lindneri	25	12	15	5	4	10	6	13	11
L. plantarum	1	3	2	1	4	2	1	1	2
L. casei/paracasei	2			6	9	5	8	4	4
L. coryniformis	3			4	11	4	1	3	6
Ped. damnosus	17	4	3	31	14	12	14	21	12
Pectinatus	4	28	21	6	3	6	5	10	7
Megasphaera	2	7	3	2	2	4	4	4	2
Saccharomyces wild yeasts	NA	5	5	7	6	11	5	2	3
Non-*Saccharomyces* wild yeasts	NA	0	0	0	3	4	5	0	2
Others	NA	2	2	0	1	1	0	0	0

[a]This table is adapted from the studies conducted by Back (1994b, 2003, 2005a) during 1980–2002. NA: not available.
[b]*Lactobacillus brevis* includes *Lactobacillus brevisimilis,* which exhibits morphological similarities to *L. brevis*. According to Back, *L. brevis* in this table consists of several types on the basis of carbohydrate fermentation profiles, arginine utilization pattern, and morphological features, suggesting that this group of lactic acid bacteria may be further divided into separate species.
[c]In 1992 and 1993 studies, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus paracasei*, and *Lactobacillus coryniformis* were consolidated into one group.

Table 7.2 Percentages of beer-spoilage microorganisms in incident reports during 2010–2013[a]

Genus/species[b]	2010	2011	2012	2013
L. brevis	51.8	52.5	42.0	44.9
L. lindneri	10.8	6.0	2.9	0.0
L. backi	4.8	10.5	10.9	9.2
L. collinoides/paracollinoides	0.0	1.0	3.6	1.0
L. buchneri/parabuchneri	3.6	4.5	0.7	15.3
L. rossiae	1.2	0.0	0.0	1.0
L. perolens/harbinensis	2.4	4.0	1.4	4.1
L. casei/paracasei	9.6	10.0	12.3	5.1
L. (para)plantarum/coryniformis	4.8	3.0	2.9	12.2
Ped. damnosus	1.2	5.0	13.0	6.1
Ped. inopinatus	0.0	0.0	0.7	1.0
Ped. claussenii	0.0	1.0	0.0	0.0
Other *Pediococcus* spp.	0.0	0.0	0.0	0.0
Pectinatus spp.	3.6	2.0	8.0	0.0
Megasphaera cerevisiae	6.0	0.5	1.4	0.0

[a]This table is adapted from the studies conducted by Hutzler et al. (2012) and Koob et al. (2014).
[b]The identification of the genus/species was performed with polymerase chain reaction analysis.

intraspecies differentiation of beer-spoilage ability in *L. brevis* is important in the brewing industry. Some strains of beer-spoilage *L. brevis*, formerly known as *Lactobacillus frigidus*, produce extracellular capsules and show resistance to disinfectants used in breweries, and may tolerate up to 25 pasteurization units (Back, 2005a). This particular subgroup of *L. brevis* strains cause severe hazes, sediments, and ropiness in beer. In a few cases, another subgroup of *L. brevis* strains, previously known as *L. diastaticus*, utilize dextrin, causing the superattenuation of worts during fermentation (Briggs, Boulton, Brookes, & Stevens, 2004). On the other hand, *L. lindneri* is highly resistant to hop compounds and grows optimally at 19–23 °C (Back, 2005a). It is also reported that *L. lindneri* is unable to grow at temperatures higher than 28 °C. Nonetheless, this species is known to tolerate relatively high thermal treatment (up to 15 pasteurization units) and sometimes survive suboptimal pasteurization process (Back, Leibhard, & Bohak, 1992). Furthermore, *L. lindneri* grows poorly on many laboratory detection media described in the brewing industry, and often causes spoilage incidents without being detected by microbiological quality control (QC) tests (Suzuki, Asano, Iijima, Kuriyama, & Kitagawa, 2008). *L. lindneri* causes relatively faint haze and sediment with very little off-odor formation in beer (Back, 2005a). The occurrence outside brewing environments has rarely been reported for this species, although it is suggested that a LAB species closely related to *L. lindneri* was isolated from wine grapes and wine-making processes (Back, 2005b; Suzuki, 2012). One striking observation is that *L. brevis* and *L. lindneri* strains grown in beer exhibit reduced cell size and more easily penetrate membrane filters used for

the removal of microorganisms (sterile filtration) in the brewing industry (Asano et al., 2007).

Beer spoilage caused by *Ped. damnosus* is characterized by acid formation and buttery off-flavor of diacetyl (Back, 2005a). The amount of diacetyl produced by *Ped. damnosus* is high and often noticeable even with the low level of contamination. Some strains of *Ped. damnosus* produce exopolysaccharides, making beer ropy and gelatinous. *Ped. damnosus* is found commonly as a contaminant in pitching yeast and beer, but not found in brewing raw materials, suggesting that this species is particularly well adapted to the brewing environment (Priest, 2003). *Ped. damnosus* has a long association with brewing microbiology and was originally known as sarcinae because their cell morphology was confused with the cubical packets of eight cells of *Sarcinia* spp. (Briggs et al., 2004). *Ped. damnosus* is also known as one of the most frequent contaminants in fermentation and maturation processes, due partly to its ability to grow at low temperatures (Back et al., 2005a). The unexpected rise in diacetyl level during the fermentation and maturation process is often caused by the presence of *Ped. damnosus*. In addition, *Ped. damnosus* is reported to adhere to brewing yeast and sometimes induce premature sedimentation of yeast cells, resulting in retardation of the fermentation process (Priest, 2003). The adherence to the brewing yeast has been observed for *L. lindneri*, as well (Storgårds, Pihlajamäki, & Haikara, 1997), suggesting that these two species tend to be latent in fermentation and maturation processes. Furthermore, *Ped. damnosus* is known as a slow grower on laboratory detection media and often requires some beer-specific components for growth (Back et al., 2005a). *Ped. damnosus* grows at rather low temperature and its optimum temperature lies around 22–25 °C. Therefore, the incubation temperature of laboratory detection media used in QC tests should be kept relatively low (typically 25–28 °C) to comprehensively detect beer-spoilage LAB species, including *Ped. damnosus* and *L. lindneri*. In addition, the *Ped. damnosus* species is known to preferentially grow under relatively CO_2-rich environments and almost exclusively isolated from beer-brewing and wine-making environments (Back et al., 2005a). On the other hand, *Ped. inopinatus* is detected in pitching yeast but rarely in other stages of brewing processes. This species is reported to grow in beer at pH values above 4.2 and with low contents of hop bitter acids and ethanol (Sakamoto & Konings, 2003). The production of diacetyl by *Ped. inopinatus* is generally weak and less noticeable than that of *Ped. damnosus* (Priest, 2003).

L. paracollinoides, *L. backi*, and *Lactobacillus paucivorans* have been recently proposed as new species (Hutzler et al., 2013; Suzuki, Funahashi, Koyanagi, & Yamashita, 2004), and the frequencies in spoilage incidents are not well known. The genetic characterization indicates that *L. paracollinoides* and *L. backi* are closely related to *Lactobacillus collinoides* and *Lactobacillus coryniformis*, respectively. Accordingly, some of the strains belonging to *L. paracollinoides* and *L. backi* might have been misidentified as *L. collinoides* and *L. coryniformis* in the past. In addition to the above-mentioned species, *Lactobacillus acetotolerans* has been recently recognized as a beer-spoilage species (Deng et al., 2014). Similar to the case with *L. lindneri*, *L. paracollinoides*, and *L. acetotolerans* show very poor culturability on many conventional culture media, which is especially true upon primary isolation

from brewing environments (Suzuki et al., 2008a). This is probably the main reason that these *Lactobacillus* species had remained uncharacterized and underreported until recently. *L. acetotolerans* and/or its closely related species are also isolated from vinegar and sake brewing environments and known as hard-to-cultivate LAB in those industries (Suzuki, 2012). In addition, *Ped. claussenii* has been reported as a new beer-spoilage species (Dobson et al., 2002). Some strains of *Ped. claussenii* produce exopolysaccharides. All the strains of *L. paracollinoides*, *L. backi*, *L. paucivorans*, and *Ped. claussenii* characterized to date have been isolated from brewing environments and therefore considered as unique LAB species to the brewing industry.

In contrast, *Lactobacillus casei/paracasei*, *L. coryniformis,* and *Lactobacillus plantarum* are ubiquitously found in nature and exhibit relatively weak hop resistance. Therefore, these *Lactobacillus* species spoil only weakly hopped beers or those with elevated pH values (Back, 2005a). Although the frequencies of spoilage incidents by these relatively hop-sensitive lactobacilli are generally low, they are known to cause diacetyl off-flavor in beer. One trend to be noted, however, is that the spoilage incidents by *L.* (*para*)*casei* appear to have increased since 2010 (Table 7.2). This trend should be watched more closely in future surveys. *Lactobacillus curvatus*, *Lactobacillus malefermentans*, and *Pediococcus dextrinicus* were also recognized as beer-spoilage LAB species (Farrow, Phillips, & Collins, 1988; Sakamoto & Konings, 2003), but the spoilage incidents by these LAB species appear to be rare and are now considered as potential beer-spoilage LAB species of less importance (Hutzler et al., 2013). Currently, approximately 20 LAB species have been recognized as obligate or potential beer spoilers (Table 7.3), but the strain-dependent differences in beer-spoilage ability within the species are often observed (Suzuki, 2012). Additionally, the beer-spoilage ability of LAB strains is substantially affected by their physiological conditions (the degree of adaptation to hop bitter acids) and the beer types (bitterness units, pH values, and ethanol contents and other antibacterial factors). These aspects of beer-spoilage LAB will be discussed later in this chapter.

Apart from spoilage incidents of beer products, certain thermophilic lactobacilli, including *Lactobacillus delbrueckii*, have been noted as contaminants of sweet wort. They are killed by the boiling process, but if the wort is kept sweet for an extended period, even stored hot (less than 60 °C), thermophilic lactobacilli spoil sweet wort by producing lactic acid (Priest, 2006).

7.2.3 Association of beer-spoilage LAB with their habitat

L. brevis, the most frequent beer-spoilage species in the brewing industry, is isolated from a diverse source of environments, including milk, cheese, silage, feces and the intestinal tracts of mammals (Kandler & Weiss, 1986). Nonetheless, it seems that beer-spoilage ability is not an innate character for *L. brevis*. In fact, *L. brevis* strains, isolated from sources other than beer-brewing environments, were reported to generally lack beer-spoilage ability (Nakagawa, 1978). In contrast, beer-spoilage *L. brevis* strains are typically isolated from brewing environments, and are suggested to possess numerous layers of hop resistance mechanisms that appear to have been acquired in a

Table 7.3 Beer-spoilage Gram-positive bacteria and their microbiological characteristics[a]

Species	Beer-spoilage ability[b]	Primary/secondary contamination[c]	Exopolysaccharide formation[d]	Diacetyl production[e]	Culturability on MRS agar[f]
L. acetotolerans	+	s>p	–	NA	Poor
L. backi	++	p>s	–	–	Presumably good
L. brevis	++	s>p	+	–	Relatively good
L. buchneri/parabuchneri	+	p>s	+	–	Presumably good
L. casei/paracasei	+	s>p	–	+	Good
L. coryniformis	+	s>p	–	+	Good
L. collinoides/paracollinoides	++	s>p	–	NA	Poor
L. lindneri	++	p>s	–	–	Poor
L. perolens/harbinensis	+	s>p	–	+	Presumably good
L. paucivorans	++	p	–	NA	Presumably good if fructose is supplemented
L. plantarum	+	s>p	–	+	Good
L. rossiae	+	s>p	+	–	Presumably good
Lactococcus lactis	–/+	s>p	–	+	Good
Leuc. mesenteroides/paramesenteroides	–/+	s>p	+	+	Good
Kocuria kristinae	–/+	s	–	+	NA
Ped. claussenii	+	p>s	+	+	Presumably good
Ped. damnosus	++	p>s	+	+	Poor
Ped. inopinatus	+	p>s	–	–/+	Poor

[a]This table is adapted from the review authored by Hutzler et al. (2013) with some modifications (Back, 1981, 2005a; Garg, Park, Sharma, & Wang, 2010). See the review (Hutzler et al., 2013) and relevant literatures for more details.
[b]++: strong beer-spoilage ability, +: intermediate beer-spoilage ability, –/+: weak or negative beer-spoilage ability.
[c]p: primary contamination, s: secondary contamination, p>s: more cases with primary contaminations observed, s>p: more cases with secondary contaminations observed.
[d]+: strain-dependent production of exopolysaccharides may be observed, which makes beer ropy.
[e]NA: information not available, –/+: most strains produce less noticeable amount of diacetyl.
[f]NA: sufficient information is not available. "Presumably good" indicates that at least some strains have been reported to grow on MRS agar. It is possible that some others belonging to the same species show hard-to-cultivate characteristics on MRS agar.

stepwise manner during their long history of beer adaptation processes (Behr, Gänzle, & Vogel, 2006; Behr, Israel, Gänzle, & Vogel, 2007; Suzuki, 2009). Interestingly, the sequencing analysis of *gyrB* indicated that beer-spoilage *L. brevis* strains form a distinct subgroup within this species (Nakakita, Maeba, & Takashio, 2003). In addition, the comparative study on electrophoretic mobilities of D-lactate dehydrogenase (LDH) supported that beer-spoilage *L. brevis* is a phylogenetically distinct subgroup that can be discriminated from nonspoilage *L. brevis* (Takahashi, Nakakita, Sugiyama, Shigyo, & Shinotsuka, 1999). The recent study using the matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) showed that the MS spectrum profiles of strongly beer-spoilage strains cluster closely together and share remarkable similarities among this specific *L. brevis* group (Kern et al., 2014), further supporting that beer-spoilage *L. brevis* forms a phylogenetically distinct subgroup within the species of *L. brevis*. From a phenotypic viewpoint, it has been shown that beer-spoilage *L. brevis* strains tend to show preference for maltose over glucose as a fermentable sugar (Rainbow, 1981), suggesting beer-spoilage *L. brevis* is well adapted to brewing environments where maltose is a more abundant sugar source. From these findings, it is conceivable that a particular subgroup of *L. brevis* strains chose brewing environments for their habitats and evolved along the history of brewing. A recent proteomic study of hop-resistant *L. brevis* TMW 1.465 showed that up to 84% of the investigated proteins were identified based on the genome sequence data of *L. brevis* ATCC 367 (Behr et al., 2007), suggesting that approximately 20% portions of the genome was not shared between these two strains. From an evolutional standpoint, these observations suggest that considerable portions of the genome have been acquired (or lost) through the long association with brewing environments, which led to the diversification of beer-spoilage *L. brevis* from nonspoilage counterparts of this species.

L. lindneri, *L. paracollinoides*, and *Ped. damnosus* are also frequently encountered species that exhibit strong beer-spoilage ability (Back, 2005a; Iijima et al., 2007). With the exception for *Ped. damnosus*, which is occasionally found in wineries, these species have been almost exclusively isolated from beer and related environments (Back, Bohak, Ehrmann, Ludwig, & Schleifer, 1996; Storgårds, & Suihko, 1998; Suzuki, 2012), indicating that *L. lindneri*, *L. paracollinoides*, and *Ped. damnosus* are brewery-specific microorganisms. Furthermore, it has also been reported that certain components in beer promote the growth of *L. lindneri*, *L. paracollinoides*, and *Ped. damnosus* strains (Back, 2005a; Suzuki, 2012), suggesting the strong adaptation of these species to brewing environments. In addition to these observations, the sugar utilization profiles of *L. lindneri*, *L. paracollinoides*, and *Ped. damnosus* are relatively narrow (Suzuki, Asano, Iijima, & Kitamoto, 2008). The narrow sugar utilization profile was also noted for the recently proposed beer-spoilage species, *L. paucivorans*, and another newly proposed *L. backi* has been reported to ferment fewer sugars than its closest species, *L. coryniformis*. This is a feature often noted with those highly adapted to a particular environmental niche. For instance, *L. delbrueckii* sub. *bulgaricus*, a yogurt-producing LAB species, is able to utilize only a few sugars, including lactose (Suzuki, Asano, Iijima, & Kitamoto, 2008). This is in contrast to other subgroups of *L. delbrueckii*, such as *L. delbrueckii* subsp. *lactis,* which can ferment a

much wider spectrum of sugars. These differences are regarded as an indication that *L. delbrueckii* sub. *bulgaricus* is deeply adapted to milk environments, in which lactose is the predominant sugar source. Therefore, beer-spoilage LAB, such as *L. lindneri*, *L. paracollinoides,* and *Ped. damnosus*, might have lost the ability to utilize a wide variety of sugars due to the deep associations with a particular environmental niche. Taken together, these findings strongly suggest the close associations of *L. lindneri*, *L. paracollinoides,* and *Ped. damnosus* with brewing environments.

Furthermore, strains belonging to these three species show hard-to-cultivate characteristics on primary isolation and often fail to grow on de Man, Rogosa, and Sharpe (MRS) agar and Raka-Ray agar, the laboratory detection media widely recommended for beer-spoilage LAB by major brewery associations (Suzuki, Asano, Iijima, Kuriyama, & Kitagawa, 2008a). Interestingly, recent studies have shown that repeated passages in beer gradually reduce the culturability of originally easy-to-cultivate strains of *L. lindneri* and *L. paracollinoides*, eventually leading to the acquisitions of hard-to-cultivate strains that mimic the state of primary isolates of these species (Suzuki, 2012). Strikingly, it was observed that highly beer-adapted strains of *L. lindneri* and *L. paracollinoides* die swiftly on MRS agar, a behavior that is in sharp contrast to that of the easy-to-cultivate counterparts of the same species that grow well on MRS agar. It has been increasingly recognized that microorganisms too deeply associated with a particular environment tend to exhibit hard-to-cultivate characteristics (Suzuki, 2012). These lines of evidence therefore suggest the profound association of these species with brewing environments and indicate that the physiological characteristics of beer-spoilage LAB living in brewing environments are drastically different from those of laboratory strains maintained in nutrient-rich media (Suzuki, Asano, Iijima, Kuriyama, & Kitagawa, 2008a). It should be also noted that, because of the hard-to-cultivate nature of *L. lindneri*, *L. paracollinoides*, and *Ped. damnosus*, these LAB species may have been underreported as causative agents of microbiological incidents in the brewing industry.

Taken collectively, these observations suggest that the beer-spoilage LAB species have long been associated with brewing environments, and a deeply beer-adapted status presumably represents their intrinsic state in nature. Therefore, these features should be taken into account when brewing microbiologists develop any QC methods for beer-spoilage LAB. This aspect of beer-spoilage LAB will be further discussed in the third section of this chapter.

7.2.4 Factors affecting the growth of LAB in beer

Growth capability of beer-spoilage LAB depends on the strain and the type of beer. In one study, the ability of 14 strains of hop-resistant LAB (*Lactobacillus* spp. and *Pediococcus* spp.) to grow in 17 different beers was assessed using a biological challenge test (Fernandez & Simpson, 1995). A statistical analysis of the relationship between spoilage potential and 56 parameters of beer composition revealed a correlation with eight parameters: pH, beer color, the content of free amino nitrogen, total soluble nitrogen, a range of individual amino acids, maltotriose, and the undissociated forms of SO_2 and hop bitter acids. Among them, the correlation coefficient of pH value and

undissociated hop bitter acids were found to be −0.72 and 0.70 respectively, suggesting that these two factors have strong influence on the sensitivity of beers to spoilage by LAB. Hop bitter acids are hypothesized to act as mobile ionophores, and their activity is pH dependent. Low pH favors antibacterial activity, but high pH reduces it. Small changes in beer pH are known to cause large changes in the antibacterial activity of hop bitter acids in beer. For instance, it has been shown that a change in pH of as little as 0.2 can reduce the protective effect of hop compounds by 50% (Simpson, 1993). However, it seems evident that factors other than pH and hop bitter acids are also influential in determining the susceptibility of beer to spoilage by LAB.

As growth inhibitors, the phenolic compounds, such as phytic acid and ferulic acid, have been shown to be antimicrobial in beer, and the antimicrobial effects of ferulic acid are significantly enhanced when it is converted enzymatically to 4-vinyl guaiacol (Hammond, Brennan, & Price, 1999). In addition, undissociated SO_2 seems to have a negative effect on the growth of LAB in beer (Fernandez & Simpson, 1995). Carbon dioxide, which is considered as a growth promoter for *Lactobacillus* and *Pediococcus* at low concentrations (<0.3 g/L), was also found to be inhibitory at the concentrations found typically in beer, indicating that beers with low carbon dioxide can be more prone to spoilage by LAB. Therefore, there is a need for particular attention to be given to hygiene when dealing with cask-conditioned beers of low carbon dioxide content and beers dispensed with nitrogen gas. It has also been suggested that the use of LAB in bioacidification of wort is beneficial in enhancing the microbiological stability of finished beer. This is because LAB produce lactic acid that lowers pH value of finished beer products (often below 4.3), and possibly form antibacterial compounds, including bacteriocins (Gänzle, 2004; Vaughan, O'Sullivan, & van Sinderen, 2005).

As growth promoters, citrate, pyruvate, malate, and arginine in beer were shown to be utilized by beer-spoilage LAB (Suzuki, Iijima, Ozaki, & Yamashita, 2005b). These four components were also found to yield ATP in beer-spoilage LAB, indicating that these energy sources help them grow in beer where nutrients are almost depleted by the fermentative activities of brewing yeast. As other nutrient sources in beer, varying amounts of maltose, maltotriose, maltotetraose, lysine, and tyrosine were found to be consumed by beer-spoilage LAB strains. In some cases, dextrins up to 14 or 15 glucose units were hydrolyzed (Lawrence, 1988). Taken together, the sensitivities of beer to spoilage by LAB are determined by various growth inhibitors and promoters present in beer, although pH value and undisocciated hop bitter acids are predominant factors.

7.2.5 Probiotic potential of beer-spoilage LAB

Increasingly *L. brevis* is recognized to possess beneficial effects on human health as probiotics. For instance, *L. brevis* KB290 was reported to be useful for early intervention in irritable bowel syndrome and to improve gut health (Waki et al., 2013), and particularly relevant for brewers are the studies conducted on *L. brevis* SBC8803 (Segawa, Nakakita, et al., 2008; Segawa, Wakita, Hirata, and Watari, 2008). This strain was isolated from barley malt for brewing and, according to the authors, exhibits growth capability in beer. The oral intake of *L. brevis* SBC8803 was suggested to

alleviate not only allergic symptoms related to type I allergy but also alcoholic liver diseases, especially the development of alcohol-induced fatty liver. Although the use of LAB is currently limited to special beers such as lambic beers, these studies indicate the promising potential of beer-spoilage LAB as probiotics. It is generally known that LAB surviving harsh environments are able to tolerate acidic conditions and bile acids encountered in human digestive systems and are more likely to show probiotic effects there. Beer can be considered as one of those harsh environments, and beer-spoilage LAB are able to survive in the brewing environments. In the future, these aspects of beer-spoilage LAB should be more vigorously studied in the brewing industry.

7.3 Hop resistance mechanisms in beer-spoilage LAB

Hop resistance in beer-spoilage LAB is a progressively evolving area of research, and many studies have been conducted to elucidate the inhibitory effects of hop and the resistance to these inhibitory effects. In this section, recent progress in this area of research is briefly summarized and, in relevant cases, the examples for spoilage LAB in sake (Japanese rice wine) and wine are described for references. This is because these alcoholic beverages represent harsh environments characterized by low pH value and high ethanol content, and, in this sense, spoilage LAB in sake and wine exhibit responses similar to those of beer-spoilage LAB. More information concerning this aspect of spoilage LAB in alcoholic beverages is available from the previous literature (Suzuki, 2012).

7.3.1 Antibacterial effects of hop bitter acids

The antibacterial activities of α-acid (humulone) and β-acid (lupulone) have been studied since before 1950. Their antibacterial activities are higher than those of iso-α-acids, but these nonisomerized hop acids dissolve to a lesser extent in beer and water, so iso-α-acids are considered as a principal antibacterial agent in beer (Sakamoto & Konings, 2003). Antibacterial effects of hop bitter acids were extensively investigated by Simpson (1993) and Simpson & Fernandez (1994). According to a series of meticulous studies, hop bitter acids were found to act as protonophores and inhibit the growth of hop-sensitive LAB strains by dissipating the transmembrane pH gradient. In LAB, transmembrane pH gradient is an important component of proton motive force (PMF), providing mechanisms by which generation of energy (ATP) and its utilization for nutrient transport can be coupled. In addition, the intracellular pH influences nutrient transport and metabolic processes. It was thus suggested that the hop-induced reduction in intracellular pH leads to the inhibition of nutrient transport and thereby the starvation of hop-sensitive LAB strains. Accompanied by the dissipation of transmembrane pH gradient, the loss of intracellular Mn^{2+} was observed, and it was suggested that hop bitter acids exchange protons for cellular divalent cations, such as Mn^{2+}. In this hypothetical model, hop anions bind with intracellular divalent cations including Mn^{2+} and diffuse them out of the cell. Thus, the ionophoric action of hop bitter acids, together with the diffusion of the hop–metal complex, result in an electroneutral exchange of cations across the

cytoplasmic membrane, leading to the growth inhibition of LAB (Sakamoto & Konings, 2003). Although Mn^{2+} is required for growth and survival of most bacteria, many LAB are known to have higher requirements of Mn^{2+} and accumulate high intracellular levels of Mn^{2+} (Groot et al., 2005; Vogel, Preissler, & Behr, 2010). Therefore, the loss of divalent cations, Mn^{2+} in particular, is presumably detrimental for the survival of LAB in hop-containing environments.

7.3.2 Hop resistance mechanisms associated with cytoplasmic membrane

Because hop bitter acids are assumed to intrude into the cells as proton ionophores, it is important for beer-spoilage LAB to alleviate the intrusion of hop compounds into the cell. The *horA* and *horC* genes, originally identified in *L. brevis*, have been shown to confer hop resistance on LAB. HorA, a product of the *horA* gene, was demonstrated to act as an ATP-binding cassette (ABC) transporter and to efflux hop bitter acids out of the cells (Figure 7.1). It was also shown that HorA confers resistance to multiple drugs that are structurally unrelated to hop bitter acids, making this protein the second member of the multidrug ABC transporters discovered in bacteria (Sakamoto, Margolles, van Veen, & Konings, 2001). On the other hand, the presumed secondary structure of HorC is similar to those of PMF-dependent multidrug transporters belonging to the resistance–nodulation–cell division (RND) superfamily (Suzuki, Iijima, Ozaki, & Yamashita, 2005a). The functional expression of HorC in *L. brevis* demonstrated that this protein confers resistance to hop bitter acids, as well as other structurally unrelated drugs. Therefore, HorC was postulated to function as a PMF-dependent multidrug efflux pump, and a defense mechanism similar to that of HorA was hypothesized (Figure 7.1; Iijima, Suzuki, Ozaki, & Yamashita, 2006). Accordingly, the activities of HorA and HorC presumably result in a reduced net influx of the undissociated and

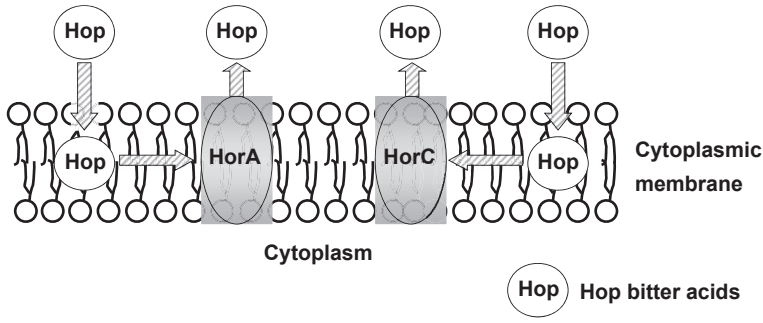

Figure 7.1 Efflux activities of hop bitter acids by HorA and HorC. HorA was shown to act as an ABC multidrug transporter and alleviate the intrusion of hop bitter acids into the cytoplasm. On the other hand, HorC was suggested to function as a proton motive force (PMF)–dependent multidrug transporter and to extrude hop bitter acids in a manner similar to that of HorA. In addition, HorC was postulated to act as a homodimer (Iijima, Suzuki, Asano, Ogata, & Kitagawa, 2009). The secondary structures of HorA and HorC were described previously (Suzuki, 2012).

membrane-permeable hop bitter acids into the cytoplasm, and thereby limit the antibacterial protonophoric effect of hop-derived compounds. However, since beer-spoilage LAB strains develop resistance against rather high concentrations of hop bitter acids, the question arises as to whether functional expression of HorA and HorC is sufficient to confer hop resistance or whether other activities could contribute to defense mechanisms against hop bitter acids.

Hop compounds are weak acids, which can cross the cytoplasmic membrane in the undissociated form (Simpson, 1993). Due to the higher intracellular pH, hop bitter acids dissociate internally, thereby dissipating the transmembrane pH gradient. As a result of this protonophoric action of hop bitter acids, the viability of the exposed bacteria decreases. On the other hand, microorganisms have been found to increase PMF-generating activities in their cytoplasmic membranes when they are confronted with a high influx of protons (Suzuki, 2012). Therefore, it is conceivable that, to defend against the antibacterial effects of hop bitter acids, beer-spoilage LAB strains respond by increasing the rate at which protons are expelled out of cells. In fact, the hop-resistant LAB strains were found to maintain a larger transmembrane pH gradient than hop-sensitive strains (Simpson, 1993), and *L. brevis* was demonstrated to increase the activity and expression level of proton-translocating ATPase upon acclimatization to hop bitter acids (Sakamoto, van Veen, Saito, Kobayashi, & Konings, 2002). These findings indicate that the extrusion of protons by proton-translocating ATPase counteracts the ionophoric effects of hop compounds and helps beer-spoilage LAB strains maintain the transmembrane pH gradient.

Given that the above defense mechanisms are energy consuming in nature, beer-spoilage LAB strains require substantial energy sources to grow in beer. Nevertheless, beer is generally considered as a poor medium to support the growth of bacteria, because most of the nutrients have been depleted by brewing yeast. Furthermore, it has been reported that the protonophoric action of hop compounds inhibits the uptake of nutrients by bacteria (Simpson, 1993). Despite these disadvantages, beer-spoilage LAB strains are still capable of growing in beer. Indeed three beer-spoilage LAB species, *L. brevis*, *L. lindneri*, and *L. paracollinoides*, were found to exhibit strong ATP-yielding ability in beer (Suzuki et al., 2005b). The inoculation tests into beer indicated that citrate, pyruvate, malate, and arginine were consumed to support the growth of beer-spoilage LAB strains in beer. The four components induced considerable ATP production even in the presence of hop compounds, accounting for the ATP-yielding ability of the spoilage LAB strains observed in beer. In general, the metabolism of organic acids and amino acids in LAB is known to directly or indirectly enhance the energy production and PMF generation in conditions in which nutrients are otherwise scarce. The putative metabolic pathways of these substrates have been discussed in the previous literature (Suzuki et al., 2005b; Suzuki, Iijima, Sakamoto, et al., 2006).

In contrast to these active hop resistance mechanisms described so far, passive defense mechanisms are also important, in which energy sources are not required once they are established. In *L. brevis*, the membrane composition was reported to change toward the incorporation of more saturated fatty acids, such as C16:0, rendering the membrane less fluid and protecting the cell against the intruding hop bitter acids (Behr et al., 2006). This phenomenon is reminiscent of sake-spoilage *Lactobacillus*

fructivorans that possesses long-chain fatty acids exceeding 24 carbons in length, which are not observed in ordinary LAB (Ingram, 1986). The proportion of these long-chain fatty acids reaches 30–40% in the entire fatty acid compositions of the membrane, when sake-spoilage *L. fructivorans* is grown in an environment containing a high concentration of ethanol. It is presumed that these unusually long-chain fatty acids prevent the intrusion of ethanol into the cytoplasmic membrane. In wine-associated lactic acid bacterium *Oenococcus oeni*, the changes in membrane fluidity coupled with the upregulation of heat-shock proteins lead to the reduction in the permeability of membrane and reinforcement of membrane structures, thereby protecting cells from the bactericidal effects of ethanol (Graça da Silveira, Vitória San Romão, Loureiro-Dias, Rombouts, & Abee, 2002; Grandvalet et al., 2008). From these observations, defense mechanisms associated with cytoplasmic membrane are generally important for LAB living in harsh environments.

7.3.3 Hop resistance mechanisms associated with cell wall

In beer-spoilage *L. brevis*, it has been shown that higher-molecular-weight lipoteichoic acids (LTAs) in cell wall increase in response to the presence of hop bitter acids (Behr et al., 2006). These changes in the compositions of LTAs are suggested to reduce the intrusion of hop bitter acids into cells by increasing the barrier functions of the cell wall against hop bitter acids. LTAs are also hypothesized to act as reservoirs of divalent cations, such as Mn^{2+}, which are otherwise scarce as a result of complexation with hop compounds (Behr et al., 2006; Vogel et al., 2010). The altered LTAs have an increased potential to bind with divalent cations and compete for them with hop bitter acids, thus reducing the detrimental effects of hop toward the cells. This type of resistance can also be considered as a passive defense mechanism that requires little energy once established. In relation to the reservoir function of LTAs for Mn^{2+}, Hayashi, Ito, Horiike, and Taguchi (2001) proposed HitA as one of the mediators of hop resistance in *L. brevis* and suggested that HitA plays a role in the uptake of divalent cations, such as Mn^{2+}, whereas hop bitter acids reduce the intracellular divalent cations. Thus HitA may modulate and maintain the levels of intracellular divalent cations in beer-spoilage LAB. In fact, many of the proteins involved in energy generation and redox homeostasis are dependent on Mn^{2+}; therefore, intracellular Mn^{2+} plays an important role in LAB (Behr et al., 2007). Accordingly, these mechanisms relating to the cell envelope may function in concert for beer-spoilage LAB to counteract the loss of intracellular Mn^{2+}.

Defense mechanisms against toxic compounds involving the cell wall are also known for LAB in other alcoholic beverage industries. In sake-spoilage *L. fructivorans* and *Lactobacillus homohiochi*, for instance, the presence of ethanol has been reported to induce the increase in cell wall thickness (Suzuki, 2012). It was thus suggested that the increase in cell wall thickness is involved in ethanol tolerance observed in sake-spoilage LAB. In another instance, the *gtf* gene that encodes glucosyltransferase is known to exist in some strains of wine-associated *O. oeni* (Dols-Lafargue et al., 2008). The presence of this gene induces the formation of the cell envelope, consisting mainly of β-glucans, and elevates the ethanol tolerance of *O. oeni* strains that possess the *gtf* gene. Accordingly, the defense mechanisms associated with the cell wall appear to play a vital role for various LAB in alcoholic beverages.

7.3.4 Other hop resistance mechanisms

It has been reported that Mn^{2+}-dependent enzymes are induced by hop bitter acids in *L. brevis* (Behr et al., 2007). These hop-inducible enzymes are suggested to be involved in energy generation and redox homeostasis. One explanation for this phenomenon is that the cells respond to Mn^{2+} limitations by upregulating these enzymes, thus compensating for the loss of Mn^{2+}-dependent enzyme activities caused by the reduced intracellular manganese availability. It has been therefore suggested that beer-spoilage LAB can cope with low intracellular manganese levels, where hop-sensitive LAB cannot maintain metabolic activities. Relatively recently, the antibacterial mechanisms of hop compounds have been suggested to involve proton ionophoric actions and redox-reactive uncoupler activities occurring in parallel (Behr & Vogel, 2010). Accordingly, it is plausible that beer-spoilage LAB have to cope with oxidative stress induced by hop compounds, in addition to PMF and intracellular Mn^{2+} depletion. Thus, the observed upregulation of Mn^{2+}-dependent enzymes responsible for redox homeostasis is most likely part of defensive responses to the oxidative stress caused by hop bitter acids (Vogel et al., 2010). In this hypothetical model, intracellular Mn^{2+} can be a target for hop-induced oxidative stress; thus hop-resistant LAB may actively adjust the intracellular Mn^{2+} concentrations to lower levels. Therefore, it is possible that beer-spoilage LAB maintain the appropriate levels of intracellular Mn^{2+} for survival in hop-containing environments. However, this aspect of hop resistance mechanisms has not been fully explored and the potential control of intracellular Mn^{2+} levels by beer-spoilage LAB will have to be further examined in future studies.

On the other hand, the morphological shifts into smaller rods were observed in beer-adapted *L. brevis* and *L. lindneri* cells (Figure 7.2; Asano et al., 2007). The diminished cell size is presumably due to the efforts by beer-spoilage LAB to reduce surface area that is in contact with beer. This is conceivable because beer contains many bactericidal factors, including hop compounds. In addition to reducing the defense perimeters, the minimized cell surface area presumably helps beer-spoilage LAB deploy membrane-bound resistance mechanisms more efficiently (Suzuki, 2012). In similar cases, it has been observed that sake-spoilage LAB remain morphologically compact in the presence of high ethanol content, whereas ethanol-sensitive LAB tend to

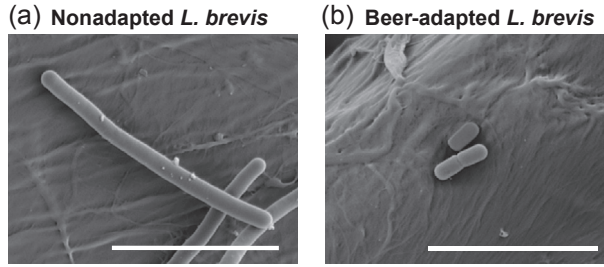

Figure 7.2 Effects of beer adaptation on morphological features of beer-spoilage lactic acid bacteria (LAB). Beer-spoilage *Lactobacillus brevis* was grown in MRS broth (a) and degassed beer (b). Cells were trapped on a membrane filter and the morphological features of beer-adapted and nonadapted strains were compared using scanning electron microscopy. Bar, 5 μm. Similar tendencies were also observed for beer-adapted *Lactobacillus lindneri* (data not shown).

exhibit elongated cell forms (Suzuki, Asano, Iijima, & Kitamoto, 2008). From these observations, the reduced surface area that is in contact with external environments is probably advantageous for spoilage LAB that must survive in hostile milieu.

The hop resistance mechanisms described above are summarized in Figure 7.3. However, it should be noted that hop resistance mechanisms are more complex than previously assumed. Presumably these multiple layers of defense systems in beer-spoilage LAB have been acquired progressively through centuries of brewing

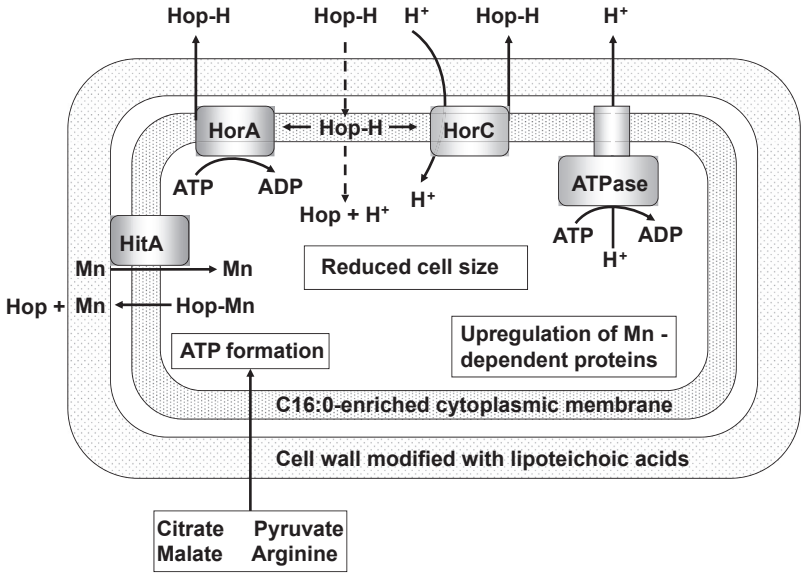

Figure 7.3 Complex hop resistance mechanisms in beer-spoilage *Lactobacillus brevis*. Hop resistance mechanisms recently reported are comprised of the following defense systems. (1) Alleviation of ionophoric actions of hop compounds: Mechanisms for prevention of hop incursions involve HorA and HorC as efflux transporters, cytoplasmic membrane modifications, and cell wall modifications. These systems presumably function together to reduce the incursion of undissociated and membrane-permeable hop compounds (Hop-H). Proton-translocating ATPase also counteracts the proton ionophoric actions of hop compounds by pumping out intruding protons. (2) Mn^{2+} homeostasis and countermeasures against the diffusions of intra cellular Mn^{2+}: Intracellular Mn^{2+} levels are maintained by the actions of the putative Mn^{2+} transporter HitA. In addition, modified cell wall functions as Mn^{2+} reservoirs, and presumably counteracts the loss of intracellular Mn^{2+}. Furthermore, Mn^{2+}-dependent proteins are upregulated in response to hop compounds. The upregulation of these proteins presumably compensates for the loss of their activities caused by reduced intracellular Mn^{2+} levels. It is also hypothesized that the upregulated Mn^{2+}-dependent proteins that are involved in redox homeostasis counteract the oxidative stress conferred by hop compounds. (3) Energy supply: Metabolisms with citrate, pyruvate, malate, and arginine supply ATP and proton motive force (PMF) for active defense mechanisms involving HorA, HorC, and proton-translocating ATPase. (4) Other defense systems: Morphological shifts into smaller rods reduce the contact areas against hostile external milieu and help beer-spoilage lactic acid bacteria (LAB) to more efficiently deploy membrane-bound defense mechanisms, such as those driven by HorA, HorC, and proton-translocating ATPase.

history. Undoubtedly these are only part of the whole resistance mechanisms of beer-spoilage LAB and novel defense mechanisms will be found in future. In addition, the inhibitory actions of hop compounds have recently been shown to involve oxidative stress. This newly found inhibitory mechanism goes beyond the proton ionophoric actions and Mn^{2+} depletion activities of hop compounds that have been traditionally accepted. It is hoped that more comprehensive pictures will emerge concerning the hop resistance of beer-spoilage LAB, which eventually leads to the development of more accurate QC tests in breweries.

7.3.5 Hop resistance genes and their distribution in beer-spoilage LAB

The distribution of hop resistance genes *horA* and *horC* has been investigated, using 167 strains that consist of various species of LAB and frequent brewery isolates. As a result of PCR and Southern blot analysis, *horA* and *horC* homologs have been detected widely and almost exclusively in beer-spoilage LAB strains (Iijima et al., 2007; Sami et al., 1997; Suzuki et al., 2005a). Among the 88 beer-spoilage LAB strains, 82 strains possessed *horA* homologs, whereas *horC* homologs were detected in 86 strains (Figure 7.4). When LAB strains with weak beer-spoilage ability were included, the presence of *horA* and *horC* homologs was found to be almost completely exclusive in beer-spoilage LAB strains, indicating that *horA* and *horC* are uniquely associated with beer-spoilage LAB. Equally interestingly, PCR and Southern blot analysis indicated that the flanking DNA regions of the hop resistance genes are detected simultaneously with *horA* and *horC* homologs in these beer-spoilage LAB

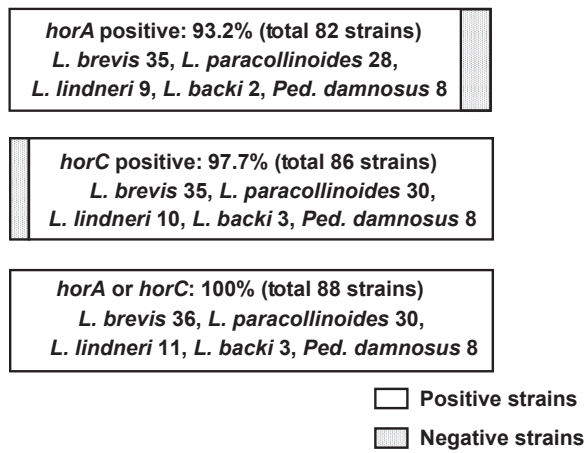

Figure 7.4 Compensatory relationship between *horA*- and *horC*-specific determination methods for beer-spoilage ability of lactic acid bacteria (LAB) strains. A total of 88 strains belonging to various beer-spoilage species were examined by polymerase chain reaction and Southern blot analysis. It was shown that beer-spoilage LAB strains possess at least one of the genetic markers, indicating that *horA* and *horC* are excellent genetic markers for comprehensibly determining beer-spoilage ability of LAB.

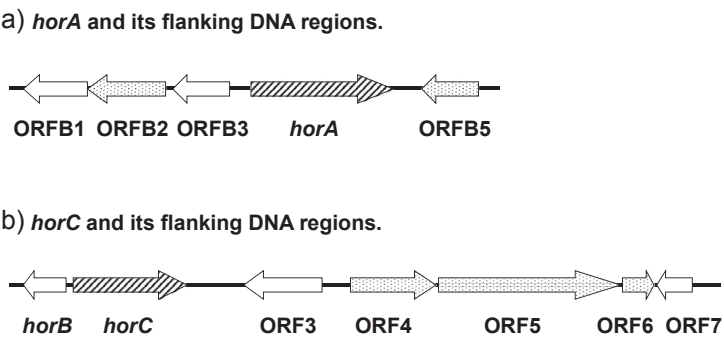

Figure 7.5 ORF structures of the gene clusters containing *horA* and *horC*. The hop resistance genes, *horA* and *horC*, are indicated by the striped arrows and the open reading frames potentially involved in cell wall synthesis are shown by the dotted arrows. (a) *horA* and its flanking DNA regions (*ca* 5.6 kb) (b) *horC* and its flanking DNA regions (*ca* 8.2 kb).

strains (Suzuki, Iijima, Sakamoto, et al., 2006). From these observations, it is quite conceivable that the flanking open reading frames (ORFs) in *horA*- and *horC*-carrying gene clusters collectively confer hop resistance on LAB, and indeed some of the ORFs in these gene clusters potentially encode proteins that may be involved in cell wall synthesis (Figure 7.5).

From a practical point of view, it is interesting to note that all of the beer-spoilage LAB strains examined in the studies were found to possess at least one of the homologs of hop resistance genes (Figure 7.4). This insight indicates that *horA* and *horC* are excellent genetic markers for the species-independent determination of beer-spoilage ability of LAB strains. In addition, the combined use of *horA* and *horC* was proposed for the detection of as-yet uncharacterized beer-spoilage LAB species, as well as the established spoilage species (Suzuki et al., 2005a). Therefore, *horA*- and *horC*-specific detection methods may find potential applications in microbiological QC in breweries. This aspect of *horA* and *horC* was more thoroughly reviewed in previous literature (Suzuki, Iijima, Sakamoto, et al., 2006). Other genetic markers for differentiating beer-spoilage ability of LAB are listed in Table 7.4. These species-independent methods have been known to be useful for detecting previously unencountered beer-spoilage species and differentiating intraspecies differences in beer-spoilage ability of LAB, but it is possible that they have some false-positive and false-negative results. Therefore, these species-independent genetic markers should be used in conjunction with conventional species identification methods to conduct comprehensive QC tests.

7.3.6 Hypothetical origin of beer-spoilage LAB

It has been known that strong beer-spoilage species are not closely related to each other (Figure 7.6). However, the recent developments in this area of research suggest that hop resistance genes, such as *horA* and *horC*, transformed originally nonspoilage LAB strains into beer-spoilage strains through plasmid- and transposon-mediated

Table 7.4 Hop resistance genes and genetic markers for the determination of beer-spoilage ability of LAB

Genes/genetic markers	Functions	Correlations with beer-spoilage ability	References
horA	ABC multidrug transporter	*Lactobacillus* spp. and *Pediococcus* spp.	Deng et al. (2014), Ehrmann et al. (2010), Haakensen et al. (2007), Pitett et al. (2012), Sami et al. (1997), Suzuki et al. (2005a)
horB	Putative transcriptional regulator of *horC*	*Lactobacillus* spp. and *Pediococcus* spp.	Fujii, Nakashima, and Hayashi (2005), Iijima et al. (2006, 2007, 2009); Suzuki et al. (2005a)
horC	Putative PMF-dependent multidrug transporter	*Lactobacillus* spp. and *Pediococcus* spp.	Suzuki et al. (2005a), Fujii et al. (2005), Iijima et al. (2006, 2007, 2009)
hitA	Putative Mg^{2+} transporter	*Lactobacillus brevis*	Behr et al. (2006), Hayashi et al. (2001)
bsrA	Putative ABC multidrug transporter	*Pediococcus* spp.	Haakensen et al. (2009)
bsrB	Putative ABC multidrug transporter	*Pediococcus* spp.	Haakensen et al. (2009)
ORF5 and adjacent DNA regions	Putative genes associated with cell wall synthesis	*Lactobacillus* spp. and *Pediococcus* spp.	Fujii et al. (2005), Suzuki et al. (2005a), Suzuki, Koyanagi, and Yamashita (2004)

horizontal gene transfer (Figure 7.7; Haakensen et al., 2007; Suzuki, Iijima, Sakamoto, et al., 2006). In addition, the comparative analysis of the gene clusters harboring *horA* and *horC* indicate that their nucleotide sequences are approximately 99% identical among distinct beer-spoilage LAB species. These findings suggest that the acquisitions of hop resistance genes by LAB strains were relatively recent events in the long history of LAB evolution (Figure 7.8).

Beer-spoilage LAB strains have been almost invariably isolated from beers and related environments, and therefore have been regarded as the microorganisms unique to brewing environments. Hop resistance genes *horA* and *horC* have also been found almost exclusively in beer-spoilage LAB (Suzuki, Iijima, Sakamoto, et al., 2006). Therefore, beer-spoilage LAB and hop resistance genes are unique to beers and related

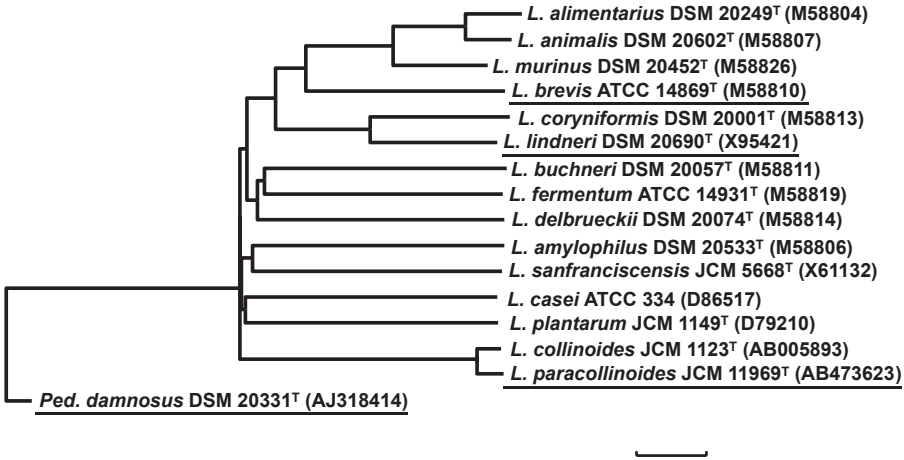

Figure 7.6 Phylogenetic tree of *Lactobacillus* and *Pediococcus* species derived from 16S rRNA gene sequence data, using neighbor-joining method for calculation. The bar indicates the number of inferred substitutions per 100 nucleotides. The accession numbers of 16S rRNA gene sequence are shown in parentheses, and the strong beer-spoilage species are underlined.

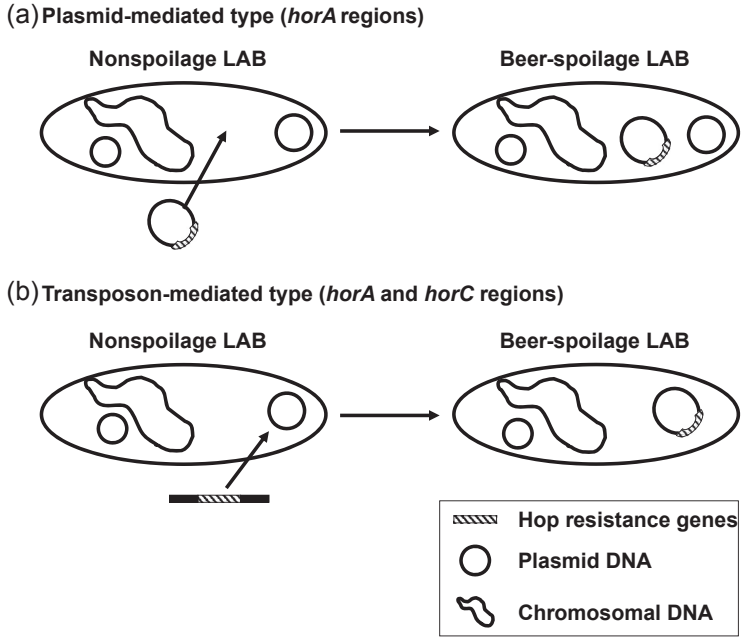

Figure 7.7 Hypothetical horizontal transfer of *horA* and *horC*. Two modes of horizontal transfer of hop resistance genes, plasmid-mediated (a) and transposon-mediated (b) types, have been postulated on the basis of the nucleotide sequence identities and open reading frame analysis of *horA*- and *horC*-containing DNA regions identified in *Lactobacillus brevis*, *Lactobacillus lindneri*, *Lactobacillus paracollinoides*, *Lactobacillus backi*, *Pediococcus damnosus,* and *Pediococcus inopinatus*. The exact mechanisms underlying the horizontal gene transfer of *horA* and *horC* are currently unknown, but several mechanisms, including conjugative transmission of hop resistance genes, are postulated.

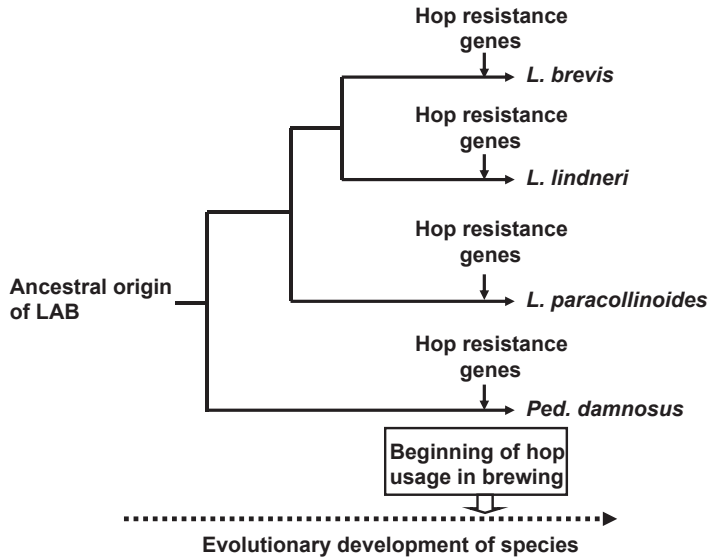

Figure 7.8 Hypothetical origin of beer-spoilage lactic acid bacteria (LAB) species. Based on the extraordinarily high-sequence identities observed with various species of beer-spoilage LAB, the acquisitions of the hop resistance genes are considered to be relatively recent events. Although the exact point of these events cannot be determined in the history of species evolution, it may have occurred as brewers widely adopted hop for a raw material in brewing.

environments. The insight that the presence of hop bitter acids is required in culture media for the maintenance of hop resistance genes further indicates that beer-spoilage LAB and hop resistance genes have been inextricably linked with the historical development of hop use in brewing. Conceivably, hop resistance genes chose LAB as companions for their own survival, and conversely LAB allowed the states of symbiosis with hop resistance genes to continue in order to gain decisive advantages in brewing environments in which almost no other microorganisms can survive. In other words, beer-spoilage LAB and hop resistance genes have developed mutually beneficial relationships along the long history of brewing.

At this time, it is difficult to determine exactly when beer-spoilage LAB emerged in the history of brewing. However, it seems increasingly likely that the progressively widespread use of hops in brewing has been responsible for the emergence and spread of beer-spoilage LAB and hop resistance genes. The origin of the use of hops in brewing is still surrounded by controversy, and many aspects of the early cultivation of hop are unclear. It is, however, reasonable to assume that the cultivation of hops began in Central Europe sometime between the fifth and seventh centuries (Barth, Klinke, & Schmidt, 1994). On the other hand, the records of hop use in brewing has existed since around 1079 (Moir, 2000), so it is plausible that the first use of hops in brewing occurred between these periods. It is therefore tempting to imagine that beer-spoilage LAB and hop resistance genes have emerged and spread with the increasingly widespread use of hops in brewing worldwide for the past 10–15 centuries.

7.4 Subculture and preservation methods of beer-spoilage LAB

As discussed earlier in this chapter, beer-spoilage LAB strains have long been associated with brewing environments, and the deeply beer-adapted status presumably represents their intrinsic states in nature. On the basis of this hypothesis, beer-spoilage LAB strains to be used for the development of QC tests in breweries should be maintained and preserved as a culture stock so that they remain in a state as if they were living in the brewing environments. Otherwise, important physiological and genetic traits of beer-spoilage LAB might be changed during the subculture and preservation processes. In this section, it will be illustrated that the subculture and preservation methods affect various aspects of beer-spoilage LAB, and a new approach will be proposed to minimize the artifacts caused by the conventional subculture and preservation methods.

7.4.1 Stability of hop resistance ability

Induction of hop resistance ability in beer-spoilage LAB strains is important, especially when biological challenge tests are conducted to evaluate the microbiological stability of beer products. The levels of hop resistance ability in beer-spoilage LAB depend largely on the preculture conditions. For example, hop resistance ability of *L. brevis* strains can be elevated by adding the subinhibitory concentrations of hop bitter acids to culture media used for preculture. It has been reported that hop-adapted strains of beer-spoilage *L. brevis* exhibit 4- to 12-fold increased levels of hop resistance ability compared with those of preadapted *L. brevis* strains (Simpson, 1993; Suzuki, Iijima, Sakamoto, et al., 2006). Conversely, strongly hop-resistant *L. brevis* strains were shown to exhibit gradually diminished hop resistance ability when subcultured with hop-free culture media. Therefore, subculture conditions substantially affect the hop resistance ability; in particular, the presence or absence of hop bitter acids in preculture media plays an important role in determining the hop resistance ability and beer-spoilage ability of the LAB strains tested. However, when laboratory culture media are used to induce hop adaptation, caution should be exercised because some components in media, such as Tween 80, inhibit or retard the hop adaptation process of beer-spoilage LAB strains (Simpson & Smith, 1992). Accordingly, MRS broth without Tween 80 was proposed as a base medium for hop adaptation. It is also the experience of this author that the use of beer with somewhat weaker microbiological stability as a preculture medium is one good way to induce hop resistance ability/beer-spoilage ability of a wide variety of spoilage LAB strains.

7.4.2 Stability of hop resistance genes

It has been reported that the repeated subculture of beer-spoilage LAB strains often leads to the loss of the hop resistance genes *horA* and *horC* (Suzuki, Iijima, Sakamoto, et al., 2006). This phenomenon typically occurs when laboratory culture media are

used without hop bitter acids for subculture. It has also been observed that the loss of these hop resistance genes is accompanied by the reduced levels of hop resistance ability and/or beer-spoilage ability. This phenomenon was originally reported to occur in beer-spoilage *L. brevis* strains, but similar observations were subsequently made in beer-spoilage *L. paracollinoides*, *L. lindneri,* and *Ped. damnosus* (Table 7.5; Suzuki, Iijima, Sakamoto, et al., 2006). The addition of hop bitter acids to laboratory culture media prevents or retards the loss of hop resistance genes. In a few cases, however, the loss of hop resistance genes was still observed after the repeated subcultures, even when laboratory culture media were supplemented with subinhibitory levels of hop bitter acids. In contrast, it was observed that the hop resistance genes are stably maintained in degassed beer even after more than 100 subcultures, suggesting that *horA* and *horC* are required for growth in beer and may be responsible not only for hop

Table 7.5 The isolation of nonspoilage variants from beer-spoilage LAB and the loss of hop resistance genes in nonspoilage variants[a]

Species	Strain no.[b]	Beer-spoilage ability[c]	Hop resistance genes	
			horA	*horC*
L. brevis	ABBC44	+	+	–
	ABBC44^{NB}	–	–	–
	ABBC45	+	+	+
	ABBC45^{CC}	–	–	–
	ABBC46	+	+	+
	ABBC46^{NB}	–	–	–
	ABBC64	+	+	+
	ABBC64^{NB}	–	–	–
	ABBC104	+	+	+
	ABBC104^{NB}	–	–	–
	ABBC400	+	+	+
	ABBC400^{NB}	–	–	–
L. paracollinoides	DSM 15502^{T}	+	+	+
	DSM 15502^{NB}	–	–	–
	LA9	+	+	+
	LA9^{NB}	–	–	–
L. lindneri	DSM 20692	+	+	+
	DSM 20692^{NB}	–	+	–
Ped. damnosus	ABBC478	+	+	+
	ABBC478^{NB}	–	–	+

[a]The nonspoilage variants were obtained by repeatedly subculturing the wild-type strains at 37 °C for *L. brevis*, 30 °C for *Lactobacillus paracollinoides*, 30 °C for *Lactobacillus lindneri,* and 35 °C for *Pediococcus damnosus*, respectively. The superscripts NB and CC indicate the hop-sensitive variants obtained from beer-spoilage wild-type strains with the same strain number.

[b]ABBC and LA: Our culture collections, principally consisting of brewery isolates; DSM: Culture collections obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen.

[c]Beer-spoilage ability was evaluated using the degassed pilsner-type beers (pH 4.2, bitterness unit: 20 B.U., ethanol content: 5.0%(v/v)).

resistance but also for the resistance to other hostile factors in beer. This notion is supported by the case with OmrA, a protein found in wine-associated *O. oeni* that shows 54% identity with HorA. OmrA has been demonstrated to confer tolerance not only to ethanol but also to multiple stress factors found in wine-making environments (Bourdineaud, Nehme, Tesse, & Lonvaud-Funel, 2004), suggesting that HorA and possibly HorC have much wider functions upon the survival in beer other than the roles as efflux pumps of hop bitter acids. At any rate, the use of beer itself as culture media seems important for maintaining the hop resistance genes and concomitantly the beer-spoilage ability of LAB strains. However it should be noted that the loss of hop resistance genes occasionally occurs when the strains are repeatedly subcultured in beers to which laboratory culture media are added for supplemental nutrient sources. One example of this failure is MRS broth prepared with beer instead of water. Presumably, nutrients contained in laboratory culture media counteract the hostile factors in beer that are important for the maintenance of hop-resistance genes.

Another important observation to be noted is that the loss of *horA* and *horC* regions also occurs with freeze drying, a method that is typically used for preserving bacterial cultures (Suzuki, Iijima, Sakamoto, et al., 2006). This phenomenon was observed when the type strain of *L. paracollinoides* was deposited in the Japan Collection of Microorganisms (JCM) and in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), and their stock cultures showed declined beer-spoilage ability. Currently JCM and DSMZ have restocked the strain at −80 °C in a frozen state with suitable protectants to avoid the loss of the hop resistance genes. Therefore, brewing microbiologists may have to be careful with the stock storage conditions of important beer-spoilage strains. In our experience, examining for the presence of *horA* and *horC* homologs is a useful indicator to determine whether the stock conditions of beer-spoilage LAB strains are suitable.

7.4.3 Culturability of beer-spoilage LAB

Culturability of beer-spoilage LAB strains is often changeable on QC detection media. As discussed earlier in this chapter, many beer-spoilage LAB strains are difficult to detect by conventional laboratory media. This is especially true for the primary isolation of beer-spoilage LAB strains, leading to the failure in the detection of beer-spoilage LAB by QC tests in breweries (Suzuki, 2012). The inability of beer-spoilage LAB to grow on laboratory detection media appears to be caused by the profound adaptation of these microorganisms to brewing environments (Back, 2005a; Suzuki, 2012; Taskila, Kronlöf, & Ojamo, 2011). There appear to be several factors involved. Some beer-spoilage strains exhibit sensitivities to nutrients typically included in laboratory media, and others require beer-specific components as either growth-promoting factors or essential growth factors. Still others seem to prefer pH environments considerably lower than those found in laboratory culture media. However, in many cases, the initially hard-to-cultivate beer-spoilage strains acquire culturability on laboratory culture media when those LAB strains are gradually and stepwisely acclimatized to the laboratory medium environments (Deng et al., 2014; Suzuki, 2012). These phenomena are observed in other fermentation industries as well. For instance,

some of the initially hard-to-cultivate wine-spoilage *L. fructivorans* strains and dressing-spoilage *L. fructivorans* strains were suggested to gain culturability on laboratory media and eventually to grow well on those media (Suzuki, 2012). Conversely, the repeated subcultures in degassed beer were found to gradually reduce the culturability of initially easy-to-cultivate beer-spoilage *L. paracollinoides* and *L. lindneri* strains, eventually leading to the acquisition of hard-to-cultivate strains that mimic the state of primary isolates of these species (Suzuki, Asano, Iijima, Kuriyama, & Kitagawa, 2008). These studies indicate that the culturability of beer-spoilage LAB can change depending on the environments to which they are adapted.

7.4.4 Subculture and preservation methods of beer-spoilage LAB

As far as the development of brewery QC tests are concerned, the most important aspects to note are the physiological/genetic traits and culturability of LAB strains to be used for the evaluation. This is because these characteristics are often changeable to a considerable extent, depending on the subculture conditions. For practical purposes, it is natural that LAB strains used for the development of a new microbiological QC test should closely mimic those actually encountered in beer products and manufacturing processes. In fact, beer-spoilage LAB rarely live in nutrient-rich environments, such as laboratory culture media, and the physiological/genetic traits and culturability of LAB strains living in the brewing environment are drastically different from those of so-called laboratory strains (Suzuki, 2009). Accordingly, if laboratory strains are to be used for the development of new microbiological QC tests, those tests may not be sufficiently suitable for practical applications. From the preceding observations, the subculture conditions have enormous impacts on the hop resistance ability and culturability of beer-spoilage LAB strains. Considering that beer-spoilage LAB prefer the brewing environments as their habitats and that these LAB are innately adapted there, beer seems to be a natural choice for a subculture medium. The subcultures in beer or on beer agar appear to maintain the hard-to-cultivate states of beer-spoilage LAB and their hop resistance ability/beer-spoilage ability. In contrast, the subcultures in laboratory culture media tend to change the culturability of the LAB strains and their hop resistance ability/beer-spoilage ability, indicating that laboratory culture media are not suitable for maintaining beer-spoilage LAB strains. Therefore, the primary isolation and subsequent subcultures should be conducted using beer and beer agar as culture media, rather than traditional laboratory culture media. However, caution should be exercised upon the preparation of beer agar, because the low pH of beer may dissolve agar matrix during the autoclaving and thereby hinder the solidification of agar. Accordingly, the pH of beer agar should be adjusted at around 5.0.

It is also important to prepare stock cultures as soon as the primary isolation process of beer-spoilage LAB strains is completed on beer agar. As previously stated in this chapter, the beer-spoilage ability of some LAB strains tend to decline by freeze drying, with the concomitant loss of hop resistance genes (Suzuki, Iijima, Sakamoto, et al., 2006). In these cases, cryopreservations are more suitable. In one study, hard-to-cultivate beer-spoilage LAB strains belonging to *L. lindneri* and *L. paracollinoides* were grown in beer and concentrated by centrifugation (Suzuki, Iijima, Asano, Kuriyama, & Kitagawa, 2006).

After the supernatants were discarded to remove toxic hop bitter acids, the cells were resuspended in 0.85% (w/v) NaCl solution. The hard-to-cultivate LAB strains were subsequently stored at −80 °C with 10% (v/v) dimethylsulfoxide or 10% (v/v) glycerol as a cryoprotectant. After 3 months of storage, the strains were reconstituted and grown in degassed beer (pH 5.0) at 25 °C. In this procedure, somewhat elevated pH of degassed beer as a recovery medium seems to improve the resuscitation rate of frozen culture stock by buffering the stress factors in beer. As a result of evaluating the reconstituted strains, no apparent changes were observed concerning the culturability and other genetic/physiological traits, suggesting this preservation method is useful for maintaining the original state of beer-spoilage LAB just as they are obtained as the primary isolates from the brewing environments. Nonetheless, the subculture and preservation method of freshly isolated beer-spoilage LAB strains has not been fully established in the brewing industry, and strain-dependent procedures may be required. It is therefore hoped that more studies will be conducted to improve these techniques.

7.5 Other Gram-positive bacteria in brewing

7.5.1 *Brewery-related LAB other than* Lactobacillus *and* Pediococcus

Some species of LAB other than *Lactobacillus* and *Pediococcus* are occasionally isolated from the brewing environments. One of the most frequently encountered species of these LAB groups is *Lactococcus lactis*. This LAB species is a common microorganism in plants but is better known for the production of diacetyl from citrate and its role in butter manufacturing (Priest, 2003). However, the hop resistance of *Lactococcus lactis* is rather low, and there have been no reports of *Lactococcus lactis* growing in beer except for those with microbiologically weak features, such as elevated pH values and low bitterness units (Back, 2005a). Another species isolated from brewing environments is *Leuconostoc* (*para*)*mesenteroides*. *Leuc.* (*para*)*mesenteroides* is acid tolerant and has been isolated from fruit mashes. As is the case with *Lactococcus lactis*, *Leuc.* (*para*)*mesenteroides* does not possess strong hop resistance and is therefore unlikely to cause spoilage incidents except in beers with microbiologically weak features (Back, 2005a). The occurrence of other LAB, such as *Streptococcus* and *Enterococcus*, seems to be relatively rare in breweries.

7.5.2 *Endospore-forming bacteria*

Due to their strong resistance to heat treatment and disinfectants, spore-forming bacteria are difficult to eradicate from the brewing environments. Therefore, endospore-forming bacteria are sometimes isolated from work-in-process products and finished beer products, especially when QC detection media possess insufficient selectivity. Major groups of spore-forming bacteria found in brewing environments have been reported to belong to the genera *Bacillus*, *Clostridium,* and *Paenibacillus* (Takeuchi, Iijima, Suzuki, Ozaki, & Yamashita, 2005). These spore-forming bacteria

are generally sensitive to low pH and hop bitter acids, and do not cause problems in normally hopped beer (Back, 2005a; Priest, 2003). Care may have to be taken, however, for beers with unusually high pH value and/or low bitterness units, since some *Clostridium* spp., including *Clostridium* (*aceto*)*butyricum*, might be able to grow in those beers. One worrisome feature is that *Bacillus cereus* group was reported as one of the species isolated frequently from brewing environments (Takeuchi et al., 2005). Some of the *B. cereus* strains are known as food pathogens, causing severe nausea, vomiting, and diarrhea. However, thanks to the microbiological stability of beer described in the beginning of this chapter, no food-poisoning incidents by bacteria have been documented in beer, including those caused by *B. cereus* (Back, 2003; Bunker, 1955; Menz, Aldred, & Vriesekoop, 2011). On the other hand, spores from enodospore-forming bacteria are present in malt and cereal adjuncts, and thermophilic or thermoduric spore-forming bacteria, such as *Bacillus coagulans*, are able to grow in hot sweet wort (*ca* 55–60 °C) (Briggs et al., 2004). However, these bacteria are generally sensitive to hop resins and can grow only slowly in media with a pH value lower than 5.0. Therefore, they do not usually cause spoilage problems in the subsequent brewing processes and finished beer (Back, 2005a).

7.5.3 Other Gram-positive bacteria relevant in brewing

Genera belonging to *Stapylococcus*, *Kocuria*, and *Micrococcus* are relatively common in breweries (Priest, 2003). These bacteria are not generally considered to be important as spoilage microorganisms, but they are known to be widely distributed in the brewing environments. Some Gram-positive cocci, such as *Staphylococcus epidermidis* and *Staphylococcus saprophyticus*, can survive in beer for long periods and are sometimes detected by QC detection media. However, these bacteria cannot grow in beer because of their hop sensitivity and their inability to grow at pH values lower than 4.5. *Kocuria kristinae*, on the other hand, is somewhat hop resistant and acid tolerant among this group of bacteria. Unlike other *Kocuria* species that are strictly aerobic, *Kocuria kristinae* is facultatively anaerobic. Although the status of *Kocuria kristinae* as a beer-spoilage bacterium is controversial, the intensity of growth seems to be affected by the oxygen content of beer because the presence of oxygen promotes the growth of this species. When *Kocuria kristinae* grows in beer, the spoilage occurs with relatively high-pH and low-bitterness beer products. It was also reported that *Kocuria kristinae* yields a fruity aroma and an atypical taste in beer.

7.6 Concluding remarks

Beer is known as a beverage with a high microbiological stability. Most bacteria cannot grow in beer due in large part to the presence of hop bitter acids and ethanol, as well as the low pH value of beer. Spore-forming bacteria that are the main source of concern in nonalcoholic beverages do not grow in beer, and foodborne pathogens, including *Staphylococcus aureus* and *B. cereus*, are also unable to grow there. These aspects are very fortunate for the brewing industries, because

all that brewing microbiologists have to do is to deal with very narrow subcommunities of microorganisms. In terms of Gram-positive bacteria, approximately 20 LAB species, belonging to *Lactobacillus* and *Pediococcus*, have been recognized as beer-spoilage microorganisms. Some of them, such as *L. brevis*, *L. lindneri,* and *Ped. damnosus*, have been traditionally known as major beer-spoilage LAB species, whereas many of the others have become known as beer-spoilage LAB since around the year 2000. Because the culturability of many beer-spoilage LAB species is poor and the spoilage incidents are often caused by the mixed populations of LAB, the fast grower, such as *L. brevis* and *L. casei*, tend to outcompete the other hard-to-cultivate LAB species on conventional QC detection media. These phenomena make the findings of as-yet uncharacterized LAB species more difficult. Partly for these reasons, it is difficult to determine whether new beer-spoilage LAB species are constantly emerging through the horizontal transfer of the hop resistance genes, or whether brewing microbiologists are dealing with the same communities of beer-spoilage LAB species as they did in the nineteenth and twentieth centuries. At any rate, the discoveries of novel beer-spoilage LAB species are still continuing, and brewing microbiologists are constantly trying to catch up with newly emerging opponents. In the face of these challenges, the recent discoveries of species-independent genetic markers, supported by the ongoing progress of hop resistance research, have been considered significant, since at least one of those genetic markers, such as *horA* and *hor*C, have been detected in the recently recognized beer-spoilage LAB species, including *L. paracollinoides*, *L. backi*, *L. acetotolerans*, *L. paucivorans*, and *Ped. claussenii* (Deng et al., 2014; Ehrmann, Preissler, Danne, & Vogel, 2010; Iijima et al., 2007; Pittet et al., 2012; Suzuki, 2012). These observations indicate that the species-independent genetic markers are useful for detecting as-yet uncharacterized species of beer-spoilage LAB. However, the hop resistance mechanisms, despite the enormous progress observed in the past 30 years, have not been fully disclosed. Because of this, the intraspecies determination of beer-spoilage ability is not always accurate enough to make a critical judgment when a LAB strain is detected in finished beer products; therefore, this area of research should be more vigorously conducted in future. It is also important to investigate the mechanisms as to why beer-spoilage LAB lapse into the hard-to-cultivate state. These studies not only help us to develop a new type of rapid and comprehensive QC detection medium but also reveal an entire spectrum of beer-spoilage LAB species, some of which may still remain undiscovered. Upon conducting the above research, it would become increasingly more important to use freshly isolated LAB strains. This is because all of the QC tests in breweries are actually carried out against beer-spoilage LAB strains latent in the brewing environments, rather than those subcultured with nutrient-rich laboratory culture media. The use of beer-spoilage LAB in the latter state often leads to an erroneous interpretation of new QC methods under development. Thus the techniques that enable us to capture and maintain beer-spoilage LAB living in the brewing environments should be more fully established in the future. The more comprehensive and accurate QC methods for beer-spoilage LAB would emerge by overcoming the above challenges.

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Gram-negative spoilage bacteria in brewing

8

A.D. Paradh
Vasantdada Sugar Institute, Pune, Maharashtra, India

8.1 Introduction: Gram-negative bacteria in brewing

Only a few Gram-negative bacteria have been found to be responsible for beer spoilage. These bacteria can be broadly classified into two categories. The first category of Gram-negative, anaerobic beer spoilers belong to the genera *Pectinatus, Megasphaera, Zymophilus* and *Selenomonas. Pectinatus* and *Megasphaera* are regarded as the most important beer spoilage bacteria, mainly in unpasteurised beer. This chapter deals with the second category, which includes Gram-negative aerobic and facultative anaerobic bacteria such as acetic acid bacteria (AAB), Zymomonas and certain *Enterobacteriaceae* species.

Previously, AAB such as *Acetobacter* and *Gluconobacter* were important beer spoilers. These bacteria metabolise ethanol to acetic acid, giving a vinegary flavour to beer (Sakamoto & Konings, 2003). However, due to implementation of effective cleaning and sanitation procedures in modern breweries and effective removal of oxygen from post-fermentation processes, these bacteria are no longer considered important, and are limited to dispense systems in public houses and cask-conditioned beers (Sakamoto & Konings, 2003).

Zymomonas mobilis is a facultative anaerobe and has been isolated from primed sugars. There has been no report of incidents of spoilage, as these microbes utilise only a narrow range of sugars (Sakamoto & Konings, 2003). Several species belonging to *Enterobacteriaceae* such as *Obesumbacterium*, *Hafnia*, *Klebsiella* and *Citrobacter* are reported to be associated with spoilage of unfermented and fermenting wort (Priest, 2006; van Vuuren & Priest, 2003). These bacteria are not normally able to grow in finished beer but are occasionally found in the initial stages of the brewing process, causing unwanted off-flavours in the final product (Priest, Hammond, & Stewart, 1994) (Table 8.1).

8.2 Acetic acid bacteria

AAB are aerobic, non–spore-forming, Gram-negative to Gram-variable bacteria having ellipsoidal to short rod-shaped cell morphology. AAB occur singly, in pairs or in chains, sometimes motile in nature. Flagellar arrangement may vary from peritrichously to polar (Gonzales, Hierro, Poblet, Mas, & Guillamon, 2005). AAB show a positive reaction using the catalase test and a negative reaction using the oxidase test (Sengun & Karabiyikli, 2011). Optimum growth of AAB occurs between pH 5 and 6.5 (Holt, Krieg, Sneath, Staley, & Williams, 1994), but their growth can also occur at

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Table 8.1 Overview of Gram-negative beer spoilage bacteria, beer spoilage effects and metabolic products

Bacteria	Occurrence in brewery environments	Off-flavour/ aroma and odour	Visual spoilage effects	Metabolic products
Acetic acid bacteria[1]				
Acetobacter[1]	Wort, beer dispenses, and cask-conditioned ales and barrel-aged ales, brewery biofilm	Sour, vinegary	Hazy, ropiness	Acetic acid
Glucano-bacter[1]	Wort, beer dispense and cask-conditioned ales	Sour, vinegary	Hazy	Acetic acid, acetate
Zymomonas[2]	Primed beers (not found in lagers)	Fruity, rotten apple, rotten egg, sulphudic	Hazy, ropiness	Acetaldehyde and H_2S
Enterobacteriaceae[3]				
Obesum-bacterium[3]	Pitching yeast and fermenting wort	Parsnip, sulphury	Hazy	Dimethyl sulphide (DMS), diacetyl, higher alcohols, *N*-nitrosamines, acetoin
Citrobacter[3]	Brewing liquor, fermenting wort	Parsnip, sulphury		DMS, diacetyl, lactic acid, acetaldehyde
Rahnella[3]	Pitching yeast, early stages of fermentation (wort)	Fruity, sulphury	—	DMS, diacetyl, methyl acetate, ethyl acetate
Klebseilla[3]	Fermenting wort, biofilm	Unpleasant odour	—	4-vinylguaicol, DMS, diacetyl
Obligatory anaerobes				
Pectinatus[4]	Low-alcohol unpasteurised beer, beer filling area, biofilm	Rotten egg, unpleasant odour	Turbidity	Acetic acid, propionic acid, lactic acid, succinic acid, H_2S, acetoin, methyl mercaptan and other sulphur compounds

Continued

Table 8.1 Continued

Bacteria	Occurrence in brewery environments	Off-flavour/ aroma and odour	Visual spoilage effects	Metabolic products
Megas-phaera[4]	Low-alcohol unpasteurised beer, beer filling area, biofilm	Unpleasant odour	Turbidity	H_2S, butyric acid, isobutyric acid, caprioc acid, valeric acid, isovaleric acid
Selenomonas[5]	Pitching yeast	Unpleasant odour	Turbidity	Acetic, lactic and propionic acids
Zymophilus[5]	Pitching yeast or brewery waste	Unpleasant odour	Turbidity	Acetic acid and propionic acid

[1]van Vuuren and Priest (2003) and Gonzalez et al. (2005)
[2]Ingledew (1979) and Swings and De Ley (1977).
[3]Priest (2006) and van Vuuren and Priest (2003).
[4]Lee, Mabee, and Jangaard (1978), Engelmann and Weiss (1985), Schielfer et al. (1990) and Juvonen and Suihko (2006).
[5]Schielfer et al. (1990).

highly acidic pH 3–4. AAB bacteria have been isolated from a variety of sources ranging from tropical fruits, rotten fruits, dried fruits, flowers, beers and wines (Sengun & Karabiyikli, 2011).

AAB bacteria are industrially important due to their ability to oxidise sugar and ethanol into organic acid, mainly acetic acid. *Gluconobacter* are industrially used in the production of vinegar due to their ability to produce high concentrations of acetic acid from ethanol under aerobic conditions. In addition, AAB bacteria are used in various biotechnological applications such as production of cellulose, sorbose and dihydroxyacetone (Gonzales et al., 2005; Gupta, Singh, Qazi, & Kumar, 2001).

AAB bacteria are also important due to their spoilage effect on alcoholic beverages such as wine and beer (Bartowsky & Henschke, 2008). Beer spoilage AAB forms a pellicle on the surface with cloudiness in beer containing oxygen. Due to the formation of acetic acid, beer tastes sour to vinegary (Ingledew, 1979; Magnus, Ingledew, & Casey, 1986). AAB are strictly aerobic bacteria, but some of the AAB isolated from draught beer have been reported to be micro-aerotolerant (van Vuuren & Priest, 2003).

8.2.1 Taxonomic status of brewery-related AAB

Taxonomic status of AAB is complex and not well established, as it has been subjected to changes on several occasions in last 40 years. Historically, AAB were mainly classified as *Acetobacter* (Beijerinck, 1898) and *Gluconobacter* (Asai, 1935). At present, AAB taxonomically belong to family *Acetobacteraceae* (Asai, 1968), which is classified under the class *alpha proteobacteria*. AAB has 15 validated genera, and only two genera; *Acetobacter* and *Gluconobacter* are reported to be associated with beer spoilage (van Vuuren & Priest, 2003).

Currently, the genus *Acetobacter* comprise 33 validated species, and the genus *Gluconobacter* has 16 validated species. Amongst the validated species of AAB, 10 species of *Acetobacter* occur in brewing environments; *Acetobacter aceti, Acetobacter liquefaciens*, *Acetobacter pastorianus* and *Acetobacter hansii* are most frequently occurring species (Priest, 2006; van Vuuren & Priest, 2003). Only one species of *Gluconobacter* (*Gluconobacter oxydans*) has been reported to be associated with brewing environments (van Vuuren & Priest, 2003; Cleenwerck, Vandemeulebroecke, Janssens, & Swings, 2002; Skerman, McGowan, & Sneath, 1980). Recently, a newly proposed species *Gluconobacter cerevisiae* was reported to be associated with brewery environments (Spitaels et al., 2014).

Gluconobacter and *Acetobacter* can be differentiated based on ethanol and lactate oxidation. *Gluconobacter* are unable to completely oxidise ethanol to carbon dioxide (CO_2) and water (H_2O) by formation of acetate, and they also are unable to oxidise lactate to CO_2 and H_2O while *Acetobacter* can oxidise ethanol to acetate and further oxidise acetate (Gonzalez et al., 2005). Moreover *Acetobacter* shows peritrichous flagellation, whereas the genus *Gluconobacter* has polar flagellation (Cleenwerck & De Vos, 2008) (Table 8.2).

Table 8.2 Important characteristics of brewery-related acetic acid bacteria

Characteristics	Genera	
	Acetobacter	***Gluconobacter***
Flagellation	Peritrichous	Polar
Oxidation of ethanol to acetic acid	+	+
Oxidation of acetic acid to CO_2 and H_2O	+	–
Oxidation of lactase to acid to CO_2 and H_2O	+	–
Growth on 0.35% acetic acid containing medium	+	+
Growth on methanol	–	–
Growth on D-mannitol	+/–	+
Growth in presence of 30% D-glucose	–	–/+
Production of cellulose	–	–
Production of levan-like mucosa substance from sucrose	–/+	–
Fixation of molecular nitrogen	–	–
Ketogenesis (dihydroxyacetone) from glycerol	+/–	+
Acid production from:		
D-Mannitol	–/+	+
Glycerol	–/+	+
Raffinose	–	–
Cellular fatty acid type	$C_{18:1}$	$C_{18:1}$
Ubiquinone type	Q-9	Q-10
DNA base composition (mol % G + C)	52–60	55–63

n, none; +, 90% or more of the strains positive; –, 90% or more of the strains negative; w, weakly positive reaction.
Source: Originally adapted from Sengun and Karabiyikli (2011).

8.2.2 Metabolic aspects of AAB

Oxidation of ethanol to acetic acid is an important characteristic of *Acetobacter* and *Gluconobacter*. Ethanol is first oxidised to acetaldehyde followed by further oxidation to acetic acid, and the reactions are catalysed by cytoplasmic membrane-bound enzymes alcohol dehydrogenase and aldehyde dehydrogenase, respectively (Figure 8.1) (Saeki et al., 1997). Under acidic conditions, the alcohol dehydrogenase activity of *Aceto-bacter* is more stable than the activity in *Gluconobacter,* which results in more acetic acid production by *Acetobacter* (Matsushita, Toyama, & Adachi, 1994).

A variety of carbohydrate sources such as arabinose, fructose, galactose, mannitol, mannose, ribose, sorbitol and xylose are utilised by AAB through the hexose monophosphate pathway (De Lay et al., 1984), Embden–Meyerhof–Parnas (EMP) and Entner Doudoroff (ED) pathway (Attwood, van Dijken, & Pronk, 1991). *Acetobacter* can also oxidise various organic acids into CO_2 and H_2O through the tricarboxylic acid (TCA) cycle, but like *Gluconobacter* lacks a functional TCA cycle, which limits oxidation of organic acids (Seo et al., 2004).

8.2.3 Occurrence and beer spoilage ability

AAB are ubiquitous and occur throughout the brewing process. However, because of the elimination of oxygen throughout the brewing process, there has been significant reduction in spoilage incidents due to AAB. AAB are highly tolerant to hop bitterness

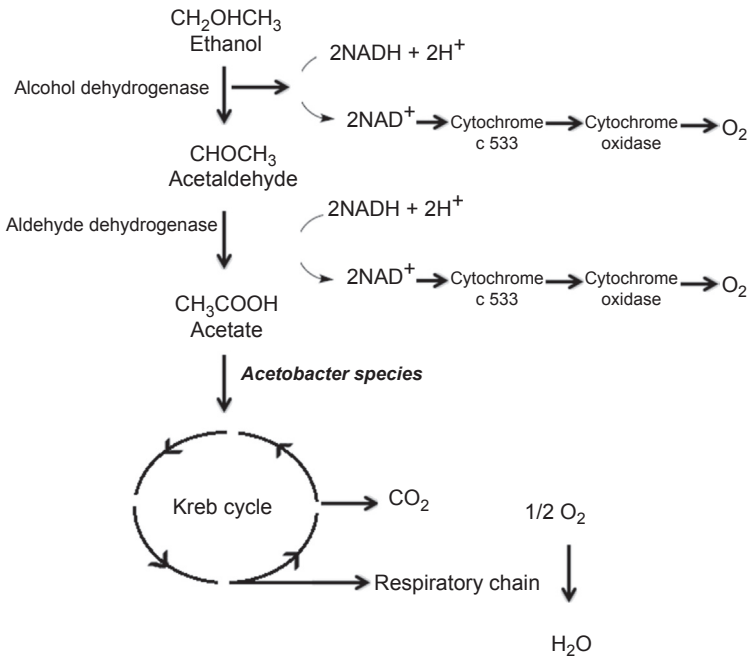

Figure 8.1 Metabolism of ethanol by acetic acid bacteria.

compounds and can survive in high concentrations of ethanol (>10% v/v) (Priest, 2006). AAB prevail in the initial stages of biofilm formation in brewery environments (Back, 1994).

AAB and some enterobacteria grow in niches and corners in brewery filling equipment, and, at later stages of biofilm development, are protected from routine cleaning due to slime formation. Yeast propagation, along with lactic acid bacteria, further provide microaerophillic and a partial anaerobic environment and substrate for growth of Gram-negative anaerobic bacteria such as *Pectinatus* and *Megasphaera* (Back, 1994; Storgårds, 2000). AAB, most frequently *A. pasteurianis sub pasteurianis,* have been reported to be isolated from the beer filling and filtration processes (Ploss, Erber, & Eschenbecher, 1979).

AAB are more commonly associated with dispense lines in pubs and public houses due to higher oxygen and high temperature at some stages in the beer dispensing system (Storgårds, 1997, 2000). Frequent incidents of beer spoilage in draught beer kegs have been reported (Ingledew, 1979). *Acetobacter* and *Gluconobacter* have also occasionally been found in samples from beer fermentation and storage tanks (van Vuuren, Loos, Louw, & Meisel, 1979). AAB are still prevalent in cask-conditioned and barrel-aged beers (Bokulich, Bamforth, & Mills, 2012) (Table 8.2).

In addition to acetic acid formation, growth of *Gluconobacter* in the beer leads to formation of a pellicle on the surface, with cloudiness in beer containing oxygen. Some strains of *Gluconobacter* produces high amounts of dextran and levan, resulting in high viscosity of contaminated beer, which finally leads to ropiness in the beer (Hornsey, 2013). Whole-genome sequencing of *Gluconobacter oxydans* revealed that it contains various membrane-bound dehydrogenases; these enzymes rapidly metabolise sugars or sugar acids from the sugar-rich substrate and can survive even in high-sugar substrates (Prust et al., 2005). *Gluconobacter* are often isolated from soft drinks and various fruit-based products (Holt, Krieg, Sneath, Staley, & Williams, 1994).

Historically, AAB such as *Acetobacter* and *Gluconobacter* have been important beer spoilers. However, due to the implementation of effective cleaning and sanitation procedures in modern breweries and the effective removal of oxygen from post-fermentation processes, these bacteria are no longer considered important, and are limited as indicator microorganisms for improper sanitation and hygiene (Sakamoto & Konings, 2003; Jespersen & Jakobsen, 1996). These microorganisms still prevail in beer dispense lines of public houses.

8.2.4 Detection of AAB

Conventional methods for detection of spoilage microorganisms in beer and other beverage generally involves pre-enrichment of sample with a nonselective medium, followed by enrichment on selective or differential agar (Hill, 2009). There are numerous media described for isolation and identification of AAB bacteria, but no single medium has been found to be effective in supporting the growth of all AAB, and selectivity of the medium is variable for different strains (refer Table 8.3).

For beer spoilage bacteria, Frateur's differential medium containing yeast extract, ethanol and calcium carbonate has been reported in the literature, *Acetobacter*

Table 8.3 Conventional and rapid detection and characterisation methods for beer spoilage acetic acid bacteria, *Zymomonas*, and *Enterobacteriaceae* species

Bacteria	Conventional detection methods	Rapid detection and characterisation methods	References
Acetic acid bacteria	1. Frateur's medium 2. Carr's differential medium 3. YPM medium 4. AE medium 5. Reinforced AE medium	1. RT-PCR 2. RFLP 3. AFLP 4. DGGE 5. FISH	Carr (1969), Priest (2006), Yamada et al. (1999), Gullo and Giudici (2008), Gammon et al. (2007), Ruiz et al. (2000), Nanda et al. (2001), Cleenwerck et al. (2009), Andorrà et al. (2008), De Vero et al. (2006) and Frank-Whittle et al. (2005).
Zymomonas	1. Beer sample with actidione 2. MYPG 3. Beer agar with lead acetate and Schiff reagent	1. ARDA 2. Duplex PCR 3. RAPD	Woodward (1982), Jespersen and Jakobsen (1996), Dennis and Young (1982), Coton et al. (2005b, 2006).
Entero-bacteriaceae	1. MacConkey agar/broth 2. UBA agar	1. PCR 2. Automated ribotyping	Jespersen and Jakobsen (1996), Maugueret and Walker (2002) and Koivula et al. (2006).

YPM, yeast extract peptone mannitol medium; AE, acetic acid ethanol medium; RT-PCR, real-time polymerase chain reaction; RFLP, restriction fragment length polymorphism; AFLP, amplified fragment length polymorphism; DGGE, denaturing gradient gel electrophoresis; ARDA, amplified ribosomal DNA restriction analysis; RAPD, random amplified polymorphic DNA.

colonies appear clear due to formation of acid from ethanol, whereas *Gluconobacter* develop chalk-white deposit colonies (Priest, 2006). Carr's differential medium (Carr et al., 1969) containing yeast extract, ethanol and bromocresol blue has also been documented. Agar (2.5% w/v) containing yeast extract (0.5% w/v) and ethanol (1.5% v/v) has been used for growth of beer spoilage AAB.

Other differential media such as yeast extract, peptone, and mannitol agar (YPM media) (Gullo & Giudici, 2008), AE medium (Yamada et al., 1999), and reinforced AE medium (Zahoor, Siddique, & Farooq, 2006) used for isolation and identification of AAB have been reported in the literature. Use of fluorescence staining techniques such as the live/dead® Baclight™ bacterial viability test has been studied for detection of viable but nonculturable AAB (Baeno-Ruano et al., 2006). The use of these microbial media is limited to the vinegar and cider industry and is rarely used in

brewing laboratories, notably because of negligible incidents of spoilage due to these microorganisms in recent years.

Various rapid detection techniques for AAB have also been described in the literature. Rapid detection of AAB using real-time polymerase chain reaction (PCR) (Gammon et al., 2007; Torija, Mateo, Guillamón, & Mas, 2010), restriction fragment length polymorphism (Ruiz, Poblet, Mas, & Guillamon, 2000; Nanda et al., 2001), amplified fragment length polymorphism (Cleenwerck, de Wachter, Gonzalez, de Vuyst, & de Vos, 2009), denaturing gradient gel electrophoresis (Andorrà, Landi, Mas, Guillamón, & Esteve-Zarzoso, 2008; De Vero et al., 2006) and fluorescence in situ hybridisation (Frank et al., 1999; Franke-Whittle, O'Shea, Leonard, & Sly, 2005) are some of the examples of molecular detection methods.

8.3 Zymomonas

Zymomonas species are Gram-negative, non–endospore-forming, catalase-positive, aerotolerant, facultative anaerobic bacteria. Morphologically, these bacteria are short plump rods that occur singly, in pairs, and sometimes in chains or rosettes (van Vuuren & Priest, 2003). *Zymomonas* is ethanol tolerant (below 10% ethanol v/v) and grows optimally at pH above 3.4 and temperature of 25–30 °C (van Vuuren, Cosser, & Prior, 1980). These bacteria metabolise glucose and fructose as a source of carbon but are unable to utilise maltose and maltotriose (van Vuuren & Priest, 2003; Yang et al., 2009b).

Zymomonas species are often isolated as a source of spoilage microorganisms from various traditional alcoholic beverages throughout the world. These bacteria are found on the glucose-rich sap of agave, sugar cane and palm trees as a naturally occurring fauna (Coton, Laplace, Auffray, & Coton, 2005). *Zymomonas* is a biotechnologically important microorganism for industrial production of fuel ethanol (Chandel, Chandrasekhar, Radhika, Ravinder, & Ravindra, 2011; Gírio et al., 2010). It has also been extensively studied as a model microorganism for genetic modification for use of ligonocellulosic biomass for ethanol production (Dien, Cotta, & Jeffries, 2003; Chandel et al., 2011).

8.3.1 *Taxonomic status of* Zymomonas

The genus *Zymomonas* belongs to the phylum *Proteobacteria*, the class *Alphaproteobacteria*, the order *Sphingomonadales* and the family *Sphingomonadaceae*. *Zymomonas* to date has only one species, cited as *Z. mobilis,* which was formerly known as *Achromobacter anaerobium,* originally isolated from beer (Shimwell, 1936). In the older literature, *Zymomonas* has been cited as *Saccharomonas lindneri* and *Pseudomonas lindneri* (Hornsey, 2013).

At present, *Z. mobilis* has three validated subspecies, namely *Z. mobilis subsp. pomaceae* (Millis, 1956; De Ley & Swings, 1976), *Z. mobilis subsp. mobilis* (Lindner, 1928; De Ley & Swings, 1976) and *Z. mobilis subsp. francensis* (Coton, Laplace, Auffray, & Coton, 2005a). All three subspecies are differentiated based on phenotypic characterisation, protein and genetic characterisation and growth at 36 °C

(Coton, Laplace, Auffray, & Coton, 2005b). Out of the three validated species, only *Z. mobilis* subsp. *mobilis* is reported to be a beer spoiler (van Vuuren & Priest, 2003).

8.3.2 *Occurrence and beer spoilage ability of* Zymomonas

The original source of contamination by *Zymomonas* species in the brewery and cider house is still unknown. Soil is suggested to be the possible source of contamination in beer (Ingledew, 1979; Coton & Coton, 2003), as incidents of *Z. mobilis* contamination are linked to times of construction of new facilities and excavation in breweries (Ingledew, 1979). *Z. mobilis subsp. mobilis* has also been reported to prevail in public houses, well-water sources, soil from brewery environments and the bottling process (Dads & Martin, 1978; Swings & De Ley, 1977).

Z. mobilis-contaminated beer has a fruity aroma (rotten apple, due to the production of acetaldehyde), which rapidly progresses to a sulphidic and rotten-egg aroma (due to the production of hydrogen sulphide) in spoiled beer (Dads & Martin, 1978). The contamination incidents due to *Zymomonas* are limited to ales supplemented with primed sugar and spoilage problems due to these bacteria have never been encountered in lager beers (Dads & Martin, 1978; Bokulich & Bamforth, 2013; Richards & Corbey, 1974).

Spoilage due to *Zymomonas* is quite a common problem in ciders; a motile rod-shaped bacterium responsible for sick cider was originally described by Barker & Hillier, 1912. Later, *Z. mobilis subsp. pomaceae* (Millis, 1956; De Ley & Swings, 1976) was isolated from spoiled cider in England and described as the causal microorganism for cider sickness in English ciders. Z. *mobilis subsp. francensis* was proposed and characterised as the causal microorganism for 'framboisé' in French ciders (Coton et al., 2005b). Similar to beer, spoilage in ciders causes off-flavour typically described as like rotten banana, grassy, rotten lemon or framboisé (due to production of acetaldehyde 100–150 mg/l), production of gas in bottled ciders to a high extent, decreased density and high turbidity in spoiled products (Coton & Coton, 2003).

8.3.3 *Metabolic aspects of* Zymomonas

It has been demonstrated through experiments conducted by Fuhrer, Fischer, and Sauer (2005) that the Embden–Meyerhof–Parnas (EMP) pathway, a common metabolic pathway, does not operate in *Z. mobilis* (ZM4). The whole-genome sequencing of *Z. mobilis* (ZM4) has revealed various interesting facts (Seo et al., 2004; Yang et al., 2009a). The gene for the enzyme *phosphofructokinase* an important glycolytic enzyme is not found in the genome, although genes for all other enzymes within the EMP pathway are present (Seo et al., 2004). *Z. mobilis* is a unique aerobic microorganism that uses the Entner Doudoroff (ED) pathway anaerobically instead of the EMP pathway. *Z. mobilis* uses the pathway ultimately to ferment glucose, fructose and sucrose to ethanol and CO_2 (Swings & De Ley, 1977; Seo et al., 2004). *Zymomonas* is unable to utilise lactose, maltose and cellobiose due to the lack of genes responsible for production of enzymes necessary for metabolism of these sugars (Seo et al., 2004).

The genes encoding two enzymes from the TCA cycle the 2-oxoglutarate dehydrogenase complex and malate dehydrogenase for the TCA cycle are reported to be

missing from the genome of *Z. mobilis* (ZM4). It is suggested that there may be an alternative pathway parallel to the TCA cycle for the synthesis of TCA intermediates such as oxaloacetate, malate, fumarate and succinate, as *Z. mobils* could synthesise all necessary amino acids except lysine and methionine (Seo et al., 2004).

8.3.4 *Detection of* Zymomonas

Conventionally, for *Zymomonas,* routine enrichment of the sample in primed beer supplemented with actidione for inhibition of yeast has been reported, although incubation of filtered membranes on an agar medium is not recommended as a satisfactory method (Woodward, 1982). Detection of *Zymomonas* in the brewery using malt yeast extract glucose and peptone (MYPG) agar supplemented with 50 ppm actidione and 3% ethanol or beer with 100 ppm actidione has been reported (Jespersen & Jakobsen, 1996). For detection of *Zymomonas,* in beer media supplemented with lead acetate (producing black colonies) and Schiff reagent (producing purple colonies) has been documented (Dennis & Young, 1982; Woodward, 1982).

Coton et al. (2005b) developed a PCR-based amplified ribosomal DNA restriction analysis method for rapid detection of *Zymomonas* at the subspecies level. A further duplex PCR method with primers specific for 23S rRNA gene for detection of *Zymomonas* species has been developed (Coton et al., 2005b). This method could detect *Zymomonas* species within 24 h with sensitivity of 10^2 CFU/ml (Coton et al., 2005b). Coton, Laplace, Auffray, and Coton (2006) characterised several strains of *Z. mobilis* with random amplified polymorphic DNA (Table 8.3).

8.4 Brewery-related *Enterobacteriaceae*

Enterobacteriaceae (Lapage, 1979) is a relatively large family of Gram-negative, facultatively anaerobic bacteria belonging to the order *Enterobacteriales* of the class *Gammaproteobacteria* in the phylum *Proteobacteria*. *Enterobacteriaceae* comprises several pathogenic and nonpathogenic genera. Several studies have shown that pathogenic *Enterobacteriaceace* such as *Salmonella*, *Serratia*, *Shigella*, *Escherichia* and *Klebsiella* are inhibited due to a combination of several intrinsic antimicrobial properties of beer and technological adaptations made during beer production (Menz, Aldred, & Vriesekoop, 2009; Menz, Vriesekoop, Zarei, Zhu, & Aldred, 2010).

Several antimicrobial factors such as low pH (3.8–4.7), presence of ethanol (0.5–10% [w/v]), presence of hop bitterness compounds (iso-α acids), low oxygen concentration (less than 0.1–0.3 ppm), relatively high CO_2 (0.5% (w/v)) (Jespersen & Jakobsen, 1996) and low levels of nutrients make propagation of contaminants difficult in beer (Sakamoto & Konings, 2003). In addition, technological and processing hurdles such as wort boiling, pasteurisation and sterile filtration ensure that most food-borne and enteric pathogens do not grow or survive in beer (Dowhanick, 1994; Menz et al., 2009).

However, several incidents of the occurrence of enteric pathogens in low alcohol and alcohol-free beers and traditional beer-like products have been reported (Menz et al., 2010, 2009; Pattison, Georgnaras, & von Holy, 1998; Shayo, Kamala, Gidamis,

& Nnko, 2000; Enikova, Kozereva, Ivanova, & Yang'ozova, 1985). Several species belonging to *Enterobacteriaceae* have been isolated from brewery environments with the propensity of wort spoilage rather than beer (van Vuuren & Priest, 2003). *Citrobacter*, *Hafnia*, *Klebsiella* and *Obesumbacterium* are notable genera associated with wort spoilage (Priest, 2006). These bacteria are indirect beer spoilage microbes that are not normally able to grow in finished beer. They may, however, grow during the initial stages of the brewing process, causing unwanted off-flavours in the final product (Priest et al., 1994).

8.4.1 *Obesumbacterium proteus*

Obesumbacterium proteus is an extensively studied beer contaminant belonging to *Enterobacteriaceae.* This bacterium is typically Gram-negative aerobic or facultatively anaerobic and a short rod, but it has been reported to show pleomorphic rod morphology in the presence of yeast in fermenting wort (Priest & Hough, 1974). This bacterium fits the general description of the family *Enterobacteriaceae* (Priest & Barker, 2010). *Obesumbacterium proteus* shows a negative reaction to the oxidase test and a delayed and weak positive reaction to the catalase test. The bacterium can reduce nitrate to nitrite in fermenting wort (Priest & Barker, 2010).

The genus *Obesumbacterium* contains only one species, *O. proteus*. This Gram-negative bacterium was isolated in pure culture from top fermenting yeast and was classified as *Flavobacterium proteus* (Shimwell, 1936). Later this bacterium was assigned to the genus *Obesumbacterium* and *O. proteus* as a sole type strain within the genus (Shimwell, 1963, 1964). As a result of detailed taxonomic studies conducted by Priest, Somerville, Cole, and Hough (1973), the genus *Obesumbacterium* was assigned to the family *Enterobacteriaceae*. In the same study, heterogeneous properties of *O. proteus* were revealed, and it was further distinguished as biogroup-1 and biogroup-2 based on phenotypic and genetic characterisation. This reclassification was later supported by data obtained from enteric repetitive intergenic consensus sequences (ERIC PCR) studies (Priest, Hammond, & Stewart, 1994).

Obesumbacterium proteus biogroup-1 are supposed to be more closely related to *Hafnia alvei*, a common pathogen, and are postulated to be the metabolically inactive strain that is adapted to brewery environments (Priest et al., 1973; Farmer, 1984). The strains from biogroup-2 are more common dwellers of brewery environments and have not been reported from any other source (Priest & Barker, 2010). Priest and Barker (2010), assigned *O. proteus* biogroup-2 to a newly proposed genus *Shimwellia* and the species as *Shimwellia pseudoproteus.*

Obesumbacterium proteus is found in pitching yeast and fermenting wort, as it cannot survive below pH 3.9, it has not been reported in beer. *Obesumbacterium proteus* in the initial stages of fermentation competes with yeast for nutrients, resulting a slower rate of fermentation and production of volatile components such as dimethyl sulfoxide (DMSO), acetoin, lactic acid, propanol, isobutanol and 2,3-butandiaol. Production of DMS imparts an undesirable parsnip flavour to the contaminated beer (Case, 1965; Priest & Hough, 1974). A fermenting wort concentration of *O. proteus* up to 1% of pitching yeast is capable of producing only 14–18 μg of DMS/l, which

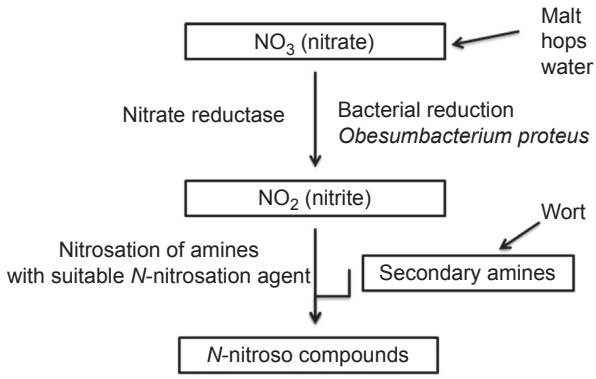

Figure 8.2 Role of *Enterobacteriaceae* in the formation of nitroamines in fermenting wort. Originally adapted from Priest (2006).

is lower than the threshold value of 30 µg of DMS/l. However, due to the practice of re-pitching, the concentration of these bacteria will eventually rise to produce off-flavour above threshold levels (Priest, 2006).

Certain species of *Enterobacteriaceae,* especially *O. proteus*, could utilise nitrates as electron acceptors for anaerobic respiration, resulting in reduction of nitrate into nitrite. Nitrite further could possibly react with secondary amines present in the wort, forming *N*-nitrosoamine (Figure 8.2). *N*-nitrosomines are carcinogenic in nature (Smith, 1994). Hence, a considerable amount of apparent total *N*-nitroso compounds (ATNCs) represents a possible risk to health, and consequently their concentration is strictly monitored and limited to 20 µg/l (Marguerite and Walker, 2002). Because of the risk of *N*-nitroso compounds, the *Enterobacteriaceae* species related to brewery environments are monitored.

8.4.2 Coliform bacteria related to brewing environments

Coliform bacteria broadly comprise *Enterobacteriaceae* species belonging to the genera *Enterobacter*, *Klebsiella*, *Escherichia Hafnia* and certain strains of *Citrobacter,* which are able to utilise lactose with gas and acid formation at 35–37 °C within 48 h (APHA, 1998). These bacteria are indicators of hygiene and sanitation in breweries. The presence of coliforms in water is an indication of the ineffectiveness of water treatment. These bacteria can be introduced to wort through contaminated water or intrusion of external fluids through connecting pipes (Vaughan, O'Sullivan, & Sinderen, 2005). Some species of coliform, such as *Citrobacter fruendii*, *Rahnella aquatilis*, *K. oxycota* and *Klebsiella terrigena,* have been associated with unfermented and fermenting wort (Vaughan et al., 2005).

Citrobacter fruendii is a facultative anaerobic, morphologically motile, slender, short rod occurring singly and in pairs and is catalase positive (van Vuuren & Priest, 2003). These bacteria are inhibited by ethanol, occur only during the early stages of fermentation, and rarely occur in beer. The effect is reported to produce enhanced fermentation rate and production of diacetyl, lactic acid, acetaldehyde and dimethyl sulphide (DMS). Two species from the genus *Klebsiella*, *K. terrigena* and *K. oxycota,* have been associated with brewery environments (Vaughan, O'Sullivan, & Sinderen, 2005).

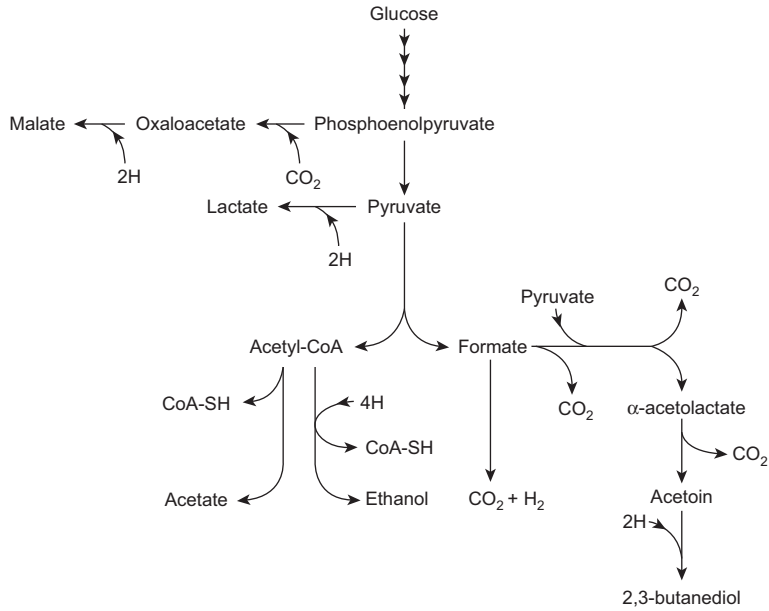

Figure 8.3 Metabolic pathways used by *Enterobacteriaceae* species for breakdown of glucose.
Adapted from van Vuuren and Priest (2003).

Klebsiella spp. are notably cited for production of phenolic off-flavour due to 4-vinylguaiacol formed from decarboxylation of ferulic acid present in the wort. A similar type of reaction is catalysed by some wild yeast (van Vuuren & Priest, 2003). *Klebsiella terrigena* is positive to the Voges–Proskauer test and produces high concentrations of acetoin and 2, 3-butanediol through the 2, 3-butanediol pathway by enhanced formation of α-acetolactate. All genes for the 2, 3-butanediol pathway in *K. terrigena* are located on a single operon, and production of 2, 3-butanediol is related to amino acid synthesis, pH and presence of oxygen (Blomqvist et al., 1993). A summary of several metabolic pathways of *Enterobacteriaceae* species is provided in Figure 8.3.

Rahnella aquatilis, formerly known as *Enterobacter agglomerans,* has been isolated from various sources such as soil, water, food, plant material and occasionally from clinical specimens (van Vuuren, Kersters, Ley, & Toerien, 1978). In brewing environments it has been reported as a contaminant in top fermenting yeast and fermented wort (Hamze et al., 1991; van Vuuren, Cosser, & Prior (1980); van Vuuren, Kersters, Ley, & Toerien, 1978). *Rahnella aquatilis* has been reported to affect the fermentation rate initially, but its growth is affected by ethanol during the later stages of fermentation. The aroma and flavour of contaminated beer has been typically described as fruity, milky and sulphury due to the production of diemthyl sulphide (DMS), acetaldehyde, methyl acetate and diacetyl in fermenting wort (van Vuuren, 1980). DMS is produced due to the reduction of dimethyl sulphoxide by *R. aquatilis,* unlike other *Enterobacteriaceae*. Because of its ability to survive through

the beer fermentation process and to accumulate in pitching yeast, it was suggested to be a potential beer spoiler (van Vuuren, Kersters, Ley, & Toerien, 1978; Hamze et al., 1991).

Mixed and uncontrolled fermentation beers such as lambic beers (Belgium) and coolship ales (United States) are produced by fermentation of wort by a mixture of brewery resident yeast and bacteria (Bokulich & Bamforth, 2013). During the early stages of fermentation, Enterobacteria such as *Klebsiella*, *Enterobacter*, *Citrobacter*, *Serratia* and *Pectobacter* are predominant and contribute to the aroma of lambic beers by producing organic acids, 2, 3-butanediol, ethyl acetate and some higher alcohols (Bokulich et al., 2012).

8.4.3 *Detection of* Enterobacteriaceae *in brewery environments*

For the detection of *Enterobacteriaceae* in wort and yeast slurries, the use of MacConkey agar supplemented with actidione (10 ppm) for suppression of yeasts is recommended by the European Brewing Convention (Jespersen & Jakobsen, 1996). MacConkey agar is a selective medium for coliform and *Enterobacteriaceae*, containing bile salts and crystal violet for inhibition of gram-positive bacteria and lactose is used as a sole source of carbon. However *O. proteus* grows comparatively slowly on MacConkey agar (Priest, 2006). Universal beer agar with actidione has been used for wort samples, and Wallerstein Laboratory Nutrient (WLN) agar has been used used for enrichment of beer (Priest, 2006). Chromogenic media used for enumeration of *Escherichia coli* and coliform bacteria simultaneously in water samples have been developed (Brenner et al., 1993), but they are rarely used in brewing laboratories (Hill, A. E., personal communication).

A PCR-based method for specific detection and discrimination of *O. proteus* biogroup-2 strains from *O. proteus* biogroup-1 and other related microorganisms has been documented (Maugueret & Walker, 2002). Characterisation of *O. proteus* biogroup-1 strains using automated ribotyping and PCR-based methods have also been reported (Koivula, Juvonen, Haikara, & Suihko, 2006).

8.5 Conclusion

AAB are still a common contaminant in beer dispense. Brewery-related *Enterobacteriaceae* need to be monitored in breweries due to the high level of hygiene required and also due to their role in production of ATNC in beer. Selective *Enterobacteriaceae* also play a significant role in flavour development in lambic and American coolship ales. *Zymomonas* seems to be limited to primed beer in the brewing industry, but strains of *Zymomonas* are still a problem in the cider industry.

Gram-negative aerobic and facultative anaerobes such as AAB, *Zymomonas,* and certain species of *Enterobacteriaceae* were of serious concern to brewers a few decades ago. As a results of technological improvement in reducing the dissolved oxygen in beer, these microorganisms are now considered less important. However, due to the extent of consumer awareness about food and beverage safety and the concern with maintaining corporate brand image, beer spoilage microorganisms remain a serious concern to breweries worldwide.

8.6 Further reading

Excellent reviews on beer spoilage bacteria have been published in recent years by Suzuki (2011), Vriesekoop et al. (2012) and Bokulich and Bamforth (2013). The Journal of the Institute of Brewing's archive (1890–present) also provides an excellent source of information on brewing microbiology aspects.

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Strictly anaerobic beer-spoilage bacteria

9

R. Juvonen
VTT Technical Research Centre of Finland, Espoo, Finland

9.1 Introduction

Strictly anaerobic beer-spoilage bacteria are a group of evolutionarily and physiologically related microorganisms. Unlike other brewery related spoilage microbes, they require a nearly oxygen-free environment to grow in beer. The first species were discovered only in the late 1970s. However, there is indirect evidence of their occurrence in breweries as early as 1946 (Haikara, 1984). It has been postulated that the improvements in the filling technology to reduce oxygen levels in the final beer, coupled with increased production of unpasteurized products, made the growth of the strictly anaerobic bacteria in beer eventually possible (Haikara & Helander, 2006). These bacteria are among the most detrimental organisms in the beer production chain and show a worldwide distribution. They mainly spoil non- or flash-pasteurized beers produced in modern breweries with effective filling technology. Spoilage is evidenced by development of turbidity and unpleasant off-odours described as rotten-egg-, rancid- or faeces-like, which render the product undrinkable. High economic losses usually ensue from spoilage due to damaging of the corporate brand and high costs of disposing contaminated batches and/or keeping the produced beer in quarantine.

This chapter will give the reader an overview of the types, evolution, occurrence and properties of the strictly anaerobic beer-spoilage bacteria. Moreover, the prevention and elimination of contaminations will be discussed, and finally an outlook of the future importance of the strictly anaerobic beer-spoilage bacteria will be given. The detection and identification methods for the strictly anaerobic beer-spoilage bacteria are presented in Chapter 10. *Zymomonas mobilis*–a specialized spoilage organism of primed beers (sugar added after bottling) and ciders produced in the UK (Van Vuuren & Priest, 2003)–is excluded, since it differs in many ways from the other strictly anaerobic beer spoilers.

9.2 The types of strictly anaerobic beer-spoilage bacteria

The strictly anaerobic beer-spoilage bacteria comprise currently nine species that are distributed between the genera *Megasphaera* (Engelmann & Weiss, 1985; emended by Marchandin, Haikara, & Juvonen, 2009), *Pectinatus* (Lee, Mabee, & Jangaard, 1978; emended by Schleifer et al., 1990; Caldwell, Juvonen, Brown, & Breidt, 2013), *Selenomonas* (Von Prowazek, 1913 as quoted by Shouche, Dighe, Dhotre,

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Patole, & Ranade, 2009) and *Propionispira* (Ueki, Watanabe, Ohtaki, Kaku, & Ueki, 2014). The analysis of brewery samples with culture-independent techniques has suggested that there are still new anaerobic spoilage bacteria to be discovered in the beer production chain (Nakakita, Takahashi, Sugiyama, Shigyo, & Shinotsuka, 1998; Timke, Wang-Lieu, Altendorf, & Lipski, 2005).

The science of classification of microorganisms is undergoing constant changes. DNA techniques, especially the sequence analysis of molecular chronometers and 16S rRNA gene in particular, have allowed the scientist to construct a classification system reflecting evolutionary (=phylogenetic) relationships between the organisms. 16S rRNA gene sequence analysis has shown that despite the Gram-negative cell envelope the strictly anaerobic beer-spoilage species originate from Gram-positive bacteria (Figure 9.1). They are currently classified in the phylum *Firmicutes* which contains otherwise mainly Gram-positive bacteria, such as *Clostridium* and *Bacillus* species (Doyle et al., 1995; Willems & Collins, 1995). Recently a new class called *Negativicutes* was established for firmicutes with a Gram-negative cell envelope (Marchandin et al., 2010). It has been suggested that these bacteria represent a line of evolution for developing a protective barrier for escaping from the lethal effects of antibiotics produced by other microbes (Gupta, 2011).

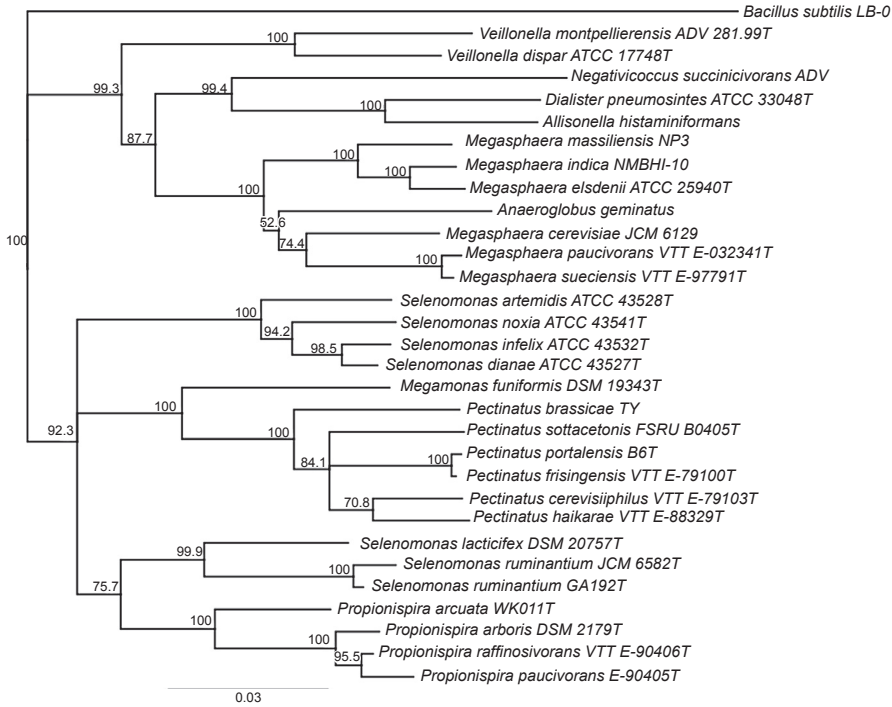

Figure 9.1 16S rRNA gene sequence-based tree describing evolutionary relationships of the strictly anaerobic beer-spoilage bacteria of the class *Negativicutes*.

9.2.1 Pectinatus

The genus *Pectinatus* ('combed bacteria') currently includes three recognized beer spoilers: *Pectinatus cerevisiiphilus* ('beer lover') (Lee et al., 1978; emended by Schleifer et al., 1990), *Pectinatus frisingensis* ('from Freising') (Schleifer et al., 1990) and *Pectinatus haikarae* ('named after Dr Auli Haikara for her many contributions to the study of *Pectinatus* bacteria') (Juvonen & Suihko, 2006). The first species, *P. cerevisiiphilus*, was named to describe an unusual strictly anaerobic bacterium isolated from spoiled beer in the USA in the late 1970s (Lee et al., 1978). A similar organism, first misidentified as *P. cerevisiiphilus*, was found shortly after from spoiled beers in Finland (Haikara, Enari, & Lounatmaa, 1981). It was eventually described in 1990 as a new species, *P. frisingensis* (Schleifer et al., 1990). In the late 1980s a *Pectinatus*-like beer spoiler genetically different from the already recognized species was deposited to a German culture collection (www.dsmz.de/). The finding of similar bacteria in Finland a few years later led (Suihko & Haikara, 2001) to the description of the third beer-spoilage species, *P. haikarae* (Juvonen & Suihko, 2006).

This millennium has seen the discovery of the first *Pectinatus* species that have not been associated with beer production. Gonzalez et al. (2005) found a new species, *Pectinatus portalensis* ('of El Portal'), from winery wastewater. However, the cultures cited as the type strain of the species do not conform to the original species description, and it has been requested to reject the name if a suitable replacement strain is not found (Vereecke & Arahal, 2008). Another nonbeer-associated species, *Pectinatus brassicae* ('of cabbage'), was recently isolated from salty pickle wastewater of cabbage production (Zhang et al., 2012). Shortly after this, another new species, *Pectinatus sottacetonis* ('of pickle'), was discovered from a commercial pickle spoilage tank of cucumbers in the USA (Caldwell et al., 2013).

Based on 16S rRNA gene sequence comparisons, the closest relative of *P. frisingensis* is *P. portalensis* (Gonzalez et al., 2005), whereas *P. cerevisiiphilus* is most closely related to *P. haikarae* (Juvonen & Suihko, 2006) (Figure 9.1). The closest known relatives of *P. sottacetonis* are *P. haikarae* and *P. brassicae* (Caldwell et al., 2013). The analysis of different molecular clocks has suggested that *P. frisingensis* is an older and more diverse species than *P. cerevisiiphilus* (Chaban et al., 2005; Motoyama, Ogata, & Sakai, 1998; Suihko & Haikara, 2001). The most closely related other bacteria to *Pectinatus* are anaerobic *Megamonas* species mainly found in caecal contents of birds (Figure 9.1).

9.2.2 Megasphaera

The genus *Megasphaera* ('a big sphere') was created in 1971 (Rogosa, 1971; emended by Marchandin et al., 2003) and is currently assigned to a family *Veillonellaceae* (Marchandin et al., 2010). It includes three beer-spoilage species: *Megasphaera cerevisiae* ('of beer'), *Megasphaera paucivorans* ('user of a few substrates') and *Megasphaera sueciensis* ('of Swedish origin'). The other species in this genus include *Megasphaera elsdenii* from rumen (Gutierrez, Davis, Lindahl, & Warwick, 1959; Rogosa, 1971), *Megasphaera micronuciformis* from clinical sources

(Marchandin et al., 2009) and *Megasphaera indica* from the human gut ecosystem (Lanjekar, Marathe, Ramana, Shouche, & Ranade, 2014). *Megasphaera massiliensis*, also from the human gut ecosystem, was not at the time of publication of this book yet officially accepted as a new species (Padmanabhan et al., 2013).

The beer-spoilage species form a distinct group in a phylogenetic tree (Figure 9.1), suggesting that they are ecologically distinct from the other *Megasphaera* species. *M. cerevisiae* shares 93.9% 16S rRNA sequence similarity with *M. sueciensis* and *M. paucivorans* (Marchandin et al., 2003). *M. sueciensis* and *M. paucivorans* have nearly identical 16S rRNA gene sequences but can be differentiated from each other using DNA–DNA reassociation and some physiological tests (Juvonen, 2009). *Anaeroglobus geminatus, Allisonella histaminiformans* and *Dialister* species are among the nearest relatives of *Megasphaera* species (Carlier et al., 2002; Marchandin et al., 2003) (Figure 9.1). Culture-independent DNA analyses have suggested that biodiversity within the genus *Megasphaera* is still largely underestimated (Juvonen, 2009; Padmanabhan et al., 2013; Shetty, Martahe, Lanjekar, Ranade, & Shouche, 2013; Zozaya-Hinchliffe, Martin, & Ferris, 2008), and in the future many new species will certainly be described.

9.2.3 Selenomas and Propionispira (Zymophilus)

The genus *Zymophilus* with two brewery-associated species, that is, *Zymophilus paucivorans* ('user of a few substrates') and *Zymophilus raffinosivorans* ('raffinose devouring') (Schleifer et al., 1990) was recently combined with the genus *Propionispira* as they were shown to have a common ancestor (Ueki et al., 2014). In addition to *Propionispira paucivorans* and *Propionispira raffinosivorans*, this genus includes *Propionispira arboris* (Schink, Thompson, & Zeikus, 1982) from wetwoods of living trees (Schink et al., 1982) and *Propionispira arcuata* (Ueki et al., 2014) from methanogenic cattle waste. The brewery-related species are evolutionarily closest to each other (Motoyama & Ogata, 2000; Schleifer et al., 1990) (Figure 9.1).

Selenomonas ('crescent-shaped') *lacticifex* ('a maker of lactic acid') is the only brewery-related species in its genus (Schleifer et al., 1990) that comprises eight other species mainly found from oral and ruminal habitats (Shouche et al., 2009; Zhang & Dong, 2009). The genus *Selenomonas* appears to originate from several ancestors and should be split and reclassified in the future (Juvonen, 2009) (Figure 9.1).

9.3 Occurrence in artificial and natural environments

The beer-spoilage species *Megasphaera, Pectinatus, Propionispira* and *Selenomonas* have been isolated from the beer production process and/or spoiled beers, and with a few exceptions their habitats in natural environment are unknown.

9.3.1 Pectinatus

The beer-spoilage *P. cerevisiiphilus, P. frisingensis*, and *P. haikarae* species have mainly been isolated from spoiled unpasteurized and flash-pasteurized beers

and from the brewing process, and their natural sources and mode of transmission to breweries are still largely unknown (see below). *P. cerevisiiphilus* and *P. frisingensis* have been found in the beer production chain worldwide (Hage & Wold, 2003; Haikara & Helander, 2006; Lee et al., 1978; Matoulková, Kosar, & Slabý, 2012; Paradh, Mitchell, & Hill, 2011; Schleifer et al., 1990), whereas *P. haikarae* findings have been restricted to the Nordic countries and Germany (Juvonen, 2009; Voetz, Pahl, & Folz, 2010).

The findings of *Pectinatus* bacteria in breweries have concentrated in filling halls (Haikara & Helander, 2006; Hakalehto, 2000; Juvonen, 2009; Matoulková et al., 2012; Paradh et al., 2011). Brewery filling halls provide a good growth environment for microbes due to their relatively high temperature and humidity and the presence of nutrients from product residues (Henriksson & Haikara, 1991). Matoulková et al. (2012) recently studied the occurrence of *Pectinatus* in 11 filling lines in 10 different brewery plants in the Czech Republic. *Pectinatus* could be isolated from all breweries regardless of their size, output and type of beer produced, the filling line capacity, rate, design, age or the method of cleaning. The most frequently contaminated areas (*Pectinatus* in more than 50% of samples) were the difficult to clean parts inside and underneath the conveyor belts and the various monoblock constructions, such as the surface of piping below the bench. Overall the highest percentage of positive samples was found in the floor sampling area, including drainage systems and cracks and crumbling joints in the floor. The occurrence in drainage systems has also been noted in many previous studies, and water has been suspected as one primary source of contamination (Haikara & Helander, 2006; Juvonen, 2009). Other reported sources within the filling halls include the air and ceiling of the filling halls and chain lubricants (for a review see Haikara & Helander, 2006; Juvonen & Suihko, 2006). *Pectinatus* has occasionally been detected in biofilms in the filling machines but also in places with no usual biofilm occurrence (Matoulková et al., 2012; Timke et al., 2005; Voetz et al., 2010).

Unlike previously thought, *Pectinatus* bacteria may contaminate all stages of the beer production process. *Pectinatus* bacteria have occasionally been detected in the fermentation area, such as in carbon dioxide collection pods of fermenters, maturing beer in the cellar and in finished beer as well as in bright beer tanks (Juvonen, unpublished data; Matoulková et al., 2012; Paradh et al., 2011). However, to our knowledge viable cells have only been recovered from the filling halls and spoiled beers. The role of brewing raw materials as contamination sources is still unresolved. Some of the early reports about the occurrence of *Pectinatus* in malt steeping water and pitching yeast have later proven to be misidentifications (Haikara & Helander, 2006).

There appears to be a seasonal variation in the occurrence of *Pectinatus* in the breweries. Spoilage incidences and *Pectinatus* findings tend to peak during the warm months of the year (Paradh et al., 2011). Despite the seasonal variation, *Pectinatus* species are considered permanent rather than occasional invaders in breweries (Hakalehto, 2000). They are typically detected from several sources in a single brewery (Hakalehto, 2000; Matoulková et al., 2012; Paradh et al., 2011; Suiker, O'Sullivan, & Vaughan, 2007). The communities can be rather complex, comprising several different genotypes (Juvonen, unpublished data). As a result several strains may be involved in spoilage incidents, which makes tracing of contaminations

challenging. Suzuki (2011) recently postulated that the beer-spoilage *Pectinatus* bacteria have adapted to live in a mutually beneficial association with brewer's yeast and lactic acid bacteria since the early times of brewing.

Since the peak in the late 1980s and early 1990s, a decreasing trend in documented spoilage incidents has been observed (Back, 2005). However, it needs to be taken into consideration that most spoilage cases are not reported and information has systematically been gathered only in Germany (Back, 2005). Suzuki (2011) recently estimated that *Pectinatus* bacteria are responsible for 20–30% of beer-spoilage incidents. *P. frisingensis* is the dominant species in spoilage incidents and is also the most frequently reported species in breweries (Matoulková et al., 2012; Motoyama et al., 1998; Paradh et al., 2011; Suihko & Haikara, 2001; Suiker et al., 2007).

During the past few years the known habitats of *Pectinatus* bacteria have widened as a result of the description of the new species from environments related to fermentation processes other than brewing, such as waste streams of wineries and distilleries and pickle production (Caldwell et al., 2013; Castelló et al., 2009; Gonzalez et al., 2005; Temudo, Muyezer, Kleerebezem, & van Loodsrecht, 2008; Zhang et al., 2012). Hence it appears that the *Pectinatus* species live in association with lactic or ethanol fermentation processes of plant raw materials. In 2013 a beer-spoilage *Pectinatus* species was for the first time isolated outside the beer production chain when *P. cerevisiiphilus* strains were found in mangrove sediments in Thailand (http://www.ncbi.nlm.nih.gov/genbank/). The available evidence suggests that *Pectinatus* species are plant-originating bacteria, which is also supported by their cell wall structures and their ability to grow with common plant sugars and in the presence of high concentrations of plant phenolic compounds (Caldwell et al., 2013; Helander, Haikara, Sadovskaya, Vinogradov, & Salkinoja-Salonen, 2004; Juvonen, 2009). On the other hand their close evolutionary relations to intestinal species (Chevrot et al., 2008) suggest an animal or human source.

9.3.2 Megasphaera

Megasphaera cerevisiae appears to be geographically more restricted compared to the beer-spoilage *Pectinatus* species. Contaminations have been reported in Australia, Finland, Germany, Norway, Sweden and the UK (Hage & Wold, 2003; Haikara & Helander, 2006; Paradh et al., 2011). *M. cerevisiae* shares its ecological niche with the beer-spoilage *Pectinatus* bacteria. It has been detected in spoiled beers and in brewery filling halls. Sporadic findings from pitching yeast and a brewery carbon dioxide line have also been reported (Haikara & Helander, 2006).

Megasphaera cerevisiae is a less frequent brewery contaminant and beer spoiler than *Pectinatus* bacteria. In Germany, *M. cerevisiae* caused 2–7% of the documented beer-spoilage cases during the time period 1990–2002 (Suzuki, 2011). *M. paucivorans* was originally isolated from spoiled beer produced in Italy, whereas *M. sueciensis* was discovered from a spoiled Swedish beer. Paradh et al. (2011) recently detected *M. sueciensis*/*M. paucivorans* (not separable by the DNA analysis used in the study) also in a brewery filling hall in the UK (conveyer belt of canning line). Moreover *M. sueciensis*/*M. paucivorans* have been found in anaerobic biohydrogen production

systems using cheese whey and anaerobic sludge as raw materials (Castelló et al., 2009; Jin, Sun, & Shi, 2010; Ning, Jin, Sheng, Harada, & Shi, 2012). Many *Megasphaera* species produce hydrogen and can be beneficial organisms in a biohydrogen production process.

9.3.3 Selenomas and Propionispira

Propionispira paucivorans and *P. raffinosivorans* as well as *S. lacticifex* have been isolated from pitching yeast samples in Germany and Finland. In the late 1980s Seidel-Rüfer (1990) examined more than 3000 yeast samples from German breweries. Of these 0–0.03% were contaminated with *S. lacticifex* and 0.12–0.7% with *Propionispira* species. Moreover brewery waste streams and drainage systems have been mentioned as possible sources of *P. raffinosivorans* (Haikara, 1989; Schleifer et al., 1990; Seidel-Rüfer, 1990).

Culture-independent analysis of microbial diversity in industrial and natural ecosystems has revealed new habitats for *S. lacticifex*. DNA sequences from *S. lacticifex* have been detected from a biomass of a continuous stirred tank reactor, sewage from a wastewater treatment plant and from the food waste hydrolysate (http://www.ncbi.nlm.nih.gov/genbank/). The close evolutionary relation of the beer-spoilage *P. raffinosivorans* to a tree pathogen *P. arboris* suggests that it may be carried to breweries with plant raw materials (Juvonen, 2009).

9.4 Appearance of cells and laboratory cultures

Properties, such as the cellular shape, motility and structures and appearance of laboratory cultures in solid and liquid media have traditionally been used as first steps to identify unknown microbes. The double staining method of Hans Christian Gram can reveal differences in the cell wall structures, and it has been used to classify bacteria to Gram-positive and Gram-negative ones. Gram-positive bacteria retain crystal violet staining purple, whereas Gram-negative bacteria decolourize and can be counterstained red with safranin. *Pectinatus, Megasphaera, Selenomonas* and *Propionispira* species and related organisms are special in that they stain Gram-negative but are evolutionarily related with Gram-positive bacteria. The cells possess features of both Gram-negative and Gram-positive bacteria being surrounded by a thick peptidoglycan layer typical of Gram-positive bacteria and an outer membrane typical of Gram-negative bacteria. The cellular and cultural characteristics of the strictly anaerobic beer spoilers are discussed below.

9.4.1 Pectinatus

Pectinatus cells are nonspore-forming, slightly curved helical rods, 0.4–1.0 by 2–50 μm or more, with rounded ends. They typically occur singly, in pairs or rarely in short chains. In older cultures elongated snake-like cells and various round cell formations can be seen (Haikara & Juvonen, 2009). A distinctive feature of the *Pectinatus*

cells is their comb-like flagellar arrangement in which flagella only emanate from one side of a cell (Figure 9.2), which leads to the formation of an X-pattern during movement.

The cell surface structures of *P. cerevisiiphilus* and *P. frisingensis* strains have been studied in detail (for a review see Helander, Haikara, Sadovskaya, Vinogradov, & Salkinoja-Salonen, 2004). Structures and composition of their lipopolysaccharides (LPS) are exceptional in many ways. LPS are unique functional components of the outer membranes of Gram-negative bacteria, consisting of a lipid and a polysaccharide part (Helander et al., 2004). Each *Pectinatus* strain appears to be capable of producing at least two types of LPS with distinct carbohydrate structures. Moreover, their LPS contain 3-deoxy-D-manno-oct-2-ulapyranosonic acid, and the lipid A linkage with polysaccharide is very acid-stable (Haikara & Helander, 2006). The outer membranes of classic Gram-negative bacteria are typically an efficient permeability barrier for cationic substances and large molecules. However the outer membranes of *Pectinatus* cells fulfil this function variably (Caldwell et al., 2013; Haikara & Juvonen, 2009). Peptidoglycan of *P. frisingensis* and *P. cerevisiiphilus* contains cross-linked meso-diaminopimelic with putrescine or cadaverine in the peptide subunit (Schleifer et al., 1990).The fatty acid composition of cells is similar between different species being dominated by odd-numbered fatty acids, that is, $C_{11:0}$, $C_{13:0}$, $C_{15:0}$, $C_{13:0\ (3OH)}$ (most probably misidentified as $C_{14:0}$ in MIDI), $C_{17:1}$ and $C_{18:1trans11}$ (Caldwell et al., 2013; Haikara & Helander, 2006).

Cultural characteristics of *Pectinatus* species have been studied in peptone yeast extract fructose (PYF) medium (http://culturecollection.vtt.fi) and MRS medium. The beer-spoilage species form circular, entire, glistening and opaque colonies on PYF

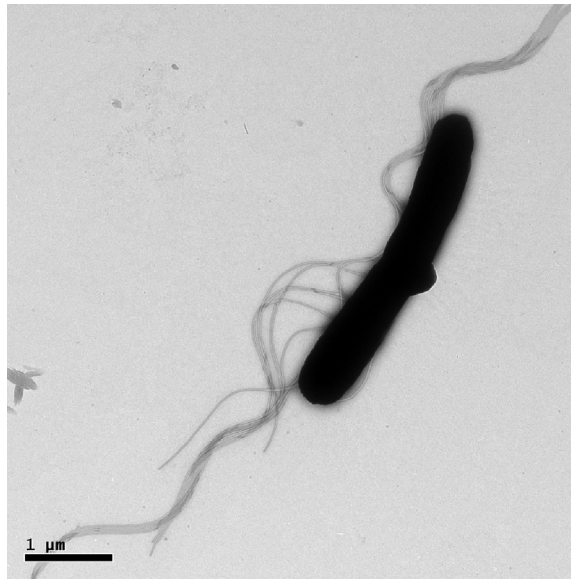

Figure 9.2 Electron micrograph of *Pectinatus haikarae* illustrating flagella on one side of the cell body. Bar = 1 µm.

medium. The colour of *P. haikarae* colonies varies from cream to greyish and in the case of the other two species from beige to white (Haikara & Juvonen, 2009).

9.4.2 Megasphaera

Unlike the other strictly anaerobic beer-spoilage species, *Megasphaera* cells are non-motile and nonspore-forming cocci (Figure 9.3). They are normally arranged singly, in pairs and sometimes in short chains. The three beer-spoilage species can be discriminated from each other by their cell size; *M. cerevisiae* is the biggest and *M. sueciensis* is the smallest (Juvonen & Suihko, 2006; Marchandin et al., 2009). The peptidoglycan of *M. cerevisiae* is of the meso-diamonopimelic acid direct type with putrescine residues (Engelmann & Weiss, 1985). The main fatty acid components are $C_{12:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:1}$, $C_{17cyclo}$, $C_{19\ cyclo}$ and $C_{14:0\ 3OH}$ (Marchandin et al., 2009). The cell surface structures of the other beer-spoilage species have not been studied.

All the beer-spoilage species grow on PYF agar medium. However, the growth of *M. sueciensis* and *M. paucivorans* is greatly improved when pyruvic acid or gluconic acid is used instead of fructose (Juvonen & Suihko, 2006). The three species differ in their growth rate on PYF agar medium. *M. cerevisiae* is normally detected within 1–2 days, whereas *M. paucivorans* and *M. sueciensis* require 3–4 days to form visible colonies (Juvonen, 2009). The colonies are circular, glossy and opaque. The colonies of *M. cerevisiae* are whitish in colour, whereas those of *M. paucivorans* and *M. sueciensis* are yellowish (Juvonen & Suihko, 2006; Marchandin et al., 2009).

9.4.3 Selenomonas and Propionispira

Selenomonas lacticifex and the beer-spoilage *Propionispira* species are motile rod-shaped bacteria. They may lose their mobility upon repeated cultivations. None of these species forms endospores. The cells of *S. lacticifex* are curved crescent-shaped

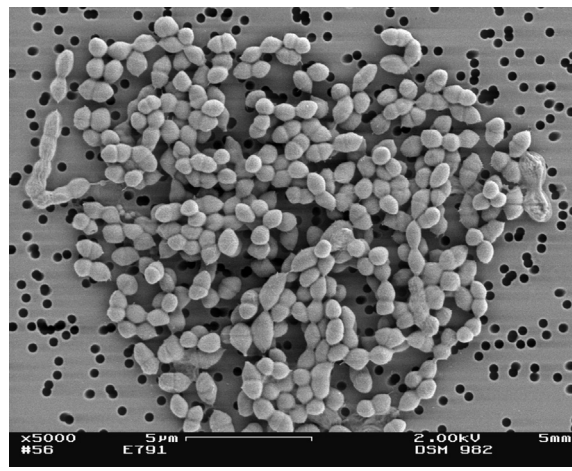

Figure 9.3 Electron micrograph of *Megasphaera sueciensis*. Bar = 5 µm.

rods, 0.6–0.9 to 5–15 µm in size (Schleifer et al., 1990). The cells of *P. paucivorans* are curved, helical or crescent shaped and up to 15 µm long. They occur singly, in pairs or in short chains. The cells of *P. raffinosivorans* are straight to slightly curved 'sausage-shaped' rods (0.8 × 6 µm). Cells in various helical arrangements may be seen even in young cultures (Seidel-Rüfer, 1990; Ueki et al., 2014). The three species share a similar peptidoglycan structure with *Pectinatus* bacteria (Schleifer et al., 1990; Ziola et al., 1999). Their other cell surface structures have not been studied.

Cultural characteristics have been determined on modified MRS agar medium in which the beer-spoilage *Propionispira* species form circular, smooth, opaque and slightly yellow colonies with a diameter of 1–2 mm after 3 days of incubation (Schleifer et al., 1990). The colonies of *S. lacticifex* are also smooth, circular, opaque and yellowish with a diameter of 2–3 mm after 3 days of incubation.

9.5 General physiology and metabolism

The strictly anaerobic beer-spoilage bacteria share a few metabolic and physiological features. For instance, they produce acetic and propionic acids from simple sugars. However, clear differences exist in their nutrition and physiology, which is reflected in their beer-spoilage ability and can be exploited for their detection and identification. The key characteristics discriminating the beer-spoilage species from each other are shown in Tables 9.1 and 9.2.

9.5.1 Pectinatus

The *Pectinatus* bacteria are fermentative organisms growing well with glucose and fructose. The different species vary to some extent in their carbohydrate utilization, which can be exploited in the phenotypic identification (Table 9.1). *P. frisingensis* grows with a wider range of carbohydrates compared to *P. cerevisiiphilus* (Schleifer et al., 1990). *P. haikarae* is the only species using lactose but not salicin. Interestingly only a few beer-spoilage strains utilize the main carbohydrate of malt, maltose (Juvonen & Suihko, 2006; Motoyama et al., 1998; Schleifer et al., 1990). Citric acid and lactic acid, which can be found in small quantities in beer, are metabolized (Haikara & Juvonen, 2009; Watier, Chowdhury, Leguerinel, & Hornez, 1996b). Due to the ability of *Pectinatus* to utilize lactic acid, contamination of beer by lactic acid bacteria may promote their growth. Ethanol or the main amino acids of beer are not metabolized by *P. frisingensis* or *P. cerevisiiphilus* (Schleifer et al., 1990; Tholozan, Membré, & Kubaczka, 1996).

The fermentation end products from simple sugars and some organic acids have been determined. Glucose is mainly fermented to propionic and acetic acids and acetoin, but succinic and lactic acids may also be produced (Haikara, Penttilä, Enari, & Lounatmaa, 1981; Juvonen & Suihko 2006; Tholozan, Membré, & Grivet, 1997; Watier et al., 1996b). The relative proportions of the end products depend on the substrate. *Pectinatus* spp. use the same metabolic pathway for propionic acid synthesis as propionibacteria (Haikara, Penttilä et al., 1981; Tholozan et al., 1994). In this pathway

Table 9.1 Phenotypic characteristics discriminating strictly anaerobic rod-shaped beer-spoilage bacteria

Characteristic	*Pectinatus cerevisiiphilus*	*Pectinatus frisingensis*	*Pectinatus haikarae*	*Selenomonas lacticifex*	*Propionispira paucivorans*	*Propionispira raffinosivorans*
Catalase	–	–	+	–	–	–
Growth at 37 °C	+	+	–	+	–	–
Acid from:						
N-acetyl-glucosamine	–	+	–	nd	–	+
Cellobiose	–	+	–	+	+	+
Inositol	–	+	+	–	–	+
Lactose	–	–	+	+	–	+
Maltose	–	–	–	d	+	+
Mannitol	+	+	+	–	+	+
Melibiose	+	–	+	+	–	+
Raffinose	–	–	–	+	–	+
Rhamnose	+	+	+	–	–	+
Salicin	+	+	–	–	–	v
Sorbitol	+	+	nd	–	+	+
Sucrose	–	–	nd	+	+	+
Xylitol	–	d	+	–	–	+
Xylose	+	–	+	+	–	+
Acetoin production	+	+	+	nd	–	–
Succinic acid production	+	+	nd	–	–	–
Lactic acid as the main metabolite	–	–	–	+	–	–

+, 75% or more of the strains are positive; –, 75% or more of the strains are negative; nd, not determined; d, delayed.
Modified from Juvonen (2009) and Haikara and Juvonen (2009).

Table 9.2 Phenotypic characteristics discriminating beer-spoilage *Megasphaera* species

Characteristic	*M. cerevisiae*	*M. paucivorans*	*M. sueciensis*
Cell size (μm)	1.5–2.1	1.2–1.9 × 1.0–1.4	1.0–1.4 × 0.8–1.2
Colonies visible on solid media	1–2 days	3 days	4 days
Acid production from fructose	+	–	–
Growth with lactic acid	+	–	–
Major metabolites[a]	**C**, iV, B	**iV**, **C**	**iV**, B, C, V

+, 75% or more of the strains are positive; –, 75% or more of the strains are negative.
[a]B, butyric acid; iV, isovaleric acid; V, valeric acid; C, caproic acid. The products in bold constitute 40–60% of the total amount.
Modified from Juvonen and Suihko (2006) and Juvonen (2009).

succinate oxidoreductase reduces fumaric acid to succinic acid. Biomass and volatile fatty acid concentrations have been found to be proportional to glucose and lactate concentrations in the medium (Tholozan et al., 1996). Propionic acid synthesis is not directly linked with growth (Watier et al., 1996b).

The *Pectinatus* bacteria are relatively acid and ethanol tolerant. In a laboratory medium, the optimal pH value for the growth of *P. cerevisiiphilus* was 6.0–6.2, whereas *P. frisingensis* grew best at pH 4.5–4.9 (Tholozan et al., 1997, 1996). The minimum pH for the growth was in the range of 3.5–4.1 (Caldwell et al., 2013; Haikara, Penttilä et al., 1981; Watier et al., 1996b). The maximum ethanol concentrations for growth varied from 5.8% to 8% (Tholozan et al., 1997, 1996; Watier et al., 1996b).

The *Pectinatus* species are mesophiles in their temperature preferences. The beer-spoilage species can grow at 15 °C, but the optimum is 30–32 °C. *P. frisingensis* and *P. cerevisiiphilus* still grow at 40–45 °C, whereas *P. haikarae* is unable to grow even at 37 °C (Haikara, 1989; Lee et al., 1978; Schleifer et al., 1990). *P. frisingensis* has been shown to be able to survive rapid temperature changes and recover quickly at suitable growth conditions (Chihib & Tholozan, 1999).

Despite being anaerobes, *P. cerevisiiphilus* and *P. frisingensis* are relatively oxygen tolerant. The decimal reduction times (D_{oxy}) at the dissolved oxygen content of 4.78 mg/L (32 °C) varied from 3.3 to 55 h (Chowdhury, Watier, & Hornez, 1995). The dissolved oxygen content in the medium affected the inactivation rates (Chowdhury et al., 1995). For instance, D_{oxy} values for *P. cerevisiiphilus* increased from 4.8 to 13.3 h when the oxygen content of wort decreased from 5.74 to 3.34 mg/L. The oxygen tolerance is also influenced by temperature, increasing with the temperature decrease. *P. haikarae* appears to also be oxygen tolerant, as it has been isolated alive from the air of brewery bottling halls (Juvonen & Suihko, 2006).

9.5.2 Megasphaera

Megasphaera cerevisiae strains form a uniform group in terms of utilized carbon sources that include arabinose, fructose, lactic acid and pyruvic acid (Engelmann & Weiss, 1985).

Table 9.3 Effects of strictly anaerobic beer-spoilage bacteria on beer quality

Genus	Effects on fermentation	Effects in finished beer		
		Metabolites[a]	Off-flavour	Turbidity
Megasphaera	Not known	Acetic, **butyric**, caproic, isobutyric, isovaleric, propionic, and valeric acids, **H_2S**	Rancid, rotten egg	+
Pectinatus[b]	Possible inhibition	Acetic, **propionic**, succinic and lactic acids, acetoin, **H_2S**, organic sulphur compounds	Rotten egg	+
Selenomonas[c]	Not known	Acetic, **lactic**, and propionic acids	Not known	+
Propionispira[d]	Not known	Acetic and propionic acids (H_2S by *P. raffinosivorans*)	Not known	+

[a]The major metabolites are in bold.
[b]*Pectinatus haikarae* may also cause foaming but are slight, off-flavour and turbidity.
[c]*Selenomonas lacticifex*.
[d]*P. paucivorans* and *P. raffinosivorans* grow at an elevated pH value of 5–6.

However, the only known carbon sources used by *M. sueciensis* and *M. paucivorans* strains are pyruvic, gluconic and glucuronic acids (Juvonen & Suihko, 2006). *Megasphaera* bacteria have a fermentative type of metabolism. The exact composition of the fermentation products depends on the energy source in the medium and may include acetic, propionic, iso- and n-butyric, iso- and n-valeric and caproic acids. Moreover, hydrogen sulphide, hydrogen and carbon dioxide are produced (Engelmann & Weiss, 1985; Juvonen & Suihko, 2006). The three beer-spoilage species can be discriminated from each other (Table 9.2) based on their volatile fatty acid metabolites, which can be determined by using gas chromatography. Metabolite analysis is a useful method to identify the growth of *Megasphaera* and *Pectinatus* in beer, especially when the cells are no longer cultivable (Table 9.3).

Megasphaera cerevisiae is moderately acid tolerant. It still grows weakly in laboratory media at pH 4.1–4.2 but not anymore at pH 4.0 (Haikara, 1989). The acid tolerance of the other beer-related species has not been studied. The growth temperature of the beer-spoilage species ranges from 15 to 37 °C (Haikara, 1989; Juvonen & Suihko, 2006). *M. cerevisiae* appears to tolerate oxygen at least at low temperatures (Juvonen, 2009). The antibiotic sensitivity of the type strains has been studied. All the type strains are resistant to vancomycin. *M. sueciensis* and *M. paucivorans* are also resistant to colistin, whereas *M. cerevisiae* is sensitive to this compound (Juvonen & Suihko, 2006).

9.5.3 Selenomonas and Propionispira

The physiological properties of *S. lacticifex* and *Propionispira* species have been little studied. *S. lacticifex* utilizes a wide range of carbon sources, including arabinose, cellobiose, glucose, lactic acid and maltose (Schleifer et al., 1990). It differs from the other strictly anaerobic beer spoilers by producing lactic acid as the major fermentation end product (Schleifer et al., 1990). *P. raffinosivorans* uses a greater variety of carbon substrates compared to *P. paucivorans* (Schleifer et al., 1990; Ueki et al., 2014). Both species ferment glucose, pyruvic, lactic and fumaric acids to acetic and propionic acids. Moreover, propionic acid is produced from succinic acid.

Selenomonas lacticifex strains from yeast samples have been shown to grow well at pH 4.3 but not at pH 4.2. The *Propionispira* strains were less acid tolerant (Seidel-Rüfer, 1990). The optimum growth temperature for all three species is close to 30 °C. *P. raffinosivorans* and *P. paucivorans* do not grow at 37 °C, whereas *S. lacticifex* strains vary in this respect (Haikara, 1989; Schleifer et al., 1990; Seidel-Rüfer, 1990). *S. lacticifex* shows a lower minimum temperature for growth compared to *Megasphaera* and *Pectinatus* bacteria and can even grow at 10 °C.

9.6 Growth and effects in beer

The strictly anaerobic beer-spoilage bacteria can be divided into absolute and potential beer-spoilage organisms. By definition, an absolute spoiler is able to grow in beer without a long adaptation period and to cause obvious quality defects. A potential spoiler does not grow in standard beers under normal conditions and does not always cause obvious quality defects or requires a long adaptation time (Back, 2005). It is currently considered that even a few viable cells of the strictly anaerobic bacteria in a package of beer may eventually lead to spoilage.

9.6.1 Pectinatus

Pectinatus cerevisiiphilus, P. frisingensis and *P. haikarae* are absolute beer spoilers. They spoil unpasteurized and flash-pasteurized beers in a package. All strains are regarded as potentially harmful, although strain-specific differences in the ability to survive and grow in beer have been noted (Suiker, Vaughan, & O'Sullivan, 2009). The spoilage results from the production of large quantities of propionic acid (up to >1000 mg/L), some acetic acid, hydrogen sulphide (20–300 µg/L) and turbidity (Figure 9.4; Table 9.3), which is evident at cell concentrations of approximately 10^5 cfu/mL (Haikara, Enari et al., 1981). Organic sulphur compounds dimethyl trisulphide and methyl mercaptan may also be produced above their taste threshold levels. The growth of *Pectinatus* too low to cause turbidity may produce metabolites in concentrations high enough to cause spoilage (Haikara, Enari et al., 1981). The spoiled beer has an odour of rotten eggs that makes it fully unfit for consumption (Haikara & Helander, 2006). The off-flavour and turbidity of beer spoiled by *P. haikarae* appear to be less noticeable compared to the defects caused by the other species (Voetz et al., 2010).

Figure 9.4 Beer spoiled by *Megasphaera cerevisiae* (left) and *Pectinatus frisingensis* (right).

The pH value, ethanol concentration and dissolved oxygen content are among the key factors controlling in concert the growth of *P. cerevisiiphilus* and *P. frisingensis* in beer. However, there are other unidentified factors that affect the susceptibility of a beer to *Pectinatus* spoilage. *P. frisingensis* and *P. cerevisiiphilus* are the most acid-tolerant species among the strictly anaerobic beer spoilers. The acid tolerance of *P. haikarae* is not known. *P. frisingensis* grows well at pH values of typical lager beers. Some growth retardation was observed at pH 4.1 (Haikara, 1984). *P. frisingensis* and *P. cerevisiiphilus* tolerate ethanol better than classical Gram-negative bacteria. *P. frisingensis* grew well in commercial beers with 3.7–4.5% (w/v) ethanol, although the growth was slower than in low-alcohol products. Strong beers with ≥5.2% (w/v) ethanol were not spoiled (Haikara, 1984; Haikara, Enari et al., 1981; Seidel-Rüfer, 1990). *P. frisingensis* and *P. cerevisiiphilus* are more hop tolerant compared to lactic acid bacteria and resist well the levels of hop bitter acids normally found in beers (EBU 33–38) (Haikara & Helander, 2006; Matoulková et al., 2012). The available information suggests that the hop tolerance may not be related to the properties of their outer membranes (Helander et al., 2004).

Despite their anaerobic nature, *Pectinatus* bacteria tolerate oxygen relatively well, especially at low temperatures, and viable bacteria have been isolated from various aerobic niches in breweries. However, low dissolved oxygen content is necessary for the growth in beer or wort. With the modern filling techniques the dissolved oxygen content of beer typically ranges from 0.3 to 0.8 mg/L. The growth of *Pectinatus* has been reported in beers with up to 1.9 mg/L of dissolved oxygen (Soberka, Sciazko, & Warzecha, 1988). The laboratory studies suggest that *P. cerevisiiphilus* may also grow slowly in oxygenated wort in the presence of brewer's yeast. In the study of Chowdhury, Watier, Leguerine, and Hornez (1997) *P. cerevisiiphilus* started to inhibit the yeast activity at fermentation temperatures above 15 °C. Hence *Pectinatus* bacteria could also cause fermentation problems.

Factors controlling the growth of *P. haikarae* have not been studied. To our knowledge, this species has only spoiled low-alcohol products (Juvonen, 2009; Voetz et al., 2010).

It grows in typical pH values of beer. *P. haikarae* appears to be better adapted to the brewing environment compared to its closest relative, *P. cerevisiiphilus*. It grows at lower temperatures and produces a catalase enzyme protecting the cells from toxic oxygen radicals. The isolation of *P. haikarae* from air samples indicates that it can survive in the air at least for short periods of time (Henriksson & Haikara, 1991).

Pectinatus brassicae, P. sottacetonis or *P. portalensis* species have not yet been found in the beer production chain. However, experiments with artificially contaminated beers have shown that *P. sottacetonis* can grow in beer containing 4 vol-% alcohol and having a pH value of 4.2 (Caldwell et al., 2013). *P. portalensis* has been observed to grow in German and Spanish beers and wines with alcohol contents ranging from 4.6% to 15% (Gonzalez et al., 2005). The beer-spoilage ability of *P. brassicae* has not been studied.

Nowadays breweries produce increasingly new types of nonbeer beverages that are expected to be more sensitive to microbiological spoilage compared to traditional soft drinks due to their higher pH value or nutrient content or milder carbonation level (Juvonen et al., 2011). We have evaluated the ability of various emerging and established beverage spoilage organisms and food pathogens to survive and grow in a range of functional drinks and modified waters (Juvonen, unpublished results). A strain of *P. frisingensis* was able to spoil a flavoured mineral water and a malt-based drink (Figure 9.5), and it could be considered a potential threat to the quality of some nonbeer beverages. It also tolerated relatively well organic acid preservatives used in soft drinks (Juvonen, unpublished data).

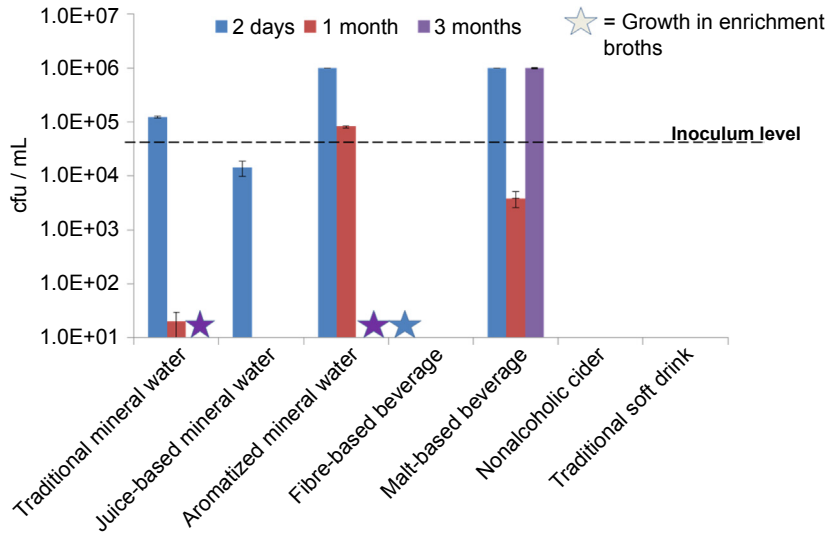

Figure 9.5 The growth of *Pectinatus frisingensis* in traditional and novel types of nonbeer beverages. Commercial beverage products were artificially contaminated with approximately 5×10^4 cfu/mL. Viable counts were determined after 2 days, 1 month and 3 months of ambient storage using the plate count technique.

9.6.2 Megasphaera

Megasphaera cerevisiae, M. paucivorans and *M. sueciensis* are regarded as absolute beer spoilers. *M. cerevisiae* spoils beer by producing copious amounts of butyric acid with minor amounts of C-5 and C-6 fatty acids and H_2S, which cause a particularly unpleasant off-flavour (Haikara & Lounatmaa, 1987). The contaminated beer normally becomes turbid in 4–6 weeks (Figure 9.4). The major organic acid produced by *M. paucivorans* in beer is also butyric acid, possibly deriving from the metabolism of pyruvic acid (Juvonen, unpublished data).

Megasphaera cerevisiae mainly spoils low-alcohol products due to its poor ethanol tolerance. In the study of Haikara and Lounatmaa (1987), the growth of *M. cerevisiae* strains was reduced in beers above 2.1% (w/v) ethanol concentration and ceased at 4.2% ethanol (w/v) concentration. The rate of spoilage was inversely proportional to the concentration of ethanol. *M. cerevisiae* is sensitive to the normal low pH of beer. In a commercial beer, no growth occurred at pH 4.0, and the spoilage rate was reduced at the pH of normal beer (Haikara & Lounatmaa, 1987). The results of our survival experiments suggest that *M. cerevisiae* can persist in nutrient poor and hostile conditions fully viable and active for long periods and initiate rapid growth when conditions improve (Juvonen, 2009).

There is a lack of data about beer-spoilage properties of the other species. *M. sueciensis* has been found as a spoilage microbe in low-alcohol beer, whereas *M. paucivorans* was found in spoiled product with an ethanol content of 3.9% (w/v). The pH of the beers spoiled by these species varied from 4.3 to 4.9 (Juvonen & Suihko, 2006).

9.6.3 Selenomonas and Propionispira

Selenomonas lacticifex is considered an absolute beer spoiler owing to its ability to grow in beer at pH 4.3–4.6 (Seidel-Rüfer, 1990). Since no beer-spoilage incidents caused by this species have been reported, it could be regarded as a potential threat to beer quality. *S. lacticifex* is more acid tolerant compared to brewery-related *Propionispira* species and less acid tolerant compared to *M. cerevisiae* or *P. frisingensis*. *S. lacticifex* is relatively alcohol tolerant. Growth has been observed in beer with an alcohol content of 4.2% (w/v) and in PYF medium containing 5–6% ethanol (w/v). Laboratory studies also suggest that *S. lacticifex* could still grow at the low temperature of yeast storage (10 °C) and lager fermentation (Haikara, 1989).

Propionispira raffinosivorans is considered to be a potential beer spoiler owing to its ability to grow in beer at pH 5.0 but not at pH 4.6. *P. paucivorans* was able to grow in beer at pH 6.0 but not at pH 5.0 and appears to be a harmless brewery contaminant (Seidel-Rüfer, 1990). There is a lack of data about other beer-spoilage properties of the *Propionispira* species.

9.7 Management of contaminations

9.7.1 Prevention

The fact that natural sources of the strictly anaerobic beer spoilers are largely unknown complicates the prevention of contaminations. Potential primary sources include plant

raw materials, such as hops and malt, bird droppings and water. It is, however, apparent that once *Megasphaera* and *Pectinatus* bacteria have found their way into breweries they can establish themselves and persist in suitable niches for years. Therefore maintenance of good factory and process hygiene, regular monitoring of critical points and rapid countermeasures in case of positive findings are key factors in preventing contaminations.

Dirty return bottles are one possible mode of transmission between and within the breweries (Haikara & Helander, 2006; Matoulková et al., 2012). Disinfection of the bottle racks and empty bottles before their entry into the filling halls, physical separation of bottle washing from the filling operations and the configuration of bottle washers so that bottle inlet and outlet are on the opposite sides have been suggested as preventive measures to reduce spreading of contaminations. Drainage systems and other anaerobic niches in the filling halls, such as broken floor structures, are often permanently inhabited by *Pectinatus* and *Megasphaera* bacteria, from where they easily spread via aerosols and human activities. Hygienic factory design and maintenance of good hygienic conditions, not only in the filling machines but also in the filling hall environment, is important to minimize the colonization of strictly anaerobic beer-spoilage bacteria in the breweries.

Filling lines are often structurally complex and contain difficult to clean areas prone to accumulation of biofilm (Storgårds & Priha, 2009). Biofilm is formed when microbial cells attach to surfaces and form complex communities that are protected by the self-produced slime. Avoiding complex constructs and regular sanitation of the complete filling lines, including dismantling and mechanical cleaning of difficult to access parts, is advised. In the study of Matoulková et al. (2012) the side ledge of the conveyor belt cover, cable line bundles beneath the conveyor belt and structural elements of the belt and monoblock parts were the critical areas, spreading contamination to the whole filling machine.

9.7.2 Elimination

Pectinatus and *Megapshaera* bacteria are able to find suitable niches in breweries where they may survive for years without causing any obvious defects (Hakalehto, 2000). Then due to some technological faults or inadequate cleaning, they may cause beer contamination and spoilage. Finding of contamination sources is the first step for their elimination. High contamination frequency of packaged products indicates that the contamination is affecting the whole production batch and sources should be looked at throughout the production process. Sporadic incidences refer to a secondary contamination in the filling stage. Frequently several contamination sites can be found in the process and it may be difficult to relate a specific source with spoilage incidents. However, occurrence of the strictly anaerobic beer spoilers in the areas where open product is handled is always a risk for the product quality and should lead to counteractions.

As counteractions, proper mechanical cleaning followed by disinfection and replacement of worn-out parts and surfaces should be promptly undertaken to eliminate

contaminations. *M. cerevisiae* and *Pectinatus* are relatively sensitive, especially to oxidative biocides such as peracetic acid, and their use is primarily recommended (Haikara, 1984). It should be borne in mind that microbes aggregated in a biofilm may have a much higher resistance towards biocides (up to 10–100 times) in comparison to planktonic cells (Storgårds & Priha, 2009).

Heat resistance studies in laboratory conditions have indicated that flash pasteurization treatments applied in the brewing process are normally sufficient to inactivate *Pectinatus* and *Megsphaera* cells (Watier, Chowdhury, Leguerinel, & Hornez, 1996a; Watier, Leguerinel, Hornez, Chowdhury, & Dubourguier, 1995). These bacteria cannot survive wort boiling. Decimal reduction time of *Pectinatus* strains at 60 °C (D_{60}) was reported to be close to 0.4 min (Watier et al., 1996b). However, *Pectinatus* cells may adapt to heat, which increases their heat tolerance (Flahaut, Tierny, Watier, Hornez, & Jeanfils, 2000). *M. cerevisiae* appears to tolerate heating better than *Pectinatus* species. The D_{60} value for this organism in wort and beer was determined to be 0.55 min. It should be remembered that any preservative method is only effective when the initial contamination level is low. In case of heavy primary contamination flash pasteurization might not eliminate the risk of spoilage by the strictly anaerobic beer spoilers (Watier et al., 1996a).

9.8 Future outlook and research needs

Naturalness and healthiness are current megatrends in the food and beverage industry. Functional nonbeer beverages and bottled water are gaining popularity, and the consumption of low-alcohol beer products is increasing. It is also increasingly popular to mix various types of drinks together to create new flavours. Low-alcohol beers are particularly susceptible to spoilage by *Pectinatus* and *Megasphaera* bacteria. Our studies have indicated that *Pectinatus* bacteria may also be capable of spoiling various new types of nonbeer beverages. Hence it may be expected that the importance of the strictly anaerobic beer-spoilage bacteria will increase in the near future. Moreover, the use of fermented vegetable juices as ingredients in the beverages could introduce new *Pectinatus* species into the brewery environment and create new spoilage risks.

The strictly anaerobic beer spoilers are still a relatively little-studied group of microbes. The spoilage properties of the latest described *Pectinatus* and *Megasphaera* species are virtually unknown and should be further studied to understand the real risks they pose to beer and beverage production. Moreover, there appear to be many factors apart from the basic variables (pH, bitterness, alcohol, oxygen content) which affect the growth of the anaerobic bacteria in beer. Understanding of the molecular basis of beer adaptation could help in developing increasingly effective control measures and new tools for screening of stress-tolerant strains. The recent studies have indicated that some of the strictly anaerobic beer-spoilage species may also be beneficial to mankind, playing a role in biohydrogen production and anaerobic wastewater treatment processes. Beneficial aspects of these intriguing organisms certainly warrant further investigation.

9.9 Sources of further information and advice

Detailed information regarding strictly anaerobic beer-spoilage bacteria can be found from the reviews of Haikara and Helander (2006), Haikara and Juvonen (2009), Marchandin et al. (2009) and Suzuki (2011). Comprehensive information regarding lipolysaccahrides of *Pectinatus* can be found from the review of Helander et al. (2004). Detection and identification methods for these organisms have been described in a doctoral thesis by Juvonen (2009).

Much of the research work in Europe on strictly anaerobic beer-spoilage bacteria has been carried out at VTT Technical Research Centre of Finland in collaboration with PBL Brewing Laboratory. VTT provides expertise, state-of-the-art methodologies and anaerobic work facilities to isolate, characterize and identify the strictly anaerobic bacteria.

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Part Three

Reducing microbial spoilage: design and technology

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Hygienic design and Cleaning-In-Place (CIP) systems in breweries

10

S. Davies, T. Sykes, M. Philips, J. Hancock
Briggs of Burton Plc, Burton on Trent, Staffordshire, England, UK

10.1 Introduction

Brewers would be extremely disappointed to find that the beer leaving their brewery was compromised in flavour and quality as a result of contaminating microorganisms. A brewer may also be disappointed to find that their brewery was unclean with respect to equipment fouling. Fouling of key processes involved in heat transfer would directly affect the heating and cooling medium temperature required and extend the process time. This has a large impact on the brewery operation, energy requirements and cycle time. Therefore to minimise the risk of contamination from spoilage microorganisms and reduce the extent of vessel and equipment fouling it is important that the brewery has been designed and engineered with hygiene in mind. Reviewed here are the fundamentals of hygienic process design and the implementation of a Cleaning-In-Place (CIP) system as applied in the brewery brewhouse. It should be noted that the actual design for an effective CIP system depends on the appropriate implementation of hygienic plant design.

The practice of CIP is thought to have been originally developed for the dairy industry as a method to effectively clean vessels and pipework without the requirement to dismantle the process equipment (Meyers, 1959). CIP technology was adopted by brewers as a method to eliminate the need for manual cleaning. This as a consequence reduced the requirement for manual labour and its associated cost. Modern health and safety regimes seek to minimise the involvement of manual labour operations and therefore reduce the risk to the plant operators. Cleaning large-scale breweries using an automated CIP system is essential today to achieve the brewery throughput and required process Turnaround Times (TAT) to meet the market demand. Fundamentally, the CIP system removes residual soil that could lead to the introduction, growth and establishment of microorganisms.

Sterilisation-In-Place (SIP) is a technology that is used in conjunction with a CIP system to provide a sterile environment. SIP is only briefly mentioned here as another process that is used to ensure a hygienic environment. An SIP system, as the name suggests, uses (sterile) steam to create an appropriately ‘sterile’ environment. This is still, however, reliant on the environment actually being clean. For example, applying steam to an unclean vessel containing residual soil would cause further physical bake on to the equipment. In the brewery, SIP systems are only found on low temperature processes, such as the yeast propagation system, where microbial contamination is most likely. It is very difficult to steam large vessels such as Fermentation Vessels

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(FV). The total steam required for this process is high and often more expensive than performing a CIP cycle. Cooling large vessels increases TAT and many of the brewery vessels do not have the pressure and temperature rating to undergo the sterilisation process conditions. Vessels must also have adequate venting systems to cope with filling with cold product. Failure to do so may cause the vessel to collapse. CIP systems are the most prevalent cleaning process in large breweries and are used to clean all major brewhouse processes and vessels, including the Mash Tun/Lauter Tun, Wort Kettle and FV. CIP also has uses in keg handling, however, this chapter is focused on the brewhouse.

From a process engineering perspective the CIP system is often more intricate than the actual main brewery process. This is predominately due to the tight integration of the CIP system around the main brewing process and also the organisation of the pipework and number of valves required to control the flow of wort and beer separately from the cleaning fluid. The implementation of a CIP system should not be simply an afterthought to the brewery process but recognised as an integral design consideration to ensure hygiene. The overall brewery process should therefore be designed for cleanability in the first instance. The presence of a CIP system in a brewery that has not been designed appropriately may still lead to equipment fouling and poor hygiene. For example, CIP systems that feature inadequate drainage, dead legs in pipework and unhygienic valve designs are each discussed here as examples of poor hygienic design practice and are recognised as likely factors that can contribute to equipment fouling and microbial contamination.

10.2 Brewery contamination

The introduction of contaminating microorganisms can occur from raw materials such as malt or hops or through airborne transmission. Contamination can also occur through the brewery process pipework or vessels if they have not been appropriately designed for hygiene. The brewery is not a sterile environment. However, the presence and prevalence of foreign microorganisms in the brewery should be minimised through appropriate brewery design and cleaning practices, as the presence of contaminating microorganisms can cause stuck fermentations and affect product yield and beer flavour, consequently affecting brewery profitability (Hill, 2009). The actual brewing process and the final product (beer) are actually quite inhospitable environments to many microorganisms. However, as is recognised throughout nature, there are a select few microorganisms that have the capacity to withstand this environment. Unfortunately for the brewmaster these undesirable, contaminating microorganisms may potentially cause undesirable off-flavours and affect the beer quality.

10.2.1 Beer is a hostile environment

From the perspective of a microorganism, the chemical composition of beer makes this product quite a hostile environment and poor growth medium. Beer typically contains ethanol in the range of 0.5–10% w/w, hop bitter compounds (approximately 17–55 ppm of iso α-acids), a high content of carbon dioxide (approximately 0.5% w/w) and a

reduced oxygen content (<0.1 ppm), a low pH (3.8–4.7) and only traces of nutritive substances such as glucose, maltose and maltotriose (Sakamoto & Konings, 2003).

In comparison, wort is a far more favourable environment for microorganisms to grow. Therefore ensuring that wort remains free from contaminating microorganisms and any toxic metabolic products that they produce is an important process consideration, especially when the presence of competing microorganisms can affect ethanol and product yields. Wort is rich in free amino nitrogen and fermentable sugars (Boulton & Brookes, 2004; Lekkas, Stewart, Hill, Taidi, & Hodgson, 2005), which are as essential to the brewer's yeast as they are to other undesirable but opportunistic fermentative microorganisms. The contamination of wort is largely minimised by the brewing process itself, along with the introduction of compounds such as iso α-acids from hops. For example, boiling wort in the wort kettle serves as a method of sterilisation. After the kettle, the wort is cooled and pitched with yeast and then transferred to the FV. Boiling wort is unique to the brewing industry. The production of Scotch Malt whisky, which has a very similar process in the preparation of wort/wash from cereal grains, does not involve wort boiling. This difference is largely due to the requirement and presence of microorganisms, such as lactic acid bacteria in the FV (washbacks) in the production of Scotch Malt whisky, which are recognised to contribute and influence the spirit flavour. Furthermore, the high alcohol content and distillation step in Scotch Malt whisky make the final product a largely unfavourable environment for microbial growth. Whilst wort boiling in the brewery is known to improve the sterility of the wort, all the downstream interconnecting pipework and process equipment should be clean. This is especially important because the wort is cooled to temperatures that are favourable for both the brewer's yeast and other potentially contaminating microorganisms.

10.2.2 Types of contamination recognised in the brewery

From a brewer's perspective, the presence of spoilage microorganisms can be detrimental to the production of beer, affecting its flavour and shelf-life through the production of unfavourable smells/off-flavours including diacetyl (Chuang & Collins, 1968) or hydrogen sulphide (Sakamoto & Konings, 2003). Spoilage bacteria are also known to affect beer turbidity and acidity (Sakamoto & Konings, 2003). Microbial infections present in breweries with less stringent hygiene and cleaning regimes often find Gram-positive anaerobic bacilli such as *Lactobacillus* spp. growing (Ault, 1965; Sakamoto & Konings, 2003; Suzuki, Funahashi, Koyanagi, & Yamashita, 2004). The predominance of this particular bacterial genus in breweries and other fermentation-based industries, such as first generation bioethanol facilities and distilleries, is due to their similarity to yeast with tolerances to an acidic pH and ethanol. A more in-depth review of these microorganisms is given elsewhere in this book.

In addition to bacterial-based spoilage microorganisms, the brewery is also susceptible to contamination by wild yeast. Wild yeast refers to yeast that were not intentionally pitched into the FV by the brewer. To a brewer, the yeast strain used in brewing fermentation is one of the key factors that contribute to beer flavour, in conjunction with the raw materials malt and hops. Therefore it is important that the brewer has confidence that the yeast strain pitched into the FV is the desired strain and that this can be achieved consistently.

Hygienic design and automation of the brewer's yeast propagation, storage and pitching systems are therefore an important consideration to ensuring only the desired yeast strain is grown. Large breweries feature on-site propagation systems to specifically manage the growth and handling of their own specific yeast strain. To reduce the potential risk of contamination from wild yeast on-site the yeast propagation systems usually feature high levels of automation and control to ensure contamination is minimised and hygiene maintained. A dedicated single use CIP system is often used to minimise cross-contamination across the brewery. The application and details of the single CIP system for yeast propagation are discussed in the overview of CIP systems later.

10.2.3 The prevalence of microorganisms in the brewery

If microorganisms have successfully infiltrated the brewery through the raw materials or poor hygienic process design, they can remain prevalent in pipework and crevices through their capacity to develop biofilms. Biofilms are essentially a community of cells that exist in a polymer network comprising of proteins, lipids and polysaccharides (Costerton, Stewart, & Greenberg, 1999; Sutherland, 2001). The establishment of a biofilm causes the contained microbial cells to undergo both morphological and genetic alterations distinct from the planktonic state, where the microbes exist in a free-floating environment. The development of a biofilm provides the microbial community with greater resistance to mechanical and chemical treatment. Microorganisms can only synthesise exopolysaccharides required for the development of biofilms if there is an available carbon and nutrient source (Sutherland, 2001). Microbially synthesised exopolysaccharides present in the biofilm are typically structurally long ($0.5–2 \times 10^6$ Da), thin and polyanionic. The structural and chemical heterogeneity of the exopolysaccharide allows various associations through electrostatic, hydrogen bonding and ionic interactions (Sutherland, 2001). With respect to cleaning, it should be noted that the biofilm exopolysaccharides feature the greatest ordered state at low temperatures and in the presence of salts (Sutherland, 2001). Therefore the application of high temperatures during the CIP operation is necessary to disrupt the exopolysaccharides' native state. The requirement for hygienic design is therefore based on minimising the opportunity for microorganisms to adhere and proliferate on surfaces, in crevices and in key process equipment.

10.3 The main principles of hygienic design as applied in the brewery

The concept of hygienic plant design evolved in the food, beverage and pharmaceutical industries. All of these industries require processes that form products that are free from contamination and are safe for human consumption or use. As mentioned previously, the implication of microbial contamination can have detrimental effects on the quality of beer. Therefore designing and engineering a brewery that is cleanable requires an appreciation of several hygienic plant design concepts. The European Hygienic Engineering and Design Group (EHEDG) and The American Society

of Mechanical Engineers (ASME) provide a set of guiding principles that have been developed over time from the contributions of their members. The EHEDG guidelines and ASME BPE 2009 bioprocess document are both extremely comprehensive in specifying and justifying best practices of hygienic process design. Discussed here are the main hygienic design principles that are most appropriate in the context of the brewery:

- pipe layout, design and overall process flow, which focuses on design considerations such as pipework dead legs; and
- the presence of crevices and imperfections in material surfaces, which arise through fabrication and material selection and are known to promote the formation of biofilms.

10.3.1 Brewery pipework design and layout to minimise contamination

Designing pipelines hygienically maximises cleanability whilst minimising the prevalence of soil or potentially contaminating microorganisms. Cleaning process pipework using the CIP system requires a combination of valves and pumps to control and direct the cleaning fluid through the brewery process. Due to their shapes, pipework fixtures, such as dead legs and T-shaped junctions, naturally lend themselves as sinks to organic material deposition that could harbour and support the growth of undesirable microorganisms (Figure 10.1). In addition to the pipework design it is also important that the

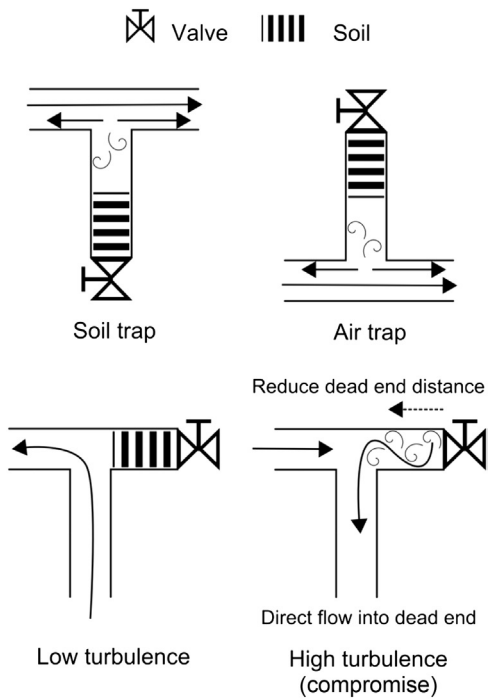

Figure 10.1 Examples of best practices in hygienic process design. Dead ends and T-shaped bends can potentially harbour soil.

Image reproduced from the Institute of Brewing and Distilling Diploma, Distilling Module 2: Plant Cleaning with permission from Brian Eaton.

CIP fluid has a turbulent flow in the process pipework to scour the surface and remove the soil. Both of these aspects are reviewed next.

10.3.2 Operating conditions required to achieve a cleaning action in pipework

The effect of surface fouling has a large impact on the heat transfer coefficient. Protein fouling creates a thermal barrier, which during wort boiling for example will increase the temperatures required by the heating medium (steam) to heat the product. Ineffective cleaning will therefore have an impact on steam usage.

A turbulent flow is required in the pipework of the CIP system to remove any residual soil. The velocity of the CIP cleaning fluid in the process pipe should be between 1.5–2.0 m/s. Practically obtaining this velocity range is dependent on the cleaning fluid flow rate (m^3/h) and the pipe diameter. A fluid is recognised as turbulent when its Reynolds number is greater than 4000 (Eqn 10.1). Fluids with a Reynolds number of 2100 and below have a laminar flow, which is not effective at scouring the pipework surface (Chisti & Moo-Young, 1994). During laminar flow the fluid has the greatest velocity (V_{max}) at the centre and lowest (zero) velocity at the pipe wall surface. Therefore there is no movement/mechanical action at the surface that you want to clean. The CIP cleaning fluids, wort and beer are all turbulent at velocities of less than 1.5 m/s due to their density (Eqn 10.1). For example, a CIP cleaning fluid at 0.3 m/s would have a Re of approximately 10,000. It should be noted that even if the fluid has a turbulent flow, if its velocity is too low it will have a thick boundary layer, which in the pipework will potentially still cause fouling and deposition. Cleaning fluid velocities greater than 2.0 m/s in the pipework are not recognised to have any additional effect on cleaning. Therefore increasing the fluid velocity beyond this value only increases the energy used to pump the fluid due to an increased pressure drop. For example, doubling the fluid velocity quadruples the pressure drop (Eqn 10.2).

Equation 10.1 Calculation of Reynolds number to determine whether the flow is laminar, transient or turbulent

$\mathrm{Re} = \dfrac{\rho u d_h}{\mu}$, where Re is the Reynolds number (nondimensional), ρ is the density (kg/m^3), u is the velocity (m/s), d_h is the hydraulic diameter (m) and μ is the dynamic viscosity (m^2/s) from The Engineering toolbox (www.theengineeringtoolbox.com).

Equation 10.2 Pressure loss in a pipe

$\Delta P = kV^2$, where ΔP is the pressure difference, k is a coefficient and V is the velocity (m/s) of the fluid travelling through the pipe from The Engineering toolbox (www.theengineeringtoolbox.com).

10.3.3 Hygienic design and operating practice of valves, fixtures and fittings

Pipework in a brewery is fundamental to moving products such as wort and beer. Therefore ensuring the pipes are arranged in a manner to promote cleanability is an important consideration for hygienic design. Dead legs in pipework should be avoided (Figure 10.1). Figure 10.1 shows several T-junctions that create environments that lead to poor cleanability and should be avoided in the brewery process design. If T-junctions are present, the cleaning fluid should be pumped in the direction of the dead leg so that sufficient turbulence action can be achieved in the dead leg space (Figure 10.1). Figure 10.1 shows how T-junctions may lead to the development of air and soil traps, which are both undesirable from a hygiene perspective. It is also important that the CIP supply routes are appropriately considered, ensuring a consistent flow through a single pipework run. Splitting the CIP supply flow across several different pipelines reduces its efficacy. Instead a single route that systematically works through the brewery pipework should be considered. However, additional pipework may be necessary to route the CIP cleaning fluids around the process through dedicated, separate pipework. Controlling the direction of cleaning fluids is achieved using double-seated mix-proof valves (Figures 10.2 and 10.3). These have become an integral part of a CIP system for routing both product and cleaning fluid. An example of a valve manifold containing an array of double-seated valves is shown in Figure 10.2.

Instrumentation such as pH probes can be fitted to the vessels either directly or, where greater hygiene is required, using hygienic housing that can retract the probe (Chisti & Moo-Young, 1994). Probes are usually directly fitted to the vessel for cost purposes. The benefit of the retractable housing is that it prevents the probe from being damaged and allows periodic cleaning, independently from the CIP system. This is important for example in yeast systems where the probe will require more frequent cleaning to reduce fouling and ensure accurate readings.

Figure 10.2 Mix-proof valves and valve array commonly used next to a chain of fermenters to hygienically control the transfer of beer and the Cleaning-In-Place cleaning fluid.

Figure 10.3 Valve manifold with hygienic mix-proof valves used in a Cleaning-In-Place system.

10.3.4 The effect of material surface finish on microbial surface adhesion

The surface finish of metals has a large impact on the capacity of microorganisms to adhere to pipes and vessels (Milledge, 2010). The surface characteristic of metal can be changed through processes such as welding and polishing. Welds for example introduce both physical and chemical changes to the metal surface from both the metal filler composition (steel) and solidified slag. Surface defects and the material topography are both known to influence the cleanability of stainless steel. The changes to the metal surface through processes such as welding are thought to facilitate the accumulation of organic material that can lead to the growth of microorganisms. The preferential colonisation of welds by microorganisms has been correlated with the material surface roughness (Sreekumari, Ozawa, & Kikuchi, 2000). As a material for vessels and pipework stainless steel benefits from the development of a passive layer when exposed to air (chromium oxides). This effectively serves as a barrier between the fluid and pipe wall itself. Periodically using acid detergents such as citric or nitric acid in the CIP system is important to re-establish this passivation layer (oxidation) and helps to ensure that the stainless steel remains rust free (BSSA–Passivation of stainless steels, 2014).

One method of evaluating the surface finishes of a metal is the roughness average (Ra) value or the root mean square (RMS) average. The development of several surface characterisation methods has arisen due to the different possibilities of representing a material's surface using an average and single digit metric. Both methods are recognised and included as part of the ASME B46.1 standard in determining the surface properties of materials. From a hygiene perspective a lower Ra value or the RMS average value indicates a reduction in the depth of crevices (peaks/troughs) across the metal surface and therefore minimises the amount of organic material that may reside on the surface. A reduction in the metal surface roughness can be achieved through more extensive polishing operations. Surface variations at the macroscopic level can be reduced using mechanical polishing and at the microscopic level using electropolishing. The pharmaceutical

Figure 10.4 Tanks used in a brewery Cleaning-In-Place (CIP) system. Left to right are the prerinse storage tank, the caustic tank and the CIP return tank.

industry has long required highly polished vessels and process equipment to improve the levels of hygiene of their plant equipment. Similarly, the food industry demands Ra values of less than 0.8 μm (Milledge, 2010). However, increasing the extent of material polishing from a manufacturing perspective increases the cost of the material. The brewing industry has never implemented the same stringent control over the material surface finish as the pharmaceutical and food industries, largely due to the additional cost and the potential to damage the material surface. The smoothest surfaces for steel are achieved through a cold rolling process, followed by chemical descaling.

10.4 An overview of CIP systems used in the brewery

Brewery maintenance is an important aspect from a hygiene perspective to prevent contamination and fouling, both of which can affect the brewery yield and process efficiency. Small breweries (<50 UK Barrel brewlength) will typically clean the process equipment by hand using brushes and spray hoses or have simple CIP systems involving a detergent tank and pump. Larger breweries would be expected to automate the cleaning process using a fully integrated CIP system. A complete CIP system used in large breweries features detergent make up tanks, interconnecting pipework, pumps, valves and heat exchangers (Figure 10.4). The whole CIP system is usually automated, relying on flow meters, temperature probes and conductivity meters to monitor the process. The complexity and functionality of the overall CIP system is highly dependent on the brewer's requirements and the brewery operation.

The cleaning fluid from the CIP system can be pumped in either the same or opposing direction to the process flow. Pumping the cleaning fluid in the reverse direction is sometimes necessary to remove soil. The number of vessels and level of automation that are required is dependent on the application of the CIP system. The insides of vessels are cleaned using cleaning machines and spray heads. The major difference in operation relates to the cleaning fluid flow rate and pressure. Cleaning machines are

Figure 10.5 Examples of vessel cleaning machines: GamJet TZ-74 (left), static spray ball (middle) and rotary spray ball (right) as applied in brewery vessels.

typically high pressure with a low flow rate. This provides greater mechanical impact and does not simply rely on the chemical action of the CIP detergent. Spray heads in comparison operate at lower pressure with a higher flow rate. Figure 10.5 shows examples of cleaning machines and spray heads that are used on brewery vessels. Cleaning machines are typically used in brewery equipment such as the mash tun and wort kettle. The application and suitability of the cleaning system is highly dependent on the vessel scale. The number and position of spray heads in the vessel is also an important consideration. Vessel equipment such as agitators can obstruct the spray ('shadowing'), which impacts the effectiveness of the cleaning fluid. During the CIP cycle the agitators should be activated to prevent shadowing from occurring. Spray heads are usually situated at the top of the vessel to allow cleaning fluid to be sprayed across the body of the tank, which then runs down the sides of the vessel. Vessels may feature several spray devices to ensure the whole surface is covered and no shadowing occurs. An internal kettle fountain for example required several spray balls to reach all the crevices in its design. To ensure sufficient mechanical action during cleaning a high pressure is required to remove soil material. Static spray heads feature no moving parts and are low cost. Spray heads use more water and energy than cleaning machines due to the higher flow rate. Cleaning fluid exiting the spray head atomises, which increases the adsorption of CO_2 by caustic, resulting in the formation of carbonates. Cleaning machines are the most effective and aggressive cleaning strategy, benefitting from the lowest energy and water usage. Both spray heads and cleaning machines can become blocked internally with soil that has contaminated the prerinse or caustic, or blocked externally, if the unit isn't self-cleaning. The internal contamination can be overcome using a strainer in the CIP supply. Ideally the solids are completely removed from the system during the initial prerinse stage, which is discharged to drain and discussed in more detail later. As an alternative a strainer may be fitted to the CIP return, which will reduce the problem and reduce any sediment reaching the CIP chemical storage tanks.

The main terminologies used to describe the operation of the CIP system relate to the direction of the CIP cleaning fluid. The CIP fluid leaving the CIP storage tank is

referred to as the CIP supply. The CIP supply is usually heated in a heat exchanger and pumped through the process pipework, reaching spray heads or cleaning machines inside the vessels. The CIP cleaning fluid that is recovered from vessels is referred to as the CIP return and is collected using a CIP scavenge pump. There are three main types of CIP system that are installed in a brewery. These include a single use, partial recovery and full recovery system. The variations of these different CIP systems relate to the extent that the cleaning fluids are recovered. The selection of the appropriate CIP system, its operation temperature and the extent of the number of CIP channels used in the recovery CIP system is based on the specific brewery operation and brewhouse design.

10.4.1 Operating conditions of a CIP system in the brewery

An effective CIP system involves three types of processes: mechanical, chemical and sanitisation. An effective CIP system is a balance between an optimum temperature, residence time, mechanical and chemical treatment. Mechanical processes physically remove materials that soil process equipment through turbulence or a scouring action. Spray balls used inside vessels and CIP pumps are necessary to remove residual particulate such as proteinaceous materials. Ineffective removal of residual soil reduces the effectiveness of brewery process equipment including plate heat exchangers and vessels. As mentioned previously, residual soil may also provide an adherence and nutrient rich site for biofilm development. Additional energy is required as the fouling of process equipment negatively affects heat transfer. In combination with the turbulent flow generated by mechanical action, the CIP system will use chemical reagents including both base and/or acid to clean. CIP systems are a relatively large user of water in the brewhouse (Reducing water use through Cleaning-In-Place (CIP) envirowise–EN894, 2008). Efforts to minimise water use in the brewery have been achieved through modifications of the CIP programme (Reducing water use through Cleaning-In-Place (CIP) envirowise–EN894, 2008).

A typical CIP cycle would include:

- A prerinse with water to remove any loose material. The wash water and any soluble material are discharged directly to the drain to eliminate any material carryover. An effective prerinse will also remove solids that cause equipment blockages.
- A hot caustic wash to chemically remove material that has soiled and fouled the equipment. A 2–3% caustic wash at 75–80 °C is used in the brewhouse and for processes involving wort. Lower strength caustic (1%) is used on lower soil environments such as bright beer. The hot caustic should digest and dissolve any soiled material. During the CIP cycle the caustic solution is recirculated several times. Heating the caustic CIP cleaning fluid is achieved using a heat exchanger, which can use waste heat to prewarm the CIP cleaning fluid.
- A further washing stage to remove the caustic.
- An acid wash which can be applied on a periodic operation of the CIP cycle. This minimises the CIP Turnaround time (TAT) and operating cost. The use of acid has several benefits in the brewhouse. It is used in cold processes such as FV where the acid serves to eliminate bacteria, effectively serving as the sanitising agent. The acid detergent does not suffer from degradation by CO_2 as recognised with caustic, which is known to reduce its effectiveness

Table 10.1 Timings of the main stages of a Cleaning-In-Place (CIP) system for both vessels and the main process systems pipework

Unit operation	Function	Vessel CIP (min)	Mains CIP (min)
Prerinse	Mechanical removal of soil	10–20	5–10
Caustic detergent	Cleaning of remaining soil	30–40	20–30
Rinse	Wash any residual detergent	10–15	5–10
Acid detergent		20–30	15–20
Final rinse	Wash any residual detergent	15–20	10–15
Sterilant		10–15	5–10

(forming carbonates). The acid wash can also serve to re-establish the passivation layer at the stainless steel surface.

- A final wash using either reverse osmosis or deionised water is applied to remove any residual detergent.

The operating cost of the CIP system is influenced by the amount and concentration of detergent used and whether it is recovered or not. Table 10.1 shows an example of typical CIP timings for the main process vessels and pipework.

10.4.2 Types of CIP systems recognised across the brewery

10.4.2.1 Single use

A single use CIP system pumps the cleaning fluid around the process pipework and vessel and on its return sends the water phase directly to the drain (Figure 10.6). The single use CIP system is the simplest cleaning system and is important for processes such as yeast handling and propagation that require the highest levels of hygiene. Brewery yeast systems are often single use CIP systems. Both the prerinse and the detergent used to clean the process vessels are sent to the drain. This essentially serves to reduce the risk of cross-contamination by CIP systems that are less than optimal in recovering and recycling the prerinse water and detergent.

10.4.2.2 Partial and full recovery cleaning systems

Partial recovery CIP systems recover the detergent for use in the next detergent step or as a prerinse. A full recovery CIP system is designed to recover the final rinse for the next prerinse and return the cleaning fluid streams back to their chemical supply tanks. Examples of recovery CIP systems are shown in Figures 10.7 and 10.8. Figure 10.8 shows a CIP system that features both caustic and acid tanks as a detergent. This is a more complicated system with respect to the number of chemical detergent tanks, valves and extent of pipework routing.

In summary, single use CIP systems are less capital intensive, require less space and reduce the risk of cross-contamination as compared to recovery CIP systems. The single use CIP system is important for specific applications such as yeast handling. In comparison, recovery CIP systems have lower energy requirements, volumes of water

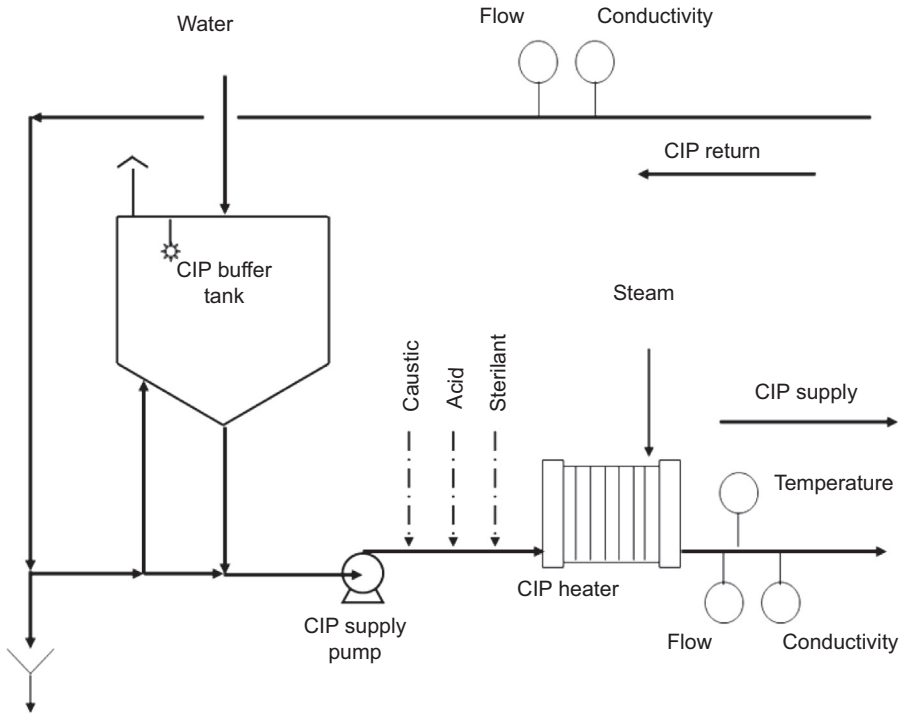

Figure 10.6 Example of a single use Cleaning-In-Place (CIP) system. In-line heating and chemical dosing.

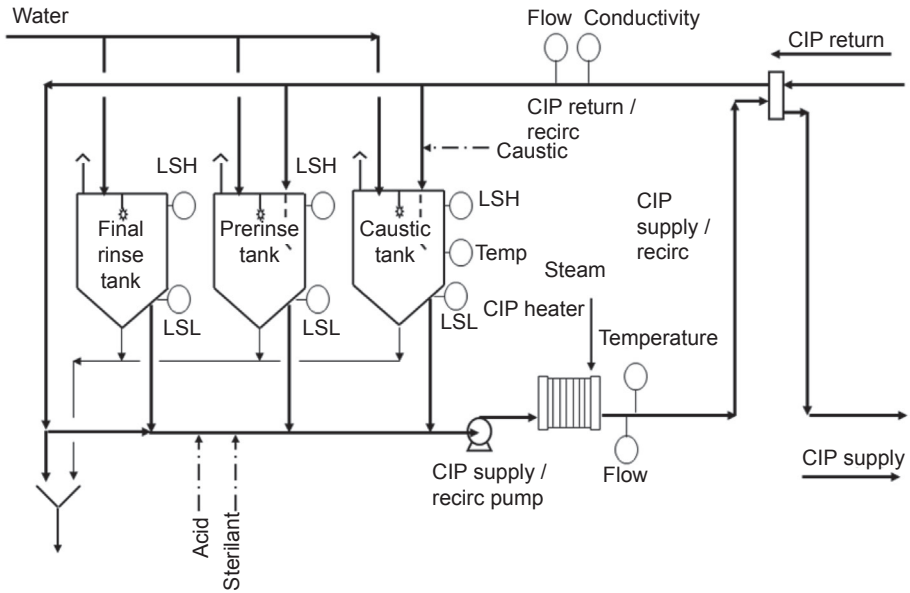

Figure 10.7 Example of a recovery Cleaning-In-Place (CIP) system with one CIP supply and three tanks.

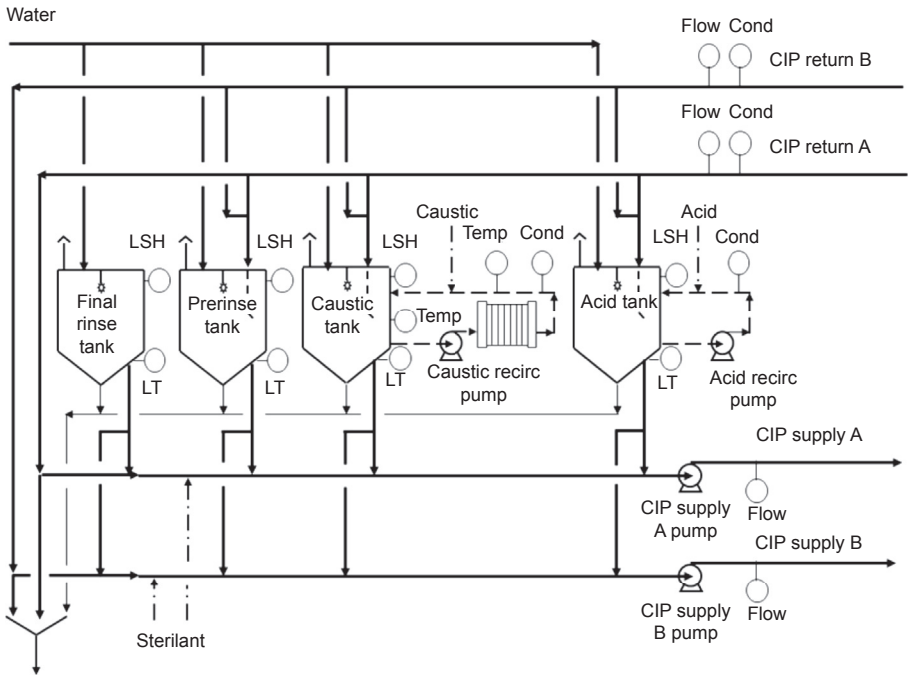

Figure 10.8 Example of a recovery Cleaning-In-Place (CIP) system with two CIP supply and four tanks. Chemical tanks feature a recirculation loop with an independent heat exchange, which allows the tanks to be heated during a CIP cycle for immediate use. Important on larger breweries to minimise Turnaround Time (TAT).

and effluent, and due to the lower chemical losses have lower operating costs with respect to the amount of chemicals required. The recovery CIP system is advantageous because the main processes do not have to wait for the CIP system to be ready. For instance, the chemical detergent tanks already contain the required detergent concentration at the necessary temperature. This allows the cleaning to begin directly after the brewhouse processes have ended and therefore minimises unproductive time.

10.4.3 CIP fluid composition

CIP systems can pump prerinse material, detergents and final rinse water to remove soil. The prerinse material (potentially dilute caustic) is used to remove any loose debris, whereas the detergents chemically remove the soil. Described below are the different detergents that can be used in CIP systems and also chemical additives such as sequestrates (chelating agents) and surfactants that improve detergent penetration.

10.4.3.1 Detergents

Detergents are used to chemically remove soil. Ideally the detergents are nonfoaming or include antifoam, free rinsing/nontainting, noncorrosive and have minimal environmental impact. Appropriately formulated detergents are effective at removing

soil. Caustic-based detergents are more effective than acid detergents on high soil environments. Sodium hydroxide reacts with proteins and oils, converting them into their respective salts, which increases their solubility and therefore their removal from stainless steel surfaces. Applying detergents at elevated temperatures using a hot water medium provides a level of disinfection. Detergents are effective at removing protein soil, which is especially important as fouling can reduce the effectiveness of heat transfer surfaces. This is especially important in high temperature processes and equipment such as the Wort Kettle or heat exchangers, where the protein can become baked onto the surface and cause fouling.

Controlling the strength of detergents can be achieved using conductivity metres. Disadvantages to caustic-based detergents are their degradation by CO_2, forming carbonates. As a result of this the brewer would require more caustic to achieve the required working concentration. This is most important in the FV where the CO_2 levels are greatest. Prerinse water can absorb some of the CO_2 present in the FV and therefore reduce the chance of producing carbonates. It should be noted that the absorption of CO_2 by caustic creates a risk of forming a vacuum that can cause the FV to collapse (Manzano et al., 2011).

Acid-based detergents are more frequently used to clean and sterilise FV, whereas caustic detergents are used to remove soil from the main brewery operations. The activity of caustic detergents is also affected by water hardness. Caustic detergents have poor rinsability compared to acid detergents and therefore require more water to remove. Caustic detergents are ineffective at removing inorganic scale such as gypsum and beerstone. Phosphoric acid and nitric acid are often used to remove inorganic scale and to regenerate the metal's passivation layer. It should, however, be noted that there are potential environmental issues of releasing phosphates and nitrates into the effluent. Selecting the appropriate detergent is based on the unit operation in the brewery, the type of equipment fouling (organic or inorganic) and the environmental impact on discharging detergents to effluent.

Detergent additives including sequestrate (chelating agents) and surfactants can also be added to the CIP cleaning fluid. Sequestrate such as ethylenediaminetetraacetic acid (EDTA), nitrilotriacetic acid, gluconates and phosphonates complex with metal ions in solution and prevent the precipitation of the insoluble salts of the metal ions. The main rationale for adding sequestrates is to inhibit the formation of scaling with the design philosophy that prevention is better than cure. Surfactants (wetting agents) added to detergents reduce the cleaning fluid's surface tension, which allows the detergent to penetrate the metal surface more effectively.

10.4.3.2 Sterilant

A sterilant can be used after the CIP process to remove any residual microorganisms, effectively serving as a low temperature SIP. Sterilants include chlorine, ionophores and peracetic acid (PAA). PAA degrades into acetic acid and hydrogen peroxide. Hydrogen peroxide is a strong oxidising agent, which can be used to enhance the CIP detergent (caustic or acid). The formation of acetic acid from the decomposition of PAA increases the organic load in the effluent waste.

10.4.4 Evaluation of the effectiveness of CIP systems

Evaluation of the brewery CIP system and its effectiveness is usually determined using off-line laboratory analysis or portable measuring devices. This typically involves dyes such as riboflavin or ATPase activity assays used to determine the presence of living microorganisms.

On-line sensors are used as part of the CIP system to check aspects such as the concentration and quality of the caustic cleaning fluid. Conductivity metres are fitted within the CIP set itself to evaluate the detergent concentration and control a dosing pump to top up the detergent as required. From an overall automation perspective, all the basic parameters for flow, temperature and time are each controlled and monitored as part of the CIP system.

10.5 Conclusions

Hygienic design is an important factor in brewery design and engineering. The brewer and beer drinker expects that resultant beer is safe to drink and consistent in quality and flavour. Reviewed here were the main design considerations that a brewery must employ to minimise the risk of contamination and fouling. A real appreciation of the intricacies of a good CIP system and the requirement for hygienic process design becomes apparent with larger breweries. Larger breweries typically require more extensive levels of automation and control to coordinate the vast array of mix-proof valves to correctly direct and route the CIP cleaning fluid. Furthermore, the amount of water and cleaning fluid reagent used in these large-scale breweries will have a large financial and environmental impact.

Efforts to minimise water and energy usage during the CIP process is an important aspect of the overall process, particularly when considering the frequency of cleaning between batches. High pressure, low flow cleaning machines are employed to remove soiled material that has fouled equipment, using less water. An effective prerinse and sufficient mechanical action are required to disrupt materials that cause fouling. Ineffective prerinsing requires more water and chemicals during the CIP cleaning cycle. Pumping cleaning fluids at lower flow rates or adopting lower cleaning temperatures are potential strategies to minimise water and energy usage during CIP operations. However, to effectively reduce water and energy use whilst maintaining an effective cleaning regime requires careful consideration of the whole CIP operation. For example, current brewery operations could simply benefit from optimisation of the CIP system schedule and cleaning detergent recipe.

The demand for greater flexibility in the brewery increases the requirement for cleanability. There appears to be a shift from operating vessels with a single function to adopting a more flexible approach, where the brewer can use equipment for a multitude of purposes to satisfy changes in market demand. For example, a contract brewer may require several yeast strains to produce different beers. Propagating and cropping several different yeast strains from a single on-site yeast propagation system is only practically achievable with hygienic process engineering and cleaning systems. As outlined in this

review, a single use CIP system would be most appropriate for this application. Expanding on the brewer's requirement to demand more from his current brewery equipment could see storage vessels and tanks repurposed to hold scrap or waste yeast, for example. Again, changing the operation of the brewery vessels without changing the equipment outright is only practical if it has been appropriately designed.

As mentioned in the introduction, the implementation and execution of the CIP used to be an afterthought to the design of a brewery. However, as hopefully highlighted here, the integration of the CIP system with the main brewery operations is fundamental to its effectiveness. The cost of contamination and equipment with respect to downtime and product loss in fermentation-based processes, such as the brewing industry, is not always considered. However, with increasing raw material costs and utility costs the brewer cannot afford to suffer from fermentation contamination or fouled equipment. As these can to lead a compromise in beer quality and higher energy requirements.

10.6 Future trends

10.6.1 Future brewery designs and the impact on water and energy recovery

Future mega-brewery designs will put additional technological pressure on cleanability. It would be expected that the larger breweries require larger diameter pipework to satisfy the greater volume capacity. Breweries featuring larger diameter pipework will require larger pumps to achieve the same flow velocities to obtain the necessary turbulent and scouring action during the CIP process. An impact of the larger pump size will be the energy required to achieve the necessary velocity of the cleaning fluid. Heat exchangers are already used to recover heat from heat intensive processes such as the wort kettle to preheat the CIP cleaning fluid. New technologies, such as electrochemically activated water generated from the electrolysis of a saline solution, could replace the requirement for the delivery of bulk caustic to a site (CIP and sanitation of process plant–SPX, n.d.). As the competition and demand for water increases in the future, the emphasis on technologies that minimise water usage and effluent discharge will become more favourable. The future for CIP is therefore expected to feature benchmarking similar to the brewery benchmarking, which compares the number of hectolitres of water per hectolitre of beer. One question would be is there still a requirement for water as part of the cleaning process or could self-cleaning materials be the future?

10.6.2 Developments in nanotechnology to provide antimicrobial surfaces and materials

The application of silver nanoparticle technology as a future antimicrobial material is an interesting area of research. Silver nanoparticles have been shown to prevent the development and establishment of biofilms (Palanisamy et al., 2014). This in principle would be an effective strategy to prevent the growth of undesirable microorganisms. The application of silver nanoparticle technology seems ideal for medical equipment,

however, due to its nonselective mechanism and its detrimental effect towards yeast it has less use in the brewery. The application of the silver nanoparticle technology in pipework would not be expected to directly replace a CIP system, owing to its role in removing both contaminating microorganisms and soil. Extensive trials would be expected to be undertaken in adding this technology to a brewery, particularly as the size of the silver nanoparticles could be a potential health risk to both the operator and consumer. It could, however, find more suitable applications on discharge pipework.

10.7 Sources of further information and advice

Further information around the guiding hygienic design standards and frameworks is included in the reference section and stated below for convenience: European Hygienic Engineering and Design Group (EHEDG) and The American Society of Mechanical Engineers (ASME) BPE 2009 Bioprocess. WRAPs Envirowise EN894 provides a useful overview to minimising water in CIP systems (Reducing water use through Cleaning-In-Place (CIP) envirowise–EN894, 2008). Chisti and Moo-Young (1994) also offer a comprehensive review of CIP in bioprocessing and fermentation-based systems.

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Reducing microbial spoilage of beer using filtration

11

G.J. Freeman
Campden BRI, Nutfield, Surrey, UK

11.1 Introduction

Filtration of beer is a challenging operation. Prefilter beer contains a significant concentration of suspended particles. Most often the volumetric bulk of these particles comprises yeast cells, many of which are joined together by the natural flocculation process. These particles, therefore, are at least several microns in size. This makes their removal relatively simple, although the volumetric bulk will add to filtration costs. Indeed, a single filtration stage can result in effectively zero yeast cells in the filtered product. This is significant because in most beer the presence of brewing yeast must be regarded as contamination. However, also present in prefilter beer are much smaller particles, many of which are as small as less than a micron. These particles mostly comprise protein–polyphenol and are known as chill haze. Beer will not be visually clear unless we remove most of these particles to as small a size as below one micron. This greatly increases the difficulty of beer filtration and limits the technologies that are suitable. However, this also means that all beer filtration technologies will reduce the count of any bacterial contamination.

11.2 Filtration technologies in brewing

Filtration processes may be classified as either depth filtration or surface filtration. Depth filtration relies on a layer of porous media in which suspended particles in the beer are trapped within the media. Examples in brewing include filter aid filtration, sheet filtration and some forms of filter cartridge. Surface filtration normally refers to membrane technology. A thin layer of membrane has pores throughout the structure. This means that it is possible to achieve very exact filtration, perhaps enabling sterilisation, but typically the quantity of suspended beer particles that may be removed is less than for depth filtration.

Fine filtration processes imply increased energy usage, most obviously increased pressure, and likely a reduced capacity for suspended beer particles. This means that there is scope to perform the filtration with more than one technology in series. As the beer progresses through the series, each filtration step becomes progressively finer. For example, a filter aid filter may be followed by a finer depth filter such as a sheet filter. A membrane filter, most commonly in the form of a filter cartridge, would normally be at least the third in a series. A filter cartridge enables the possibility of guaranteed

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sterile filtration. A filter sheet of suitable grade, although a depth filter, may enable effective sterile filtration to the satisfaction of the brewer.

There is a need to design a sequence of filtration operations to maximise the throughput. For example, a relatively coarse initial stage will increase the duty on the second stage and therefore may not increase the total throughput. There is scope to perform this optimisation on the pilot or laboratory scale (Freeman, 1996).

The process sequence may be designed to enable sterile filtration. Other technologies to achieve microbiological stability in product include pasteurisation or maintenance of a yeast culture in product to prevent the growth of damaging contaminants. The relative advantages of sterile filtration and pasteurisation pertain to both process costs and product quality. Pasteurisers, either in-line (flash) or for small package (tunnel), are expensive capital items compared to a sterile filtration unit. However, the ongoing need to replace the sterile filtration media means that, typically, the operating costs for sterile filtration are higher. It may be, therefore, that sterile filtration is more viable for a small brewery and pasteurisation for a large brewery. The relative merits for product quality are a matter of some dispute (White, 2008). Thermal treatment of beer accelerates chemical reactions and therefore reduces flavour stability and produces 'cooked' off-flavours, although good operations in a modern brewery have reduced the extent of thermal treatment required. Sterile filtration of course does not include thermal treatment at all. However, all filtration processes, especially fine filtration processes, result in some removal of positive beer characteristics such as colour, foam stability bitterness and 'mouthfeel'.

11.3 Filter aid filtration

The majority of the volume of beer in the world is processed by filter aid filtration. This technology relies on adding powders to the beer that form a very porous bed when they impinge on the filter surface. This is achieved by slurrying the filter aid in water, deaeration and pumping the slurry into the beer. Thus the prefilter beer on reaching the filter surface encounters a fresh layer of filter aid as the bed develops. This prolongs the filtration run.

The most common filter aid employed is kieselguhr (diatomaceous earth). This comprises the fossils or skeletons of fresh or salt water algae known as diatoms (Figure 11.1). The highly porous nature of the particles enables effective liquid flow but also entrapment of particles. Other types of filter aid that are commonly used include perlite (volcanic glass) and cellulose fibres. However, the porous internal structure of the diatoms makes them more effective than the alternatives.

Filter vessel technologies include plate and frame, leaf and candle. The latter two are preferred today because they are easier and faster to clean and restart than the plate and frame. Candle filters are simpler constructions but leaf filters are probably more flexible (Hermia & Brocheton, 1994).

The filtration operation is preceded by precoating of the filter. This comprises recirculation of deaerated water around the vessel while adding filter aid slurry. Thus an initial bed of filter aid exists at the start of beer filtration. This means that the brewer has options to optimise the process other than simply the selection of the main

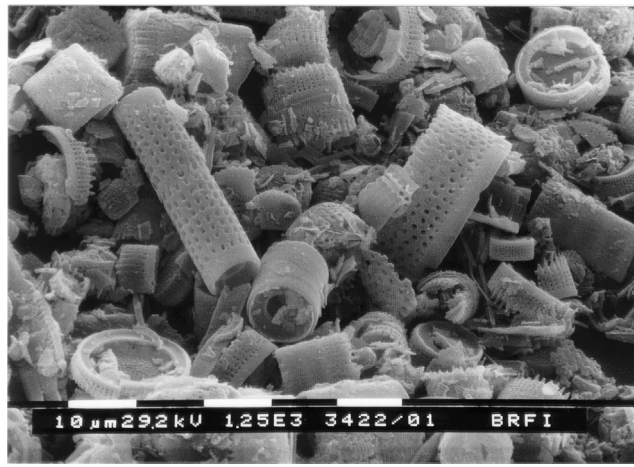

Figure 11.1 An electronmicrograph of a kieselguhr filter aid.

filtration grade. For example, if the precoat is a finer (smaller) grade than the bodyfeed (admixed to the beer) grade, then we have a genuine two-stage process. Beer clarity would be improved at the expense of some run time.

An optimised filter aid filtration process is capable of removing the vast majority of particles down to as small as half a micron. This means that contaminating bacteria that typically have a minimum dimension of half a micron are removed in significant quantities by a filter aid filter. Indeed, it is likely that a well-operated operation will reduce bacteria by a factor of 1000 (log reduction value of three). This has several beneficial implications. It reduces the requirement for microbiological stabilisation. For example, the brewer can pasteurise with less intensity. If sterile filtration is employed, excellent beer clarity post filter aid filter increases the run time of the sterile filtration process. To this end there is generally an advantage to using kieselguhr as opposed to other filter aids because of the superior beer clarity obtained.

11.4 Crossflow microfiltration

Filter aid filtration was in many ways the only viable option for bulk filtration of large volumes of beer for many years. However, there are significant problems with the technology. In particular, the material in kieselguhr (the most efficient filter aid) comprises crystalline silica (cristabolite). This is carcinogenic if inhaled, causing the disease known as silicosis. Alternative filter aids such as perlite are not crystalline silica but as mentioned are not as effective. Thus, it may be a hazard to brewery personnel if packaging fails or during transfer from the package to the slurry tank. Also, disposal of the spent filter aid cake usually has landfilling as the only viable option. Depending on location landfilling is becoming increasingly expensive and restricted.

Another significant issue pertains to beer quality. Although the kieselguhr manufacturing process includes a calcination (furnacing) step, which is designed to remove metal

ions other than silicon, these are still present in the kieselguhr. Transition metal ions such as iron, copper and manganese instigate oxidative damage to the beer that causes turbidity and stale flavours. Kieselguhr filtration commonly results in a doubling of the concentration of these ions. Thus, especially in these times of increasing corporate social responsibility, there is real incentive to use technologies that do not employ filter aids.

At the latter end of the twentieth century, a technology emerged that was competitive in terms of cost with filter aid filtration. Crossflow microfiltration, alternatively known as tangential flow microfiltration, utilises a membrane filtration process in a single stage. Deposition of particles as a 'cake' on the membrane surface would normally cause a membrane to foul very quickly and make the process impractical. However, this effect is minimised by causing the prefilter beer to flow across the membrane surface. This causes the 'cake' to be re-suspended back into the prefilter beer and helps to maintain a satisfactory flow of filtered beer. The down side is that the operation requires a lot of pumping energy, and as a consequence, requires a lot of refrigeration energy also.

The membrane format currently employed in brewing is a tube, with the beer flowing longitudinally down the inside so that filtrate passes through the tube wall to the tube set housing. The membrane composition may be either polymeric or ceramic. Polymeric membranes underwent a step-change improvement with the development of the ability to manufacture membranes in polyether sulphone (PES). This material demonstrably improved the flow rate performance of the membranes because PES is less inclined to adsorb beer components such as colour and proteins. Ceramic membranes currently achieve slower rates of filtration per unit area of membrane than polymerics. However, they have a key advantage in that if operated responsibly they are extremely long-lasting, perhaps for well over 10 years. Whereas polymeric membranes have to be replaced more frequently, currently every one to two years (a significant operating cost) typically, the ceramics are more rugged. It is unclear as to which type will evolve to become more attractive in the future, but it is clear that membrane efficiency and reduced effective cost will continue to improve. Compare this with filter aid filtration where costs will increase, in particular for disposal of the used filter aid.

The polymeric membranes are installed, several hundred at a time, in housings of suitable hygienic material. Figure 11.2 shows the top of an example of such housings. The pipe and valve arrangements mean that flow through the individual housings can be separated from the other housings. Thus, it is possible to clean one individual housing while continuing to filter beer through the others. Therefore, crossflow filtration plants are compatible with continuous processing.

The pore-size ratings of the crossflow membranes are most commonly in the range of 0.4–0.8 microns. It should be noted that a 0.45 micron membrane is regarded as being capable of removing all beer spoilage organisms. Thus at first sight the brewer has the potential to perform sterile filtration in a single stage. However, there are problems with this approach. Sterile filtration necessitates integrity testing (see later) of filter modules before the run. Although this is feasible, the complexity of the highly modularised (Figure 11.2) and large filtration plant makes it an engineering challenge. Also, many brewers have further processes between the filtration and packaging lines, for example, stabilisation processes comprising adsorbents such as polyvinyl polypyrollidone or agarose gels. However, there are options for stabilisation upstream or

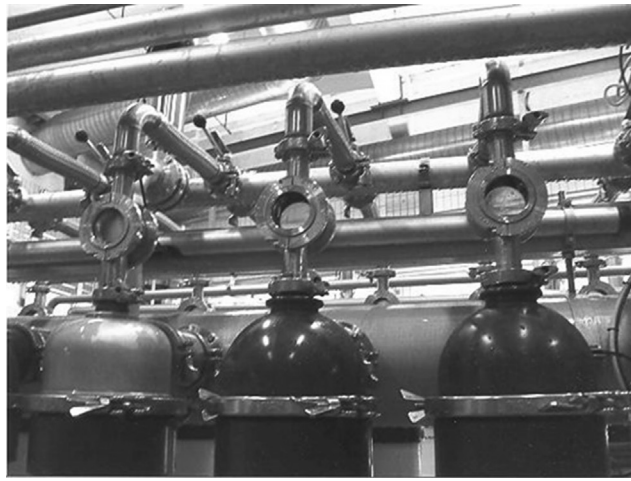

Figure 11.2 The upper part and pipe and valve arrangements of a polymeric crossflow filtration plant.

even employment of the additions in the prefilter beer that is recirculating around the crossflow plant. Furthermore, conventional brewing employs a process step of holding the beer immediately after filtration in a 'bright beer tank' before microbiological stabilisation and packaging. This step is mainly to perform the last quality control checks on the product, enabling adjustments or blending as required, and is not normally maintained to a high level of sterility. Therefore improvements to product consistency and process hygiene would need to be achieved to employ crossflow microfiltration as the sterilising process. In the author's opinion these difficulties could be overcome in the future with potential advantages in process simplicity and costs.

11.5 Sterile filtration

11.5.1 Cartridge filtration

At the time of writing the most important technique to achieve sterile filtration in a brewery is through the use of cartridge filters. These comprise a membrane or depth filter that is a relatively thin layer and is pleated within the support structure to provide a high filtration area in a compact volume (Figure 11.3).

The unit needs to be readily cleanable and disinfectable. Ideally the unit may also be backflushed. This means that cleaning fluids or rinses can be flowing in the reverse direction to normal filtration. This enhances the removal of filtered particles from the cartridge. The lifetime of the cartridge is affected by both the filtration duty that is placed upon it and the consequent number of aggressive cleans. The cartridges are relatively expensive and if the lifetime is short then costs become significant.

As described earlier, to manage the filtration of turbid beer from maturation and conditioning to sterile product requires several stages. Typically, these stages will resemble

Figure 11.3 A cartridge filter showing the pleated membrane and support structure. This is contained in a stainless steel housing and filtration is from outside to inside this unit.

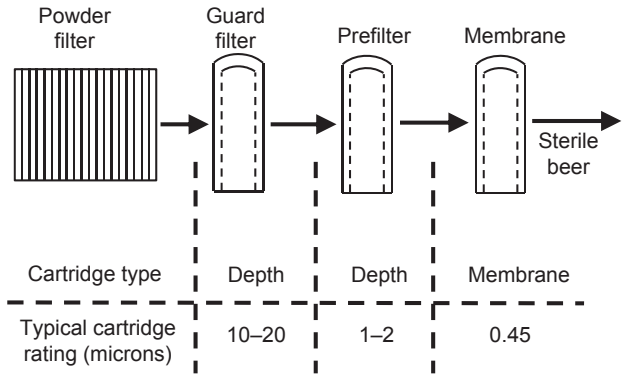

Figure 11.4 A typical sterile filtration process utilising cartridge filtration.

something akin to that described in Figure 11.4. The main beer filter in this process is a powder (filter aid) filter. This filter will remove the vast majority of the volume of suspended material, but some very fine particles including some bacteria may remain. Most often the filter aid filter is followed by a relatively coarse guard or trap filter cartridge. The purpose of this is to catch any filter aid that leaks through the main filter. The next filter in the sequence is a cartridge filter that operates as a depth filter, so that the active layer in the cartridge is a fibrous mass, perhaps of polypropylene. The rating as shown is most likely to be in the range of one to two microns, although more recently many brewers will use a smaller rating, perhaps below half a micron, to ensure that the relatively

expensive final filter performs negligible filtration duty. For a depth filter that does not exhibit an absolute cutoff size this is known as a nominal cutoff or nominal pore size. It represents the size above which effectively all particles are removed. However, it is a feature of depth filters that they will also remove a lot of particles below the nominal cutoff. Hence, the beer that leaves this cartridge will be of 'sparkling' clarity with few particles that will require removal by the final membrane (surface filter). This is essential because the particle removal capacity of the surface filter is much less than that of the depth filter. The final 0.45 micron membrane cartridge acts only as a guarantee of total removal of bacteria (effectively sterility for the brewer) prior to packaging.

To maximise the efficiency of the process, it is necessary for the operators to monitor the whole system performance. Maximising value is largely an issue of maximising the run times through the cartridges. There is a risk that different beer products will affect the filtration sequence in different ways. For example, if a batch contains a great deal of very small particles it may increase the loading on the final two cartridges, especially damaging if it is the final membrane, and the run time may become very suboptimal. The most obvious monitoring, which is often overlooked, is to have pressure gauges or transmitters on either side of each of the filtration steps. It is then simple for the operator to identify the step that is taking more of the filtration load than is optimal for that step. Actions that may optimise the process, depending on the pressure drop characteristics that are achieved, include

- changing the filter aid specifications in the filter aid filter,
- preventative maintenance on the filter aid filter to prevent leakage of filter aid and blinding of the trap filter,
- changing the rating of the cartridge(s) that protect the final membrane, and
- simply adding or removing filtration area from the step as appropriate.

11.5.2 Integrity testing

One requirement of sterile filtration is that the final membrane filter must be integrity tested. This refers to ensuring that the membrane pore structure is still intact and will remove the microorganisms as required. In the food and beverage industry sectors where pathogens are an issue, it is likely that integrity testing is a legal requirement, as required by the Food and Drug Administration (FDA) in the United States. However, in brewing there are not pathogens in conventional beer and the concern is about spoilage. Nevertheless, the threat is still significant enough to make integrity testing an important business requirement. Integrity testing of a membrane in process involves wetting the membrane and then draining it. The housing is full of air or gas, but the microscopic pores of the membrane are still full of water. The most common integrity test is known as bubble point and involves increasing the gas pressure upstream of the membrane until the pressure pushes the water out of the pores and gas flow occurs. The pressure difference must exceed a certain value or the integrity of the membrane has been compromised. Similar tests involve measuring the small flow rate of gas at a small pressure drop caused by diffusion, known as the diffusional flow technique, or a similar test that measures pressure difference decay, known as the pressure decay test. These tests can be performed manually although many brewers employ automated systems that are compatible with modern brewery automation.

11.5.3 Other sterile filters

Sterile filtration can potentially be achieved by fine depth filtration. As mentioned previously, sheet filters have been used to filter beer to a satisfactory microbiological stability. Sheet filters are essentially compressed pads of (normally) cellulose fibres that are arranged into a plate and frame filter press. Performance is sometimes enhanced by incorporating kieselguhr into the structure. Colloidal stabilisation may also be achieved by the presence of polyvinyl polypyrollidone. Process efficiency can be improved by making use of the fact that the plate and frame pack may be arranged so that the beer passes through relatively coarse grades of sheet before a finer grade. Such a two-stage process enables the finer of the two grades to be effectively a sterilising grade. Sheet filters operate less effectively if a maximum flow rating is exceeded. Also, best performance is gained by minimising interruptions to the flow of beer that cause "dislodging" of previously trapped particles.

11.5.4 Downstream process

One issue that arises with sterile filtration is the requirement to fill into package in a sterile manner. Sterile filling of large containers (kegs) has been performed for many years. In this case the high beer flow rates in the filling machinery greatly reduce, but do not eliminate, risks. Filling small pack such as plastic or glass bottles and cans requires many more filling heads and a greater opportunity for contaminants to get into product if operation is poor. Sterile filling lines rely on techniques such as positive air pressure around the sensitive areas to prevent airborne bacteria approaching and tightly controlled hygienic practices by the operators. It should be noticed, however, that there is a distinct trend away from tunnel pasteurisation of small pack products. Tunnel pasteurisers are much more energy expensive and also water expensive than in-line flash pasteurisers. The latter of course will also require sterile filling.

11.6 Improving filtration performance

As discussed above, sterile filtration is capable of producing beer of high quality. However, because the process is multistage the costs become significant. Some opportunities for optimisation have already been outlined. However, ultimately the limiting factor for performance of a filtration step is the characteristics of the beer requiring filtration. Therefore, there is scope for the introduction of technologies upstream of the filtration steps that improve the 'filterability' of the beer.

11.6.1 Centrifugation

Disc stack centrifuges are powerful solid–liquid separators. In years past they were often problematic. They often gave very undesirable effects such as warming the beer up and drawing in oxygen. Today, however, superior engineering design features such as hermetic sealing have largely eliminated these problems. In addition, disc stack centrifuges are available that are more powerful than previous versions. Some may remove some of the colloidal particles as small as one micron. In the context of filtration

it may be viable to use the centrifuge upstream of the bulk beer filter. In the case of a filter aid filter this will allow increased run time because of the reduced solids loading. However, an often overlooked benefit is that the brewer could employ finer grades of filter aid. This improves the filtered beer clarity and in the case of a sterile filtration sequence will reduce the loading onto the subsequent stages, prolonging run time.

11.6.2 Flocculents (finings)

There are several types of flocculents employed in brewing. One example is isinglass, which is a suspension of collagen, derived from the swim bladders of fish, in weak mineral acids. The collagen macromolecules unusually form a net positive charge in the mildly acidic beer. Thus they can interact electrostatically with the suspended beer particles that almost exclusively exhibit a net negative charge. This results in coagulation and flocculation processes that cause the particles to group into large flocs, making sedimentation and removal more simple and rapid. Indeed, this process can be so effective that isinglass can be used to produce acceptable beer clarity on its own. An example is traditional UK cask ale, which is a clear product that is not filtered.

However, the real opportunity for the employment of flocculents such as isinglass in the context of sterile filtration is that flocculents are very effective at the removal of small, colloidal particles. If compared with a centrifuge, which has a mode of operation that makes it more effective at removing relatively large particles, flocculents can be seen to be effective at all particle sizes. Hence it is clear that application of flocculents will reduce the loading of colloidal material that will in particular curtail the run length of the sterilising filters (such as the membrane) at the end of the process. Isinglass may be employed in the cold storage stage that precedes beer filtration. Improved colloidal (clarity) stability of the final product in package is a benefit as well as improved performance of the filters.

Other flocculents are often employed in the brewhouse. Copper finings are employed in the wort boiling stage. They comprise carrageenan derived from certain seaweeds. Their main mode of operation is that they very significantly increase the precipitation of protein–polyphenol material when the wort is subsequently cooled, ready for fermentation. Removal of this material greatly reduces the potential for ‘chill haze’ formation in beer processing and package. Hence copper finings have a much greater effect on both colloidal stability and beer filter performance than is often realised. An alternative to copper finings is silica sol, which is an aqueous suspension of colloidal silica. There are similar stabilising and precipitating effects, although usually silica sol is only preferred in beers that aim to obey Reinheitsgebot, the German Beer Purity Law.

11.6.3 Enzyme treatments

Exogenous enzymes are those that are added by the brewer rather than those that occur naturally from the malted cereals and yeast. Enzymes are available that exhibit proteolytic, cytolytic (plant cell wall material) and carbohydrase activity. Cost-effective enzyme preparations are not in general pure enzymes, however, a blend is often an advantage, enabling more than one activity to occur.

Enzymes may be employed throughout the process from brewhouse through to cold storage to eliminate problematic components or in some cases significantly

change beer flavour and quality. A common example is beta-glucanase. This originates from the cell walls of the raw materials most obviously from barley malt. Its substrate beta-glucan is capable of causing problems throughout the process. In particular, late in the process it is likely to precipitate from the beer as the wort sugars, which stabilise it in solution, are reduced by fermentation. Worse still in some cases it will form very high molecular weight gels that will seriously impede the beer filters. Application of beta-glucanase at a judicious point in the process may eliminate the detrimental effects. Similar applications may be found on occasions for proteases, pentosanases (xylanases) and other carbohydrases such as amylases.

Membrane filters, including both final sterilising filters and crossflow filters, are often found to be difficult to clean to a suitable standard for effective processing. In some cases it is viable to use specialised enzymatic cleaners even though they may be relatively expensive compared to 'non-biological' detergents. It should be noted that different beer compositions may call for different enzyme activities, depending upon what is fouling the membrane (Taylor, Faraday, O'Shaughnessy, Underwood, & Reed, 2001). Several suppliers of membrane technologies also supply their own propriety enzymatic cleaners.

11.7 Future trends

The pressure to eliminate kieselguhr from the brewing process on the grounds of health and safety and environmental friendliness will continue to grow. The latter will directly increase the costs of usage. Zuber (2009) describes an example of some developments in man-made, regenerable filter aids, thus reducing landfill disposal requirements. Often these may be readily retrofitted to existing plants. However, in the medium-long term it seems likely that crossflow microfiltration will become the bulk filtration process of choice. This is because membranes are likely to come down in cost (in real terms) and improve in efficiency. At this time there is a need for membrane suppliers to increase the ruggedness of, in particular polymeric, membrane modules. This will reduce repair or replacement costs and also reduce the risk to potential purchasers of crossflow plants.

At the time of this writing, it is clear that there has been a divergence in the brewing industry. On the one hand, there are multinational brewing companies with large, global brands. On the other hand, there are smaller brewing companies that produce so-called 'craft' beers. As described earlier, the economics of sterile filtration over pasteurisation are favourable for smaller brewers. Sterile filtration may give them the option to produce beer in conventional bottles and cans on their own site. It could be that sterile filtered beer brands will become more common in the future.

11.8 Sources of further information and advice

An excellent practical guide to filtration processing is available from the European Brewery Convention (EBC Manual of Good Practice, 1999). Further learning materials are available from the Institute of Brewing and Distilling (IBD). The IBD is a

UK-based international professional body for personal development and learning. It has a free-to-access search facility for material that is then free to IBD members but can be purchased by nonmembers (www.ibd.org.uk).

Another body that readers may be interested in is the Filtration Society (a mixture of academics, commercial suppliers and users) at www.filtsoc.org.

Campden BRI operates a commercial database on all aspects of brewing and can be contacted for information support at www.campdenbri.co.uk.

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Reducing microbial spoilage of beer using pasteurisation

12

E. Wray
Campden BRI, Surrey, UK

12.1 Introduction

Pasteurisation has long been used for the preservation of beer, following on from the work of Louis Pasteur. It is a process of applying heat to preserve food and drinks. It is effective in stabilising beer with regard to microbial contaminants and is in widespread use across the brewing industry. A number of concepts have been developed to measure and analyse the degree of pasteurisation which can show wide variation depending on time, temperature, drink composition and organisms present. These concepts include the D value, the z value and Pasteurisation Units (PUs).

The composition of beer makes it an inherently stable product and factors such as alcohol content, low pH, low nutrients and anaerobic conditions mean only a low degree of pasteurisation needs to be applied to achieve microbial stability, though differences in beer composition mean these factors vary across brands. Different microorganisms also have different degrees of heat resistance, so selecting the level of pasteurisation to apply to beer is not always a straightforward matter.

Two main methods are used to pasteurise beer: tunnel pasteurisation, where bottles or cans are passed through a series of water jets applying heat, and flash pasteurisation, where the beer is heated rapidly in a plate heat exchanger and holding tube before packaging.

The application of heat can affect the flavour of beer in a number of ways, particularly if oxygen levels are high. Nevertheless, with good practice and attention to quality control a high-quality product can be produced with minimal flavour changes and a high degree of microbial stability.

12.2 History

The term ‘pasteurisation’ takes its name from the great French scientist Louis Pasteur. Prior to his work heat preservation of some foods and drinks was already employed and had been for some time. However, this was carried out on an empirical basis and it was Pasteur who was able to elucidate the scientific reasoning behind how it works.

In 1865 he patented a heat treatment for wine preservation and in 1866 he published his *Studies on Wine* where he stated that heating to as low as 50 °C could preserve wine by killing the microorganisms that caused spoilage. The potential for preserving beer in a similar way was of immediate interest to brewers, though it was not until after the

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Franco-Prussian war of 1870–71 that Pasteur himself turned his attention to beer. He hoped to gain revenge against the German victors by improving beer production in other countries to such an extent that it would undermine German beer exports. Working with other scientists and prominent French brewers he developed means of producing beer with much less risk of infection, which were published as *Studies on Beer* (1876) and included details on pasteurising beer, though not without reservations.

A number of large breweries rapidly adopted pasteurisation of their bottled beers, and various methods were developed for carrying it out in cabinets using steam and hot water. These had high energy use so ways of recovering heat were developed. Methods of moving crates through zones of water at different temperatures were developed in the early twentieth century, as were using water sprays (European Brewery Convention, 1995).

The development of walking beam technology and improvements in mechanisation improved the technology further and led to modern tunnel pasteurisers. Walking beams are stainless steel strips that run the length of the pasteuriser. Alternate beams can be lifted and moved forward a short distance before lowering and returning, having moved the can or bottle slowly forward in the process (Wilson, 1981).

The introduction of keg beer leads to the next main method used in beer pasteurisation as in-package pasteurisation of such large containers is impractical. By passing beer though a plate heat exchanger it can be rapidly heated and held in a holding tube at the required temperature before cooling for packaging (flash pasteurisation). As the beer is cooled before packaging scrupulous hygiene must be maintained to ensure the pasteurised beer does not become re-infected. Automation and control improvements from the 1970s onward improved this process and it is now also used for bottles and cans.

12.3 Principles of pasteurisation

Pasteurisation is a means of achieving microbial stability in food and drinks by applying enough heat to destroy organisms capable of growing during the subsequent storage period. It works in conjunction with other parameters of the product to ensure microbial stability. The other parameters which contribute to beer stability include low pH, alcohol content and the presence of hop compounds. These will be discussed further in the section on hurdle technology (Section 12.5.1).

Pasteurisation is not full sterilisation; it is what has been termed ‘practical sterility’ (European Brewery Convention, 1995). Microorganisms that are able to grow in beer need to be killed in order to achieve stability, but the heat treatment does not have to be to such a level to kill heat resistant spore forming bacteria, as they are unable to grow in beer. Applying the minimal amount of heat necessary to achieve stability ensures that both the effect on flavour and energy use is minimised. Pasteurisation will not, however, solve problems in beer quality that poor hygiene can cause before pasteurisation is carried out!

The temperature applied during pasteurisation and the length of time it is applied for are of critical importance in pasteurisation, as is which microorganisms are present in the beer and the quantity in which they are found.

12.4 D value, z value, *P* value, process time, Pasteurisation Units and L value

A number of concepts have been developed to explain and quantify the effects of pasteurisation on a product.

The D value is the time required at a set temperature for a decimal reduction (i.e. one log or 90%) in the population numbers of a known organism. The size of the D value depends on the temperature, the microorganism and the other parameters in the beer that affect microbial grown (see Section 12.5.1 on hurdle technology). A higher temperature or lower pH will lead to a lower D value. A D value can be expressed in minutes or seconds.

The z value is the change in temperature required to bring about a 10-fold change (i.e. one log) in the D value. The z value is expressed in degrees. In the brewing industry a z value of 6.94 °C (often rounded to 7 °C) is generally used after the work of Del Vecchio, Dayharsh, and Baselt (1951).

The *P* value is the time required to achieve a stated reduction in numbers of a microbial population at a given temperature and z value. It is a function of the D value and the z value to give a total pasteurisation value. A *P* value must therefore have the temperature and the z value specified for it to have meaning. A *P* value expressing the pasteurisation value at 60 °C for a z value of 6 °C is written as P^{6}_{60}. *P* value is used to give the time for a specified log reduction in organism numbers. For example, when the D value is 3 min at 60 °C, with a z value of 6 °C, to achieve a 6 log reduction will require 18 min and the *P* value will be written as $P^{6}_{60} = 18$.

In the brewing industry things have been simplified somewhat and the Pasteurisation Unit (PU) is routinely used. The PU is based on the temperature of 60 °C, and a z value of 6.94 °C is used. One PU = 1 min at 60 °C.

As the effect of pasteurisation is highly temperature dependent and process temperatures will not be fixed, the Lethality rate at a given temperature (L_T) can be related to the D value at 60 °C (as in PU) using the equation

$$L_T = D_{60}/D_T \tag{12.1}$$

The Lethality rate is usually expressed as the time in minutes which will give one PU (i.e. is equivalent to the 1 min at 60 °C).

To calculate the PU of 1 min at any given temperature (*T*) the following equation can be used:

$$PU = 1.393^{(T-60)} \tag{12.2}$$

Total PU can be obtained by multiplying the result by the number of minutes at that temperature.

As a useful approximation, a temperature increase of 2 °C doubles the PU and an increase of 7 °C increases the PU 10-fold. Lethality tables can be produced showing the PUs for a range of given times and temperatures, and can be used to calculate the

Table 12.1 The effects of different temperatures on Pasteurisation Units

Temperature °C	PU/minute
60.0	1.0
62.1	2
67.0	10
70.0	28
73.9	100

total effect of a pasteurisation process. The Table 12.1 shows the effects of different temperatures on PU at certain key temperatures.

A more detailed look at how PUs alter with temperature is shown in Table 12.2.

12.5 Spoilage hurdles

Pasteurisation is only effective in conjunction with other factors that make a product inhospitable to contaminating organisms. The numerous hurdles present in beer have a synergistic effect and mean that only mild pasteurisation is required for practical sterility and the effects of heat treatment on flavour are minimised.

Mashing and boiling during wort production produce the required degree of sterility prior to yeast pitching and the fermented beer has a number of hurdles (Vriesekoop, Krahl, Hucker, & Menz, 2012; Table 12.3):

12.5.1 Ethanol

The ethanol content of beer (generally between 3.5–5% by volume) is a large hurdle to microbial growth. Ethanol inhibits cell membrane functions and induces cell membrane leakage. Increased cell membrane permeability increases the effects of low pH on the cell by increasing proton passage into the cytoplasm and reducing the ability of the cell to maintain pH homeostasis.

12.5.2 Low pH

The low pH of beer (generally 3.7–4.1) inhibits the growth of many microorganisms. At low pH entry of organic acids into the cell is enhanced, causing intracellular acidification. This leads to the destruction of enzyme systems and the reduction in nutrient uptake.

The microorganism will attempt to maintain pH homeostasis by using energy to pump cations across the cell membrane. When the ability of the cell to do this is overwhelmed starvation and cell death follow.

The low pH of beer also has a synergistic effect with the antimicrobial properties of hop resins. Low pH is an important reason why beer is generally considered to be

Table 12.2 Lethality table showing Pasteurisation Units for 1 min at the given temperature (degrees given in column one, decimals in row one)

T °C	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
50	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.05	0.05	0.05
51	0.05	0.05	0.05	0.06	0.06	0.06	0.06	0.06	0.07	0.07
52	0.07	0.07	0.08	0.08	0.08	0.08	0.09	0.09	0.09	0.10
53	0.10	0.10	0.10	0.11	0.11	0.12	0.12	0.12	0.13	0.13
54	0.14	0.14	0.15	0.15	0.16	0.16	0.17	0.17	0.18	0.18
55	0.19	0.20	0.20	0.21	0.22	0.23	0.23	0.24	0.25	0.26
56	0.27	0.27	0.28	0.29	0.30	0.31	0.32	0.33	0.35	0.36
57	0.37	0.38	0.40	0.41	0.42	0.44	0.45	0.47	0.48	0.50
58	0.52	0.53	0.55	0.57	0.59	0.61	0.63	0.65	0.67	0.69
59	0.72	0.74	0.77	0.79	0.82	0.85	0.88	0.91	0.94	0.97
60	1.00	1.03	1.07	1.10	1.14	1.18	1.22	1.26	1.30	1.35
61	1.39	1.44	1.49	1.54	1.59	1.64	1.70	1.76	1.82	1.88
62	1.94	2.01	2.07	2.14	2.22	2.29	2.37	2.45	2.53	2.61
63	2.70	2.79	2.89	2.99	3.09	3.19	3.30	3.41	3.52	3.64
64	3.77	3.89	4.02	4.16	4.30	4.44	4.59	4.75	4.91	5.07
65	5.25	5.42	5.60	5.79	5.99	6.19	6.40	6.61	6.84	7.07
66	7.31	7.55	7.81	8.07	8.34	8.62	8.91	9.21	9.53	9.85
67	10.18	10.52	10.88	11.24	11.62	12.01	12.42	12.84	13.27	13.72
68	14.18	14.66	15.15	15.66	16.19	16.73	17.30	17.88	18.48	19.11
69	19.75	20.42	21.10	21.81	22.55	23.31	24.10	24.91	25.75	26.61
70	27.51	28.44	29.40	30.39	31.41	32.47	33.56	34.70	35.87	37.07
71	38.32	39.61	40.95	42.33	43.76	45.23	46.76	48.33	49.96	51.64
72	53.38	55.18	57.04	58.97	60.95	63.01	65.13	67.33	69.59	71.94
73	74.36	76.87	79.46	82.14	84.91	87.77	90.73	93.78	96.94	100.21
74	103.59	107.08	110.69	114.42	118.28	122.26	126.38	130.64	135.04	139.60
75	144.30	149.16	154.19	159.39	164.76	170.31	176.05	181.98	188.12	194.46
76	201.01	207.78	214.79	222.03	229.51	237.24	245.24	253.50	262.05	270.88
77	280.01	289.44	299.20	309.28	319.70	330.48	341.62	353.13	365.03	377.33
78	390.05	403.20	416.78	430.83	445.35	460.36	475.87	491.91	508.49	525.63
79	543.34	561.65	580.58	600.15	620.37	641.28	662.89	685.23	708.32	732.20
80	756.87	782.38	808.75	836.00	864.18	893.30	923.41	954.53	986.70	1019.95

Table 12.3 Hurdles to microbial growth

Hurdle	Mode of action
Ethanol	Inhibits cell membrane function
Low pH	Affects enzyme activity
	Enhances inhibitory effect of hop resins
Hop resins	Inhibits cell membrane function in Gram-positive bacteria
CO_2	Creates anaerobic conditions
	Lowers the pH
	Affects enzyme activity
	Affects cell membranes
Low O_2	Anaerobic conditions inhibit the growth of obligate aerobes
Low nutrient content	Starves cells

unable to support pathogen growth. For example, *Clostridia* are unable to grow below pH 4.5 and *Salmonella* are unable to grow below pH 4.

12.5.3 Hop resins

The antimicrobial effect of hop resins is mainly derived from the isomerised alpha-acids. Though beta-acids also have antimicrobial effects, their low solubility means they contribute little to this in beer. The isomerised alpha-acids cause the cell membranes of many Gram-positive bacteria to leak, dissipate the transmembrane pH gradient, deplete the proton motive force, inhibit the uptake of nutrients, deplete divalent cations and cause oxidative stress. However, beer spoilage bacteria have a number of mechanisms to resist these effects.

12.5.4 Carbon dioxide

Carbon dioxide is one of the main products of wort fermentation and extraneous CO_2 is commonly added to beer to increase carbonation. It creates an anaerobic environment, lowers the pH, affects reactions in the cell and inhibits cell growth.

12.5.5 Low oxygen level

The low oxygen levels found in beer inhibit the growth of many microorganisms, preventing some growing entirely and slowing the growth rate of others.

12.5.6 Low nutrient content

As beer is a fermented beverage many of the nutrients present in wort are utilised by yeast during the production process. This leaves a nutrient depleted environment for spoilage organisms; the more attenuated the beer the more the nutrients are depleted and the less are available for other organisms to utilise.

The degree of protection from microbial growth that hurdles will provide in beer will vary with the composition of the beer. High pH, low attenuation, low CO_2, low ethanol and low hopping rates will all make beer less inherently microbiologically stable. Low alcohol and nonalcoholic beers in particular will require more PUs to be applied for practical sterility to be obtained.

12.6 Microorganism heat resistance

Pasteurisation must be effective against the most heat resistant organism likely to be present as a contaminant. As has been stated previously practical sterility, not absolute sterility, is required and the hurdles to microbial growth mean the pasteurisation of beer is a mild process compared to many other beverages (Table 12.4).

Del Vecchio et al. (1951) based the z value used in PUs as 6.94 °C because the most heat resistant organism they found was an abnormal yeast with this z value. It has since been found that some beer contaminants have a higher z value than this, though in practice for most common contaminants it is less (O'Connon-Cox et al., 1991a).

D values similarly show variation between organisms at the same temperature, and the changes to the D value with temperature changes also vary greatly (Boulton & Quain, 2006). As is often the case caution is required when utilising the calculations in practice. The different compositions of each beer brand will further complicate matters, as the level of protection provided by hurdle technology will vary. It has been suggested that pasteurisation regimes will need to be established for each beer according to its composition (Garrick & McNeil, 1984) and common contaminants found in a plant (O'Connor-Cox, Yiu, & Ingledew, 1991b).

Despite these complications PUs remain in widespread use in the brewing industry and in practice reflect well actual microbial destruction at the temperature range

Table 12.4 D and z values for a number of microorganisms

Organism	D_{60} value (min)	z value (°C)
Saccharomyces cervisiae	0.01	4.6
Saccharomyces pastorianus	0.004	4.4
Saccharomyces diastaticus	0.06	7.8
Lactobacillus paracasei	0.02	6.5
Aspergillus niger	0.04	3.7
Pediococcus sp.	0.00073	4.0
Hansenula anomala	0.0039	4.6
Pichia membranaefaciens	0.00025	2.8
Lactobacillus frigidus	0.44	15
Lactobacillus delbrueckii	0.091	12

Source: European Brewery Convention (1995), Gaze (2006), Kilgour and Smith (1985).

Table 12.5 Typical PU values for different brewery products

Product	Typical minimum PU	Typical maximum PU
Pilsner and lager beer	15	25
Ale and stout	20	35
Low alcohol beer	40	60
Nonalcoholic beer	80	120
Lemonade	300	500
Fruit juices	3000	5000

(60–72 °C) used in beer pasteurisation (Zufall & Wackerbauer, 2000). To relate these figures to PU it has been reported that one PU is sufficient to achieve practical sterility with regards to brewers' yeast and *Pediococcus* sp., five PUs are required for *Lactobacillus* sp. and 10 PUs are required for wild yeast (O'Connor-Cox et al., 1991b). In practice more are often used for security reasons, though the general trend has been for the amount of PU applied to come down over time.

Though 10–12 PUs should be adequate and has been confirmed as satisfactory in some breweries (Wackerbauer & Zufall, 1997) the EBC Manual of Good Practice (1995) on Beer Pasteurisation makes the following recommendations (Table 12.5).

The inclusion of low- and nonalcoholic beers as well as lemonade and fruit juices shows how much the properties of beer minimised the amount of PU that are required for beer pasteurisation.

12.7 Tunnel pasteurisation

With tunnel pasteurisation the beer is filled into a container (bottle or can) which is sealed before pasteurisation. The container is then transported through the tunnel pasteuriser. Water is sprayed on the containers in stages as they pass through the tunnel, at first heating them until the desired holding temperature is reached and then cooling them to the required discharge temperature (Figure 12.1).

Tunnel pasteurisers are divided into zones where water is sprayed at different temperatures, with the total transit time and temperature profile calculated to provide the required degree of pasteurisation. It is common in practice to have a 'superheat' zone before the holding zone to ensure the required temperature has been reached. Heat recovery is important to ensure maximum energy efficiency and minimise costs. Each zone of the tunnel pasteuriser will contain a water tank, a pump and a water distribution system. Heat recovery is achieved by water being moved to different zones where its temperature is appropriate.

Heat transfer to beer inside bottles or cans takes place through the walls of the beer container which causes a lag in the heating process, bottles having a longer lag than cans. Convection currents are also generated in the beer being heated. Because of this there will be a point near the base of the container, known as the cold spot, where the lowest rate of heat transfer occurs. It is this point that calculations of PU applied to the beer must be made. The temperature rise cannot be too rapid or there is a risk of

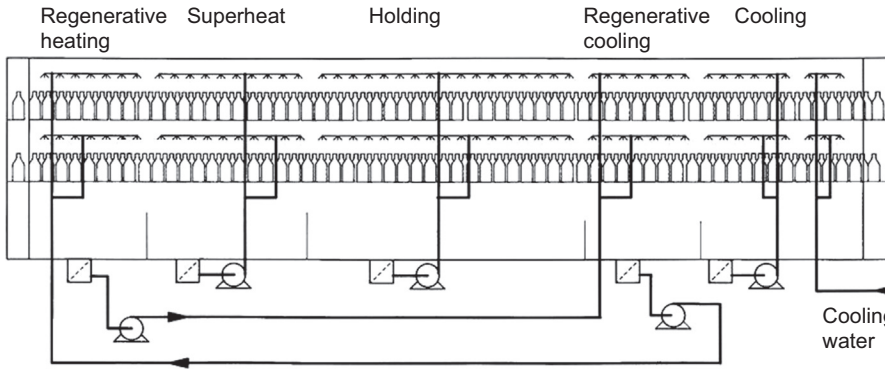

Figure 12.1 Tunnel pasteuriser.

glass bottles breaking or the pressure rise in the carbonated beer causing the container to burst. The amount of headspace has a large effect on the internal pressure generated during pasteurisation and must be carefully controlled. Pasteurisation in a tunnel pasteuriser can take up to an hour in total.

The first heating stage will gently warm the container approximately 10 °C and subsequent stages will steadily raise the temperature to at least 60 °C. The most important stage is the superheat zone which must be accurately controlled at 61–65 °C to ensure the container has reached 60 °C as it enters the holding zone.

To prevent over pasteurisation, and associated deterioration in product flavour and risk of producing haze, it is important that controls are in place to adjust the heat delivered in the event of a stoppage. Modern tunnel pasteurisers can calculate the total PUs delivered to containers as they travel though the pasteuriser.

Travelling recorders are also routinely used, consisting of a dummy bottle or can on a base plate with sensors and recorders. This can be passed through the pasteuriser to measure the temperature profile that containers are exposed to both as they pass through different zones of the pasteuriser and in different positions that containers may occupy. This means 'cold spots' due to blocked spray jets or other problems can be detected.

Each section of the pasteuriser will have a water tank, a water pump and spraying system (spray nozzles or spray pans). A float valve and overflow maintain a constant water level in the tanks. Heaters bring the water to the required temperatures on start up and for adjustment, though during normal operation are only needed in the superheating and holding sections.

The different heating and cooling sections are interconnected and therefore make efficient use of the water at different temperatures. For example, cooling zones toward the end of the pasteuriser will pick up heat from warm containers leaving the holding zone. This warmed water will be pumped to the front of the pasteuriser where it can start to warm cold containers entering the pasteuriser. This will cause it to lose heat so it can be pumped back to carry out more cooling duty. This can be repeated several times as the containers pass through the various stages. Excess heat in cooling sections is reduced by letting hot water overflow drain and bringing in more cold water. Heat recovery in tunnel pasteurisers is around 50% (European Brewery Convention, 1995; Table 12.6).

Table 12.6 Typical temperature time profile for a seven zone pasteuriser

Stage number	Function	Spray temp (°C)	Spray time (minutes)	Container temperature in (°C)	Container temperature out (°C)
1	Preheat	22	6	2	9
2	Preheat	32	7	9	21
3	Superheat	65	14	21	60
4	Holding	60	6	60	60
5	Cooling	40	10	60	43
6	Cooling	32	7	43	36
7	Cooling	22	6	36	28

Source: Dunn (2006).

Movement of the containers through the pasteuriser can be by a 'walking beam' system or on a conveyor belt through a flat bed system. The former copes well with broken fragments from glass bottles and the latter was originally developed for cans, though has now been found to cope well with glass bottles, too. Double deck tunnel pasteurisers are available offering greater capacity and/or saving space.

Products passed through a tunnel pasteuriser can have a shelf life of up to a year (Boulton & Quain, 2006).

12.7.1 Cleaning

The warm and wet conditions inside a tunnel pasteuriser make it a good environment for slime to grow and corrosion problems to develop. The water used for spraying may be treated with biocides, softened to prevent scaling, and treated to minimise corrosion. Care must be taken with addition rates, however, to ensure compliance with local regulations, the effects on the equipment and personnel and even possible effects on cans.

A 'strainer box' will be present at the outlet from water tanks to trap any material that may cause nozzle blockages. These will require frequent cleaning. Water in the tanks will also need to be changed frequently due to build up of broken glass and other solid materials and spilt beer from damaged containers.

A key design parameter for tunnel pasteurisers is ease of cleaning. Water tanks, spray jets and strainers must have easy access so scale, broken glass and other foreign bodies can be easily removed. Spray nozzles must have large bores and be easily removed and replaced.

Spraying all sections of the pasteuriser with water at >80 °C can be used as part of the cleaning process to limit biological growth (European Brewery Convention, 1995).

12.8 Flash pasteurisation

In flash pasteurisation the beer is rapidly heated in a plate heat exchanger and held in holding tubes where the required number of PUs is applied in a matter of seconds. Typically the beer will be heated to around 72.5 °C and held for 20 s, which will give 20 PUs (Figure 12.2).

The plate heat exchanger consists of a series of sealed metal plates linked by connections in the corners and clamped together at the ends. Beer will be allowed to flow through one side of the plates and pass though the corner ports to the next and heating liquid will flow in a similar manner through the other side of the plate. When the beer is at the required temperature it will enter a holding tube in order to give the time at the appropriate temperature to give the desired degree of pasteurisation. The flow through the pasteuriser is highly turbulent which aids rapid heat exchange and little temperature difference across the diameter of the holding tube, though beer in the centre will flow slightly faster than that close to the wall of the tube. The rapid passage of beer through a flash pasteuriser means that most of the PU is applied in the holding tube section. A regeneration section with flash pasteurisers allows for a high degree of heat recovery (over 90%) as beer is cooled from the pasteurisation temperature (Dunn, 2006; Table 12.7).

As flash pasteurised beer is not pasteurised inside a sealed container a higher degree of hygiene and sterility is required than for tunnel pasteurisation. It is particularly critical once the beer has left the pasteuriser, and the buffer tank, filling equipment and all associated pipework are all areas of particular risk.

A thorough microbiological monitoring system should be in place to ensure that hygiene is maintained with samples taken before and after pasteurisation. According to Boulton and Quain (2006) typically a shelf life of six to eight weeks is given to flash pasteurised beer, though in trade keg beer with a shelf life of up to six months is commonly seen.

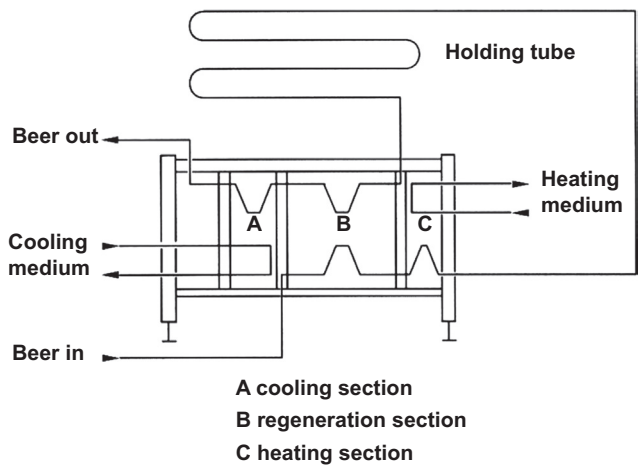

Figure 12.2 Flash pasteuriser: heating section, cooling section, regenerative section.

Table 12.7 A typical time–temperature programme

Beer inlet temperature	**3.0 °C**
Outlet from regenerative heating section	65.6 °C
Outlet from heating section/entry into holding tube	70.3 °C
Outlet from regenerative cooling section	7.7 °C
Outlet from cooling section	3.0 °C
Holding time	30 s

Source: European Brewery Convention (1995).

12.8.1 Process control

The short time that flash pasteurisation takes means that maintaining the correct temperature and holding time is critical.

PUs can be calculated using the following equation (European Brewery Convention, 1995):

$$PU = (V/Q) \cdot 1.393^{(T-60)}$$

V = Volume of holding tube

Q = Flow rate in m^3/minute

T = Temperature (12.3)

Even though the flow through the holding tube is turbulent the beer in the centre will still flow faster than the beer adjacent to the pipe. To ensure that even beer flowing at the fastest rate obtains the correct number of PU the calculated holding time needs to be increased by a factor of 1.25 (European Brewery Convention, 1995).

Flow rate is controlled by a valve on the outlet, which can be either manually or automatically controlled. In the case of automatic control it can adjust in response to the level in the buffer tank. The temperature of pasteurisation is controlled by adjusting the temperature of the heating medium. This can be linked to a PU controller which calculates the temperature required in relation to the flow rate. The outlet temperature of the beer needs to be at a level suitable for packaging and is controlled by adjusting the flow rate of coolant.

Flash pasteurisers work best at a constant flow rate but packaging operations mean that this is not always possible and pasteurisers will need to be able to work at variable speeds. It is usual to have an outlet buffer tank to smooth out the flow rate prior to filling (Dunn, 2006).

If the temperature at the pasteuriser outlet is low the system should be designed to ensure no under processed product leaves the pasteuriser by diverting the flow back to the inlet until the correct operating temperature is restored. In the case of serious faults prolonged recirculation should be avoided by shutting down the system.

12.8.2 Gas breakout

At the temperatures used in flash pasteurisation CO_2 has very low solubility so the beer needs to be kept under high pressure to prevent gas breakout and fobbing. If fobbing does occur it increases the flow rate of the beer leading to under pasteurisation, a problem compounded by the possibility of microorganisms avoiding heating inside gas bubbles. Collapsing foam can lead to haze formation and can also bake onto the holding tube, increasing the risk of infection. A pressure monitor needs to be installed to detect when problems have occurred so the process can be stopped. The pasteuriser will need to be cleaned and sterilised before being restarted (Dunn, 2006).

12.8.3 Plate failure

Another potential problem with flash pasteurisers is that of plate failure, when corrosion causes a hole to develop. This can allow unpasteurised beer or coolant to leak into the pasteurised beer. A good maintenance programme will minimise occurrence. Also a booster pump is typically used to ensure pasteurised beer is maintained at the highest pressure in the system (at least 0.5 bar higher than the product) so that in the event of any leakage it will be of pasteurised beer into unpasteurised beer or coolant, ensuring that product is not contaminated (Hyde, 2001).

12.9 Flavour change

Pasteurisation can affect the flavour of beer and Pasteur himself had concerns about the effects of pasteurisation on beer (Pasteur, 1876):

> *To preserve bottled beer from deterioration, some bottlers employ, at the moment of filling, a small quantity of bisulphite of lime [calcium bisulphite]. Others heat the bottles to a temperature of 55 °C (131 °F) in the north of Germany and in Bavaria, this practice has been widely adopted since the publication of the author's 'Studies on Wine', and some of M. Velten's writings. The process has been termed* pasteurization *in recognition of the author's discovery of the causes of deterioration in fermented liquors, and of the means of preserving such liquors by the application of heat. Unfortunately this process is less successful in the case of beer than in that of wine, for the delicacy of flavour which distinguishes beer is affected by heat, especially when the beer has been manufactured by the ordinary process.*

A more recent study (O'Connor-Cox, Yiu, & Ingledew, 1991a) has described the detrimental effects of pasteurisation:

> *Perhaps the worst effect may be the off-flavours accompanying the processing. Pasteurization flavour has been described as oxidized, bread crust-like, or possessing a cooked quality. These off-flavours have been shown to be associated with a wide range of carbonyl compounds including unsaturated aldehydes. Prolonged pasteurization and/or exposure to oxygen have both been*

shown to have a significant effect on the development of carbonyl compounds. If oxygen is present, pasteurization generally also results in darkening of beer color.

The effects of pasteurisation are more apparent in lighter flavoured beers, which is why less PUs are typically applied to lagers than to ales and stouts.

The negative effects of pasteurisation on beer flavour are a particular problem when oxygen levels are high or excessive pasteurisation is employed. The presence of metal ions in beer, particularly copper, also increases the staling effects of pasteurisation. Keeping oxygen levels in the beer below 0.3 ppm (O'Connor-Cox et al., 1991a), not using copper brewing vessels and only applying the level of pasteurisation required minimises these effects. It has been found with flash pasteurisation that applying the required PU with shorter time and higher temperature has the least effect on flavour (Meilgaard, 2001).

Pasteurisation can also cause hazes to form in beer. It is mainly due to prolonged heat treatment causing proteins to denature (O'Connor-Cox et al., 1991a).

12.10 Good practice and quality control

Once a pasteurisation process has been established resulting in a stable product of the desired quality the time and temperature of the process will need to be routinely monitored (Gaze, 2006). It should be included in the HACCP plan as a Critical Control Point. Monitoring devices will need to be regularly calibrated and calibration records must be maintained.

Verification of the process will need to be based on likely errors that may occur and the organisms that are most likely to cause problems. The different processes employed in tunnel pasteurisation and flash pasteurisation mean they have different potential problems and monitoring must take this into account. For example, blocked jets or gas breakout are specific to particular methods though poor maintenance or calibration can apply to both.

For tunnel pasteurisers time temperature indicators that travel through the tunnel recording the temperatures that containers are subjected to, and for what time, can be used to monitor the process.

Figure 12.3 illustrates data from a time temperature indicator showing the temperature profiles during low (10.8 PU) and high (36.8 PU) levels of pasteurisation.

For flash pasteurisers the pasteurisation process needs very close control as the potential risks of both errors and infection are greater. The recirculating system must be assessed to ensure that the correct level of pasteurisation has been reached before any product leaves the system. If microbiological monitoring indicates under pasteurisation is taking place the calibration of the monitoring instruments must be checked to ensure that correct temperatures, timings and flow rates for the required PU are being achieved. Similar checks must be made if over pasteurisation is suspected.

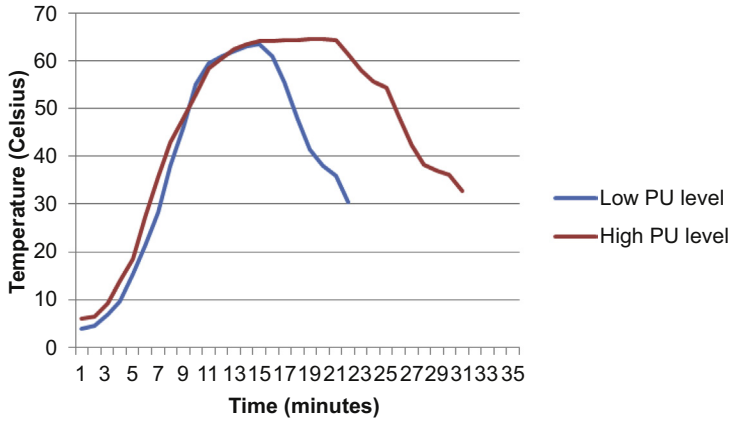

Figure 12.3 Graph to show termperature during travel through a tunnel pasteuriser.

It is good practice to ensure pasteurisers run steadily and process interruptions are as limited as possible. The correct sizing of equipment and buffer tanks can be used to help ensure this (European Brewery Convention, 1995).

12.10.1 Microbiological problems

To ensure the effectiveness of pasteurisation a sampling plan will need to be developed for the process, taking into account likely places where microorganisms will be able to grow and using specific culture media and conditions for the microorganisms in question. Efforts must be taken to maximise recovery of microorganisms, even if the cells are damaged, otherwise contaminated product may be undetected. This is particularly important for flash pasteurisation as the product is at risk of infection once it has left the pasteuriser.

Enzyme activity has also been used to monitor pasteurisation in beer (European Brewery Convention, 1995). Yeast cells excrete cell materials, including enzymes, into beer and as enzymes are highly temperature sensitive measuring their activity can be used to determine the degree of pasteurisation. The ability of invertase to produce glucose from sucrose has been used in this way, though as the enzyme is quite heat labile this method is only appropriate up to about 5 PUs. The ability of enzyme melibiase, found in the cell walls of lager yeast, to produce glucose from melibiose can be used to determine the degree of pasteurisation and this method works up to about 80 PUs.

12.11 Future trends

As breweries strive to reduce their water and energy usage the trend has been to move away from tunnel pasteurisation to flash pasteurisation. It has been estimated that the costs of flash pasteurisation are only 15% of that of tunnel pasteurisation

(Hyde, 2001). The capital expenditure required for replacement and the durability of tunnel pasteurisers means their replacement will proceed slowly but it has been predicted that they will be gone by 2030 (Nelson, 2009). There has also been an increase in using sterile filtration as an alternative to pasteurisation.

A number of novel methods for nonthermal pasteurisation of beer have also been investigated (Hill, 2009). Pulsed electrical fields have been used to inactivate microorganisms by electroporation. This has the benefit of causing little or no change to the organoleptic properties of the product. High levels of hydrostatic pressure (100–1000 MPa) have also been used successfully to enhance microbial stability in beer to a level similar to heat treatment. This works by increasing the permeability of the cytoplasmic membrane and inactivating hop resistance mechanisms. No chemical changes to the beer were found after this treatment.

High-pressure homogenisation as a means of inactivating spoilage microorganisms in beer has recently been investigated (Franchi, Tribst, & Christianini, 2013). This is a continuous process where the fluid is forced under pressure through a narrow gap where it undergoes rapid acceleration (200 m/s at 340 MPa) followed by an extreme drop in pressure. This can lead to microbial inactivation by causing cell permeability changes and a reduction in fluid viscosity. However, a number of chemical changes also occur which can alter the colour of the beer and increase haze (Franchi, Tribst, & Christianini, 2011). Whether any of these novel processes make it into production only time will tell.

12.12 Sources of further information and advice

The European Brewery Convention Beer Pasteurisation (Manual of Good Practice, 1995) remains the best source of detailed information on the pasteurisation of beer. *Excellence in Packaging of Beverages* (2001) also contains a wealth of information. Wilson's chapter on microbial stabilisation in the Master Brewers' Association of the Americas book *Beer Packaging* (1981) provides a good overview of the process, as does Dunn's (2006) section on pasteurisation in *The Handbook of Brewing*.

The pair of articles by O'Connor-Cox et al. (1991a, 1991b) provides useful information on what happens during pasteurisation and actual industrial practice.

The Lemgo D and z value Database for Food provides an excellent resource for finding these values for a number of beer contaminant microorganisms:
http://www.hs-owl.de/fb4/ldzbase/index.pl.

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Traditional methods of detection and identification of brewery spoilage organisms

13

A.E. Hill
The International Centre for Brewing & Distilling, Heriot-Watt University, Edinburgh, Scotland, UK

A proactive approach to microbial spoilage, such as implementation of good manufacturing practices and HACCP process control systems, has made a significant impact in reducing incidences of waste batches and product recall. However, as shown in Figure 13.1, opportunities for microbiological contaminants to enter the brewing process are available at all stages and their amazing ability to adapt to seemingly hostile conditions makes them a tenacious threat.

The most commonly encountered spoilage microbes are detailed in Figure 13.2 (Bokulich & Bamforth, 2013; Quain & Storgårds, 2009). Methods employed by breweries to detect and/or identify both yeast and bacteria are constantly changing and vary depending on the scale of operations. Costs for rapid detection methods are decreasing and most breweries routinely use adenosine triphosphate (ATP) testing. However, traditional methods, such as plating, remain the first approach in detection and identification of microbes. In this chapter the traditional methods available to detect and identify microbes at each stage of the brewing process are detailed.

13.1 Detection of brewery spoilage organisms

13.1.1 Raw materials

13.1.1.1 Cereals

Fungi from the genera *Alternaria*, *Cladosporium*, *Epicoccum*, and *Fusarium* are the main hazards in terms of barley and malt infection. Tolerance of fungal growth on cereals is less than 10 colony forming units (cfu) per gram and zero tolerance of wild yeast. Malt should also be free of mold. Typically malt is sampled at each intake and at regular intervals during storage and tested for bacteria, fungi, wild yeast, and mycotoxins. Air samples may also be taken.

The simplest way to evaluate the internal microflora of grain is by direct plating. Grain is immersed in full strength or 50% household bleach for 1 min to kill surface microflora then rinsed in sterile distilled water. Either grind the grain before adding to molten agar (express per gram) or place individual grains on the surface of the agar in a Petri dish (express per grain) and incubate at 25–30 °C to allow microbes located in the interior of the grain to grow out. The level of internal infection is an indicator of

Brewing Microbiology. http://dx.doi.org/10.1016/B978-1-78242-331-7.00013-7

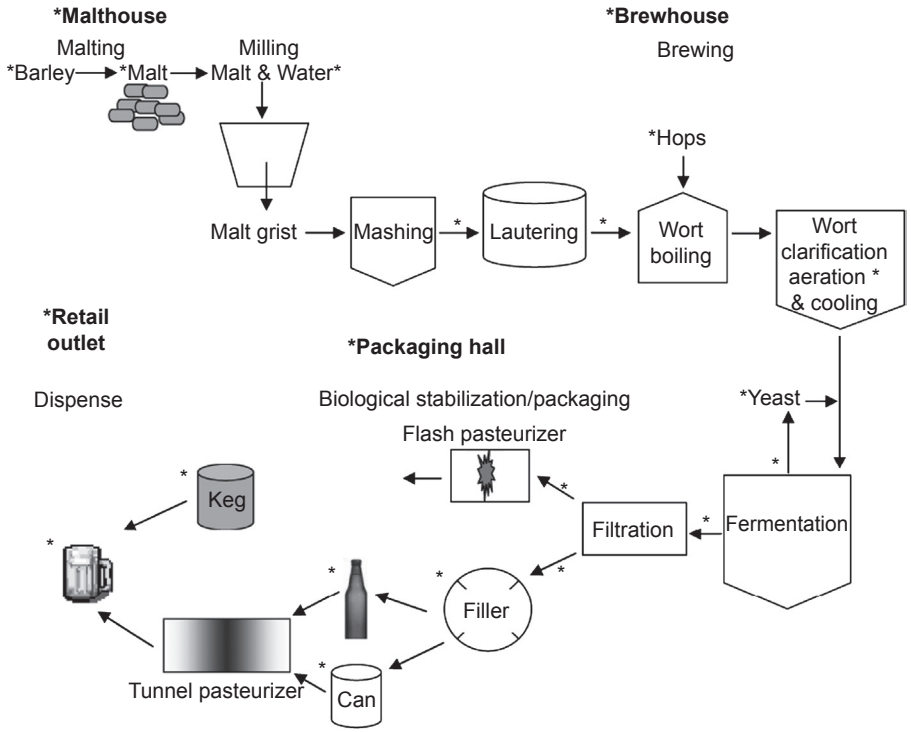

Figure 13.1 Schematic of the brewing process. Potential sources of microbiological contamination are indicated by* (Vaughan, O'Sullivan, & van Sinderen, 2005).

quality and storability of the grain. If *Aspergillus flavus* and *Aspergillus parasiticus* or Czapek-Dox Iprodione Dichloran Agar (see Table 13.1) are used this technique can also give some information about the safety of the grain by indicating whether or not potentially toxic *A. flavus*, *A. parasiticus*, or *Fusarium* species are present.

Grain is susceptible to mycotoxins produced either while the crop is growing by *Fusarium* species or during storage by *Penicillium* species. High quality malting barley should be free from deoxynivalenol (DON) from Fusarium head blight and free from disease. For disease there is no compromise; diseased crops will be rejected and reducing the price will not make them acceptable. Barley from areas with conditions conducive to Fusarium head blight is routinely screened for DON and barley with DON levels over 0.5 ppm will normally be rejected for malting purposes (tolerance is 1 ppm if for human consumption). Unprocessed common wheat and barley are also usually screened for Zearalenone (ZON) (tolerance is up to 100 ppb). DON and ZON are both included in the Home Grown Cereals Authority (HGCA) Grain Passport.

Methods for detecting mycotoxins are summarized in Table 13.2. Chromatographic methods are frequently used for detecting, quantifying, and confirming the presence of mycotoxins. These methods include thin-layer chromatography, high-performance liquid chromatography, liquid chromatography combined with mass spectrometry, gas chromatography, and gas chromatography combined with mass spectrometry.

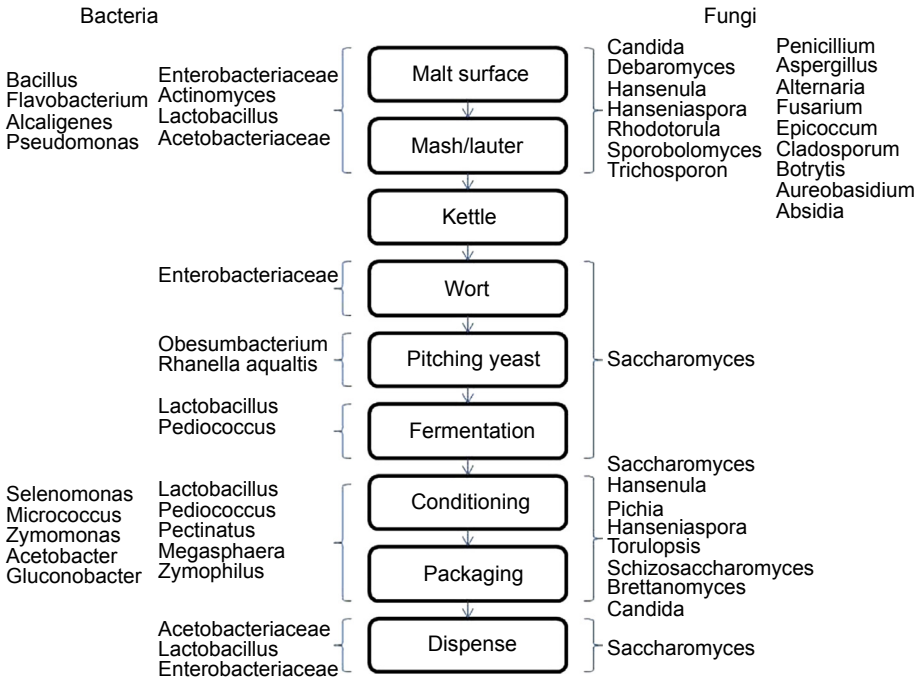

Figure 13.2 Microbial contaminants within the brewing process. Adapted from Bokulich and Bamforth (2013) and Quain and Storgårds (2009).

Table 13.1 Detection media for grain analysis

Growth medium		Incubation conditions
Aspergillus flavus and *parasiticus* Agar (AFPA)	*Aspergillus flavus* and *Aspergillus parasiticus*	28 °C 7 days (morphological analysis), 10 days (toxin production)
Czapek Peptone Yeast Extract Agar (CZPYA)	Actinomycetes	
Czapek-Dox Iprodione Dichloran Agar (CZID)	*Fusarium* species	
Dichloran Chloramphenicol Peptone Agar (DCPA)	*Fusarium* species	25 °C 4 days (initial examination), 6 days (morphological analysis)
Dichloran Glycerol Agar (DG18)	*Fusarium* species	
Dichloran-Rose Bengal-Chlortetracycline Agar (DRBC)	*A. flavus* and *A. parasiticus*	
Pentachloronitrobenzene Peptone Agar (PPA)	*Fusarium* species	
Potato Dextrose Agar (PDA)	Fungi	

Source: Hocking and Pitt (1980), Mostafa, Barakat, and El-Shanawany (2005), Thrane (1996).

Table 13.2 Methods for detection of mycotoxins

Mycotoxin(s)	Method(s)
Aflatoxins	TLC, HPLC, ELISA, immunoaffinity column
Deoxynivalenol	GC, HPLC, ELISA, immunoaffinity column
Fumonisins	HPLC, ELISA, immunoaffinity column
Moniliformin	HPLC
Ochratoxin	TLC, HPLC, ELISA, immunoaffinity column
Zearalenone	TLC, HPLC, ELISA, immunoaffinity column

Source: Mirocha and Christensen (1986).

Various detection methods, such as fluorescence, ultraviolet absorption, and others have been combined with chromatographic methods. New methods based on the production of antibodies specific for individual mycotoxins have also been developed and include enzyme-linked immunosorbent assays and immunoaffinity columns. These methods allow for specific and precise detection and quantification of specific mycotoxins. This has led to the development of test kits for mycotoxins, such as VICAM®, which are rapid and simple to use and can be used in the field and throughout the processing stages.

13.1.1.2 Water

As the main ingredient of beer and a utility in the production process, water quality is central to brewing. Algae, protozoa, fungi, yeasts, and bacteria may all be present in water, but fortunately very few waterborne microbes are able to cause serious problems to brewers. Typically, water from boreholes contains fewer micro-microorganisms than surface water, that is, rivers, ponds, and tanks, and public water supplies are of course rigorously tested. Microbiological tests on water predominantly involve detection of an indicator organism. The primary fecal indicator organism is *Escherichia coli* which is abundantly common and has similar survival qualities to *Salmonella*. Water used for human consumption can have no more than one positive sample (>1 coliform/100 ml) in 40 samples tested in a month and the concentration of fecal coliforms must be zero. It is good practice to monitor nonpublic supplies (borehole, spring, etc.) seasonally.

Brewing water should be tested before entering the hot liquor tank, or any mashing vessels. Similarly, water for dilution should be tested prior to use. Clean-in-place (CIP) and rinse water should be checked every cycle. Generally, however, most supplies are checked weekly or upon encountering unstable wort. Sampling points should be uniformly distributed throughout a piped distribution system and the number of sampling points should be proportional to the number of links or branches. The points chosen should generally yield samples that are representative of the system as a whole and of its main components.

The traditional method for detection of waterborne microbes is direct plating. Samples may also be filtered either on- or off-line and filters placed directly on the surface of an agar plate. A range of media for the detection of coliforms is available (Table 13.3) and confirmation of thermotolerant *E. coli* is possible by incubation at 44 °C.

Table 13.3 Microbiological media for water analysis

Medium	Target microorganism(s)	Incubation conditions
Azide	Intestinal enterococci	40–48 h at 36 ± 2 °C
Bismuth Sulfite NPS	*Salmonella typhi* and other *salmonellae*	40–48 h at 36 ± 2 °C
Chromocult NPS	Total coliforms and *Escherichia coli*	20–28 h at 36 ± 2 °C
ECD NPS	*E. coli*	16–18 h at 44 ± 2 °C
ENDO NPS	*E. coli* and coliform bacteria	18–24 h at 36 ± 2 °C
Heterotrophic plate count		
LMC broth	Coliforms and *E. coli*	1–2 days at 30–35 °C
Lysine	Wild yeast	3–5 days at 30–35 °C
MacConkey	Coliform bacteria and other enterobacteriaceae	18–72 h at 30–35 °C
Malt extract	Yeasts and molds	3–5 days at 20–25 °C or at 30–35 °C depending on the target of the investigation
Meat extract-peptone	Total count	<5 days at 30–35 °C
mFC	*E. coli* and fecal coliform bacteria	18–24 h at 36 ± 2 °C
MLGA (membrane Lactose Glucuronide Agar)	Coliforms and *E. coli*	30 °C for 4 h, then 37 °C for 14 h
R2A	Heterophilic organisms	>5 days at 30–35 °C
Rainbow agar	*E. coli*	18 h 37 °C
Sabouraud	Yeast and molds	<5 days at 20–25 °C
Schaufus Pottinger (m green yeast and mold)	Yeast and molds	2–5 days at 20–25 °C or at 30–35 °C depending on the target of the investigation
Soybean-Casein Digest medium (Caso)	Total count	Bacteria: <3 days at 30–35 °C Yeasts and molds: <5 days at 30–35 °C
Standard TTC	Total count	<5 days at 30–35 °C
Teepol (Lauryl Sulfate medium)	*E. coli* and fecal coliform bacteria	18–24 h at 36 ± 2 °C
Tergitol TTC NPS	Coliform bacteria and *E. coli*	18–24 h at 36 ± 2 °C
Tryptone glucose extract	Total count	<5 days at 30–35 °C
Wallerstein (WL nutrient)	Microbiological flora of brewing and fermentation processes	2–5 days at 30–35 °C aerobic or anaerobic depending on the target of the investigation
Wort	Yeast and molds	3–5 days at 20–25 °C or at 30–35 °C depending on the target of the investigation
Yeast extract	Aerobic bacteria	44 ± 4 h at 36 ± 2 °C 68 ± 4 h at 22 ± 2 °C

Techniques available to rapidly detect bacteria include fluorescence microscopic methods (e.g., Epifluorescence microscopy using acridine), detection of specific metabolites, antibody methods, and DNA-based methods. However, many of these methods are expensive, require an enrichment step, sophisticated equipment, and expertise, and/or are not suitable for routine analysis. The determination of ATP with a bioluminescence assay has emerged as the main method for rapid detection of viable bacteria in breweries. Despite ATP being considered a robust monitoring parameter for microbial drinking water quality, a significant increase in ATP should be accompanied by methods for detection of specific bacteria in order to validate whether or not contamination has occurred. Therefore, the best approach for monitoring microbial drinking water quality, in order to enhance water security and safety, is to combine rapid methods with methods targeted for specific bacterial detection (Vang, Corfitzen, Smith, & Albrechtsen, 2014).

13.1.1.3 Yeast

Of all the raw materials, the most likely source of contamination is from yeast because it is added after wort boiling. Yeast handling plants also tend to be very complex and difficult to clean (Briggs, Brookes, Stevens, & Boulton, 2004). Acid washing can be used to reduce or remove bacterial contaminants but this process does not remove wild yeast. The most common contaminants are the lactic acid bacteria (LAB) Lactobacilli and Pediococci and the microbiological media employed to check pitching yeast reflects their nutritional requirements. Yeast should be checked before pitching (preferably 2–4 days prior to brewing) and monthly checks should also be carried out to test for nonbrewing strains that may populate over time. Tolerance is less than 10 cfu/ml for bacteria and zero tolerance of wild yeast.

Media employed in traditional plate checks typically include inhibitors and/or stimulators (Table 13.4). Such chemicals might include lysine, a nitrogen source that brewing yeast cannot utilize but wild yeast can; copper sulfate, which is also inhibitory to strains of *Saccharomyces*; or plates containing Actidione, which selectively promotes bacterial growth. Such techniques typically require a two-day 25° incubation period (EBC Analytica Microbiologica: Part II Continued, 1984).

When attempting to identify particularly hard-to-culture LAB strains, Suzuki (2011) maintains that "advanced beer-spoiling detection" media is the quickest and most effective media, primarily because of the low pH levels which it employs. When seeking to identify LAB, anaerobic incubation at around 28 °C is typically employed (Suzuki, 2011). Supplementing MRS with catalase can potentially speed up growth of LAB (Deng et al., 2014).

For breweries using flow cytometry to determine yeast count and viability, it is possible to extend use of this method to detect beer spoilers such as *Zygosaccharomyces*, *Dekkera* (*Brettanomyces*), and *Lactobacillus* (Bouix & Leveau, 1999; Donhauser, Eger, Hubl, Schmidt, & Winnewisser, 1993; Jespersen, Lassen, & Jakobsen, 1993). The principle of flow cytometry is based on fluorescence staining or labeling and the cells are brought in a fluid stream within a thin capillary where the fluorescence molecules are excited by a laser and the emission is detected. The laser is also used to count the particles and determine the size. All data are collected and a report is generated with the result of live/dead cells or detection of beer spoilers.

Table 13.4 Microbiological media for pitching yeast analysis

Medium	Target microorganism(s)	Incubation conditions
$CuSO_4$	Wild yeast	48 h, 25 °C
Lysine	Enteric, acetic and lactic bacteria, wild yeast	48 h, 25 °C
MacConkey + Actidione	Enteric bacteria	48 h, 25 °C
MRS (de Man, Rogosa, Sharpe)	Enteric, acetic and lactic bacteria, wild yeast	48 h, 25 °C
MYGP (yeast extract glucose peptone)	Wild yeast	48–72 h at 37 °C; lager strains will not grow
NBB®-Broth	Lactic acid bacteria	72 h, 25–30 °C; aerobic and anaerobic
Nutrient Agar/Broth	General purpose medium for bacteria although many yeasts will grow	48 h, 25 °C
WLN (Wallerstein Laboratory Differential)	General purpose medium for bacteria	48 h, 25 °C
Yeast Morphology Agar	Assessment of yeast colony morphology	48 h, 25 °C

13.1.1.4 Hops

The antibacterial properties of hops are one of the main reasons for their use in brewing. Hops are dried down to 8–10% moisture to prevent spoilage but nonetheless remain susceptible and, as with other raw materials, checks should be made for each batch as a matter of quality control. A number of breweries carry out dry hopping post-brewhouse which increases the risk of introducing contaminants. For hops the most likely microbes are fungi, molds, and mildew and the tolerance is less than 10 cfu/g, with zero tolerance of wild yeast.

For traditional plating, a weighed sample is rinsed in sterile water and the rinse water is either mixed with molten agar or spread on the surface of an agar plate. Media for the detection of common spoilage organisms is given in Table 13.5.

A range of molecular based techniques are available to detect major fungal crop pathogens, including PCR and DNA microarray methods.

13.1.1.5 Sugars and syrups

Noncereal adjuncts and priming sugars are commonly employed in brewing. Irrespective of the point of addition, any materials added to the process should be checked as a matter of quality control. For sugars and syrups the low water potential prevents growth of contaminants, but the main threat is survival of spores from *Bacillus* species. A range of media is available to selectively culture *Bacillus* species (Table 13.6). A sugar solution may be mixed with molten agar or spread directly on the surface of an agar plate.

Table 13.5 Microbiological media for hops analysis

Medium	Target microorganism(s)	Incubation conditions
Lysine	"Wild" yeast	3–5 days at 30–35 °C
Malt Extract Agar	Fungi, e.g., *Podosphaera castagnei*, mold, mildew	48 h, 25 °C
Sabouraud	Yeast and molds	5 days at 20–25 °C
Schaufus Pottinger	Yeast and molds	2–5 days at 20–25 °C or at 30–35 °C
WLD (Wallerstein Differential Broth)	Flora of brewing and fermentation processes	2–5 days at 30–35 °C; aerobic or anaerobic
Wort agar	Yeast and molds	3–5 days at 20–25 °C or at 30–35 °C

Table 13.6 Microbiological media for analysis of syrups and sugars

Medium	Target microorganism(s)	Incubation conditions
BACARA®: chromogenic media	*Bacillus* species	48–72 h at 25–30 °C. Combined anaerobic (3 days) followed by aerobic (2 days)
Bacillus Differentiation Agar	Differentiation between *Bacillus cereus (colorless)* and *Bacillus subtilis (yellow)*	35–37 °C for 18–24 h
Malt Extract Agar (MXA)	All microorganisms	22–25 °C for 3 days
MYP AGAR	*B. cereus*	30 °C. 24 h incubation

Rapid methods of analysis tend not to be used for detection of contaminants in sugars and syrups due to the difficulties in extracting genetic material from the complex medium, but PCR primers for *Bacillus* species are readily available.

13.1.2 Brewing process

As we move toward fermentation it is essential that the integrity of the system is maintained and that all raw materials and adjuncts are appropriately stored and transferred under sterile conditions. The first challenge for the microbiologist is to ensure that all vessels and pipework are tested.

13.1.2.1 Brewery surfaces

As the beer progresses through the brewery it comes into contact with vessel surfaces, pipes, and fittings, all of which can harbor infection, particularly on the cold side of the brewery after wort clarification. Any surface that comes into contact with wort, beer, or yeast should be thoroughly cleaned and sterilized, that is, vessels, piping,

Table 13.7 Brewery ATP testing schedule

Location	Sample type	Frequency	Target organism(s)
Hot liquor tank	Liquid water sample Final rinse CIP	Weekly Each CIP	All microorganisms
Cereal cooker	Swab Liquid (CIP final rinse)	Each use/ cleaning cycle	All microorganisms
Mash tun	Swab Liquid (CIP final rinse)	Each use/ cleaning cycle	All microorganisms
Lauter tun	Swab Liquid (CIP final rinse)	Each use/ cleaning cycle	All microorganisms
Mash filter	Liquid (CIP final rinse)	Each use/ cleaning cycle	All microorganisms
Kettle	Swab Liquid (CIP final rinse)	Each use/ cleaning cycle	All microorganisms
Paraflow/chiller	Liquid (CIP final rinse) Liquid (wort)	Each use/ cleaning cycle Each batch	All microorganisms

and implements. Soiled surfaces can support a microbiological growth which can be introduced into the beer. Any recurrent contamination may indicate the presence of a biofilm. Biofilms are particularly difficult to clean as they can bind strongly to the vessel or pipe.

Almost ubiquitously, ATP tests are employed to check plant hygiene. This includes both swab tests of vessels and pipework and liquid tests of CIP final rinse water. Typical sample locations and types are shown in Table 13.7.

Secondary testing (either traditional plating or rapid methods) tends to only be used if ATP levels are consistently breaching tolerance limits and/or are not reduced by additional cleaning. Malt extract agar or similar may be used for the detection of acetic and lactic bacteria and wild yeast.

13.1.2.2 Air and process gases

Microorganisms are ever-present in the air, often in association with dust particles or airborne moisture droplets. They can also be introduced to the environment by insects and other pests. Every effort must be made to keep the brewing environment as clean as possible and to minimize the ingress of outside contamination. Wherever possible all vessels should be covered to reduce the risk of aerial contamination.

Process gases such as oxygen used during pitching and carbon dioxide applied during packaging may also provide a route for contaminants to enter the system. Any hosing should be inspected for leaks and all gases and air checked. The method involves either exposing an agar plate to the air for a set period of time or filtration of the air through a sterile filter which is then placed on the surface of an agar plate. Typically a nonspecific medium is used, such as malt extract agar, and incubated for 48 h at 25 °C.

Table 13.8 Microbiological media for wort analysis

Medium	Target microorganism(s)	Incubation conditions
Carr's Bromocresol Green Medium	Gram-negative bacteria	27 °C for 1 day
Hsu's *Lactobacillus*/*Pediococcus* Medium (HLP)	*Lactobacillus*, *Pediococcus*	25 °C for 2 days
Lee's Multi Differential Agar (LMDA)/Schwarz Differential Agar (SDA)	Lactic acid bacteria	25 °C for 2 days
Lin's Cupric Sulfate Medium (LCSM)	Wild yeast	25 °C for 1–3 days
Lins Wild Yeast Media (LWYM)	Wild yeast	25 °C for 1–3 days
Malt Extract Agar (MXA)	All microorganisms	22–25 °C for 3 days
MRS	Enteric, acetic, and lactic bacteria, wild yeast	25 °C for 2 days
MRS + Actidone	Enteric, acetic, and lactic bacteria	25 °C for 2 days
Sabouraud Dextrose Agar	Yeast	22–25 °C for 3 days

13.1.2.3 Wort

As the temperature falls following wort boiling, any bacteria or wild yeast present on surfaces will multiply in the nutrient rich medium. Problems can also arise if wort is saved and allowed to stand (such as with weak wort recycling). Contaminated wort can result in a decreased fermentation rate, off-flavors/odors, and haze and therefore should be checked at each batch. Enterobacteria are the most common contaminants and the tolerance is less than 10 cfu/ml, with zero tolerance of wild yeast.

Following boiling the likely prevalence of spoilage organisms is low and the wort will therefore need to be filtered through a sterile filter and placed on an agar plate. Typical media are given in Table 13.8.

It is from this stage forward that rapid methods of analysis are increasingly likely to be used, simply due to the increased cost of spoilage as we move toward final product. Such methods are described in Chapter 15, but a faster "traditional" method includes the microcolony method which employs microscopy to detect growing cells that have not yet reached visibly discernible colonies. Several systems are available, including Rapid Micro Biosystem's Growth Direct™ test, which uses digital imaging technology to automatically enumerate microcolonies. The system captures the native fluorescence (autofluorescence) that is emitted by all living cells. More advanced systems use 96-well microplate formats and automated plate handling systems. The μFinder Inspection System (Asahi Breweries, Tokyo, Japan) involves trapping cells onto 0.4 μm pore-size polycarbonate membrane filters followed by anaerobic incubation on Advanced Beer Detection (ABD) agar medium at 25 °C. The incubated filters are then soaked with carboxyfluorescein diacetate (CFDA) staining buffer for 30 min at 30 °C. Flourescent stained cells may be discriminated from other

Table 13.9 Microbiological media for fermentation analysis

Medium	Target microorganism(s)	Incubation conditions
Hsu's *Lactobacillus*/*Pediococcus* Medium (HLP)	*Lactobacillus*, *Pediococcus*	25 °C for 2 days
Lee's Multi Differential Agar (LMDA)/Schwarz Differential Agar (SDA)	Lactic acid bacteria	25 °C for 2 days
Lin's Cupric Sulfate Medium (LCSM)	Wild yeast	25 °C for 1–3 days
Lins Wild Yeast Media (LWYM)	Wild yeast	25 °C for 1–3 days
Malt Extract Agar (MXA)	All microorganisms	22–25 °C for 3 days
MRS	Enteric, acetic, and lactic bacteria, wild yeast	25 °C for 2 days
MRS + Actidone	Enteric, acetic, and lactic bacteria	25 °C for 2 days

particles based on their morphological characteristics and fluorescence intensities. In general, the microcolony—CFDA method lacks the selectivity for spoilage strains and largely depends on the selectivity of media used for microcolony formation. However, it discriminates beer-spoilage strains from nonspoilage strains upon detection of microcolonies on ABD and enables the intraspecies differentiation of beer spoilage ability of LAB species, such as *L. brevis*, *L. lindneri*, and *L. paracollinoides* (Asano et al., 2009).

13.1.2.4 Fermentation

Fermentation conditions are ideal for bacterial growth and contamination can retard or extend fermentation and cause off-flavors and odors. Typically specific gravity, pH, and flavor are checked while brewing and microbiological analysis is only carried out if issues arise during fermentation. Lactic and acetic bacteria and wild yeast are the main threats with a tolerance of less than 10 cfu/ml and zero count, respectively. Media for their detection is given in Table 13.9.

13.1.3 Product

Once we reach the final stages of the brewing process the sample volume is very low in relation to the batch volume (typically 250 ml from 1000 hl) and levels of beer-spoiling microbes are extremely low. Filtration is therefore needed to improve the likelihood of detecting contaminants, and this can be carried out either in- or off-line.

13.1.3.1 Bright beer

Lactic and acetic bacteria present in bright beer cause vinegary, sour astringent off-flavor and odor, excessive gassing, and strong head retention. Every batch should be

Table 13.10 Microbiological media for bright beer analysis

Medium	Target microorganism(s)	Incubation conditions
Beer agar	All microorganisms	22–25 °C for 3 days
Malt Extract Agar (MXA)	All microorganisms	22–25 °C for 3 days
MRS	Enteric, acetic, and lactic bacteria, wild yeast	25 °C for 2 days
MRS + Actidone	Enteric, acetic, and lactic bacteria	25 °C for 2 days
NBB®-Broth	Lactic acid bacteria, *Pectinatus* and *Megasphaera*	25–30 °C for 3 days; aerobic and anaerobic
Raka-Ray	Lactic acid bacteria, *Pectinatus* and *Megasphaera*	30 °C for 3 days; anaerobic

tested and the tolerance is less than 10 cfu/ml. Commonly used media for analysis of bright beer are given in Table 13.10.

The complex nature of beer results in difficulties in sensitivity/interference for many rapid methods; however, a number of kits are available and discussed in Chapter 15.

13.1.3.2 Packaging

The packaging process exposes bright beer to a range of new surfaces from the buffer tank through the filling machine and the eventual container. The potential for secondary contamination is high, with this stage representing one of the most common points for entry of spoilage organisms, and as such strict attention should be paid to both hygienic design and cleaning regimes. Process quality control parameters should include turbidity (haze), dissolved oxygen, CO_2 content, original extract or alcohol, and the presence of acetic and lactic acid bacteria. A range of bacteria and yeasts may be present in biofilms, such as *Pseudomonas* and *Enterobacteria* species, *Rhodotorula* and *Cryptococcus*, and molds including *Geotrichum* and *Aureobasidium* (Back, 2005). However, the most significant spoilage organisms for brewers are *Pectinatus* and *Megasphaera*; improvements in packaging to reduce oxygen in the headspace of bottled beers has led to an increase in incidence of anaerobic beer spoilage bacteria, which are able to survive within filler heads and CO_2 recovery systems (Paradh, Mitchell, & Hill, 2011).

As with other brewing surfaces, ATP testing is the most common method for detection of microbial activity within the packaging area. Media used for traditional plating tests are also identical to those described for bright beer, with Raka Ray and NBB the most common media used within the UK brewing industry (Paradh et al., 2011).

13.1.3.3 Dispense

At the dispense stage product security is usually outside of the control of the brewery. The most common spoilage organisms are acetic and lactic acid bacteria, which

Table 13.11 Media for the detection of dispense spoilage organisms

Medium	Target microorganism(s)	Incubation conditions
MRS	Enteric, acetic, and lactic bacteria, wild yeast	25 °C for 2 days
Rainbow Agar	Enteric, acetic, and lactic bacteria, *E. coli, Salmonella, Shigella*, and *Aeromonas*	18 h 37 °C
WLN (Wallerstein Laboratory Differential)	Enteric, acetic, and lactic bacteria, yeast	2–5 days at 30–35 °C; aerobic or anaerobic depending on the target of the investigation

cause surface film, haze, and vinegary flavor/odor. Wild yeasts may also proliferate in dispense lines where biofilms are a common hazard. Cask dispensing introduces oxygen into beer, adding further risk, and therefore line cleaning should be carried out at least biweekly. Tolerance is less than 10 cfu bacteria and 0 cfu wild yeast.

As with packaging, ATP testing is commonly employed. Detection media for traditional plating are detailed in Table 13.11.

13.2 Identification of brewing spoilage organisms

Once a colony has been isolated from any of the stages above the next step is to identify it. Identification is necessary to determine whether or not it represents a risk in terms of spoilage. Preliminary identification of many of the microbes of significance in brewing has traditionally been made on the basis of the following few simple characteristics of the cells:

- growth requirements,
- ability to grow under aerobic or anaerobic conditions,
- colonial characteristics and cell morphology (Figure 13.3), and
- gram reaction (for bacterial colonies).

Further identification is made on the basis of biochemical properties such as

- ability to produce enzymes that can be detected by simple tests,
- ability to metabolize sugars oxidatively or fermentatively, and
- ability to use a range of substrates for growth, for example, glucose, lactose, and sucrose.

These tests can be carried out individually, for example, in broth media containing the specifically required nutrients and/or reagents, but they are more commonly performed using commercial kits or automated systems which have the potential to give a rapid identification based on biochemical profiles.

Bergey's Manual of Determinative Bacteriology is the standard reference for laboratory identification of bacteria. Dichotomous keys, which incorporate information

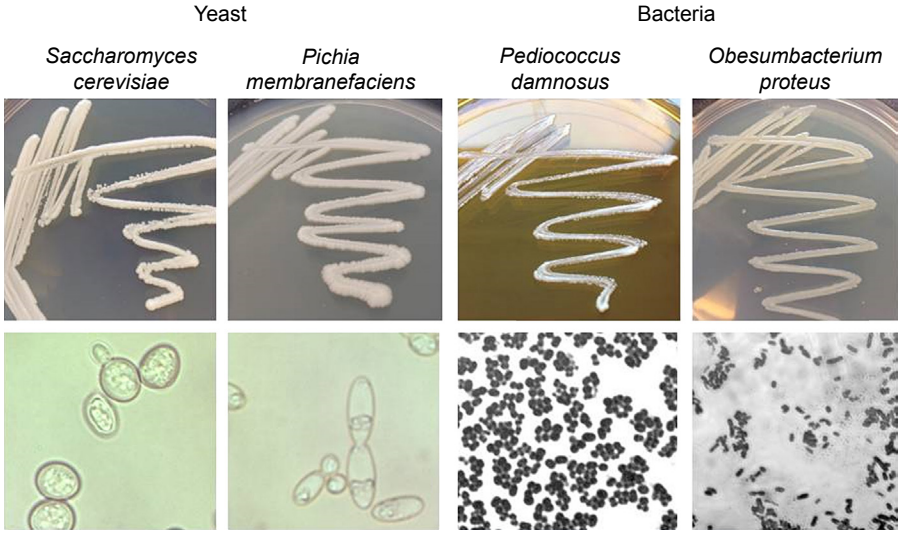

Figure 13.3 Colony and cell morphology of common brewery yeast and bacteria.

from a variety of identification methods, are also commonly used for the identification of organisms. Dichotomous keys for brewing fungi and bacteria are given in Figure 13.4.

13.3 Summary

The combination of hygienic plant design, effective CIP, and quality assurance of raw materials represents a sensible strategy for minimizing the risk of microbial contamination during the brewing process. However, "reactive" testing throughout is also essential in quality control. Plate counting and enrichment remain the principal methods for detection of microbes in breweries during the brewing process and in final product analysis. The wide range of media and methods available may seem overwhelming, but there are common media and tests that may be used for several/all stages, making effective control and maintenance reachable for all regardless of budget.

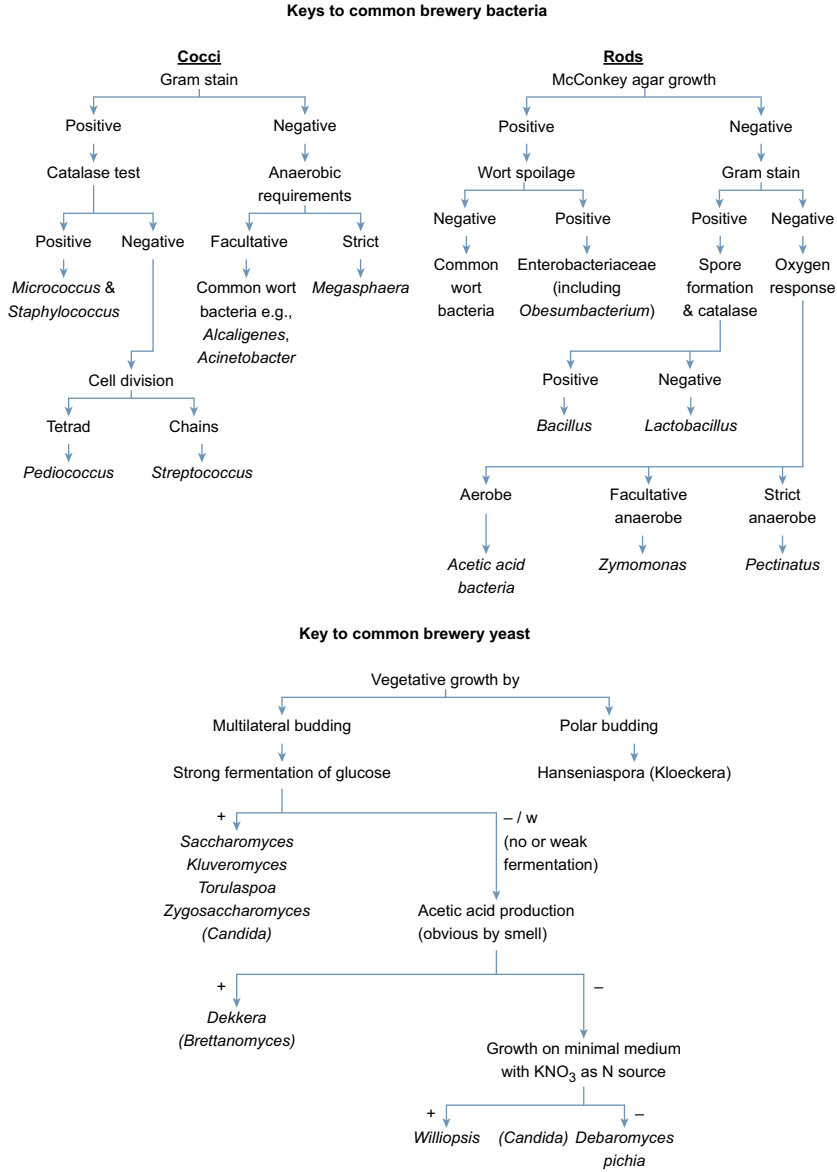

Figure 13.4 Dichotomous keys for identification of brewing spoilage microbes.

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Rapid detection and identification of spoilage bacteria in beer

14

J. Siegrist[1], *M. Kohlstock*[2], *K. Merx*[2], *K. Vetter*[2]
[1]Sigma-Aldrich, Buchs, Switzerland; [2]Scanbec, Bitterfeld-Wolfen, Germany

14.1 Introduction

Traditional methods in microbiology are primarily culture based, and it often takes several days to detect, identify and confirm organisms. In the case of the brewing industry, the problem is that the range of spoilage organisms is extensive, and often only a very low concentration is present. This requires the use of many different classical tests and at minimum, a culture enrichment step. Additionally, in a brewery, large batches are fermented and must be properly stored somewhere in a blocked stock or they will spoil and eventually end up being recalled. Therefore, the rapid release of a guaranteed high quality new batch of beer saves a lot of money and prevents getting a bad reputation, as well as avoiding an expensive recall. The main drivers for the trend to rapid methods are:

1. Increased awareness of product quality and safety
2. Increased competition and declining beer consumption
3. More regulations
4. New products: trends towards nonpasteurized beer, low or nonalcohol beers and alcopops (sweetened beer mixtures)
5. Availability of improved technology
6. Time and cost savings.

In recent years, diverse rapid methods have been publicized and there are several systems in use today. For some applications and organisms, optimized methods are available which can detect bacteria within 8 h and most of them can do it within 24 h. Some rapid methods are even able to detect several organisms in one step.

Adenosine triphosphate (ATP) measurement, the direct epifluorescence filter technique (DEFT), *in situ* hybridization systems, polymerase chain reaction (PCR) methods and MALDI-TOF (matrix-assisted laser desorption ionization time of flight mass spectrometry) mass spectroscopy are some of the rapid methods used to detect beer spoiling bacteria and are likely to become the standard quality control tools of the future. For hygiene monitoring, the ATPase test is already widely used but there are also other methods such as protein detection or the oxidoreductase test. DEFT is used for total viable cell counts and to determine the viability of cells. With the antibody and oligo DEFT, a modified version of the method is provided which can be used for specific detection of pathogens or beer spoiling organisms. PCR technology has developed significantly in recent years, resulting

Brewing Microbiology. http://dx.doi.org/10.1016/B978-1-78242-331-7.00014-9

in diverse methods for various applications. With the *in situ* hybridization, a molecular biology system was found which does not need PCR. It is more robust and detects no dead cells as it uses rRNA as the target. In recent years microbiologists have also discovered MALDI-TOF for the identification of microorganisms since a pure colony can be identified within a few minutes.

14.2 Hygiene tests (ATP bioluminescence, oxidoreductase)

ATP (adenosine triphosphate) is the stored form of energy in microorganisms and is central to many biochemical reactions in the metabolism of organisms. Therefore it is an excellent indicator for the presence of living microorganisms (Webster, Walker, Ford, & Leach, 1988). It is used in applications where hygiene control is needed, such as food and beverage production and cooking areas. The ATP test is mainly used to check equipment, surface and material sanitation or the effectiveness of treatments from such material or fermenters with biocides and detergents. In the brewing industry the ATP test is a good indicator to have some evidence that fermenters and used equipment are presumably free of any spoiling organisms and the cleaning process was successful.

Today's systems are in most cases based on the bioluminescence with ATP and luciferase from the firefly. As an alternative system it is also possible to use a colour test: nicotinamide adenine dinucleotides (NAD/NADH) and nicotinamide adenine dinucleotide phosphates (NADP/NADPH), which are also compounds used for the energy transfer in the metabolism in living cells or compounds found in food debris.

14.2.1 Principle of test

14.2.1.1 ATP test

The ATP test is based on the presence of ATP from living cells, which delivers the energy needed for the reaction catalysed by firefly luciferase. In the presence of luciferase, luciferin and ATP react to form luciferyl adenylate and phosphate. Then the luciferyl adenylate and oxygen react, forming oxyluciferin and AMP (adenosine monophosphate). The oxyluciferin is formed in an electronically excited state and therefore a photon is released, which is visible by emitting a yellow-green light. The oxyluciferin returns back to the ground state (luciferin) (Rhodes & McElroy, 1958). This reaction is called bioluminescence and can be seen in fireflies and some other creatures. Light is a positive reaction for living organisms, including bacteria, and is therefore a sign of insufficient cleaning. The sensitivity is stated as 10^{-16} ATP per litre (Anand, 2004) (Figure 14.1).

14.2.1.2 Oxidoreductase tests

If NAD(P) and/or NAD(P)H is present in the sample, glucose dehydrogenase converts β-D-Glucose into D-gluconolactone, then diaphorase converts a tetrazolium salt into a coloured formazan salt (HY-RiSE system from Merck). Any colour development on the test strip indicates a positive result (not clean).

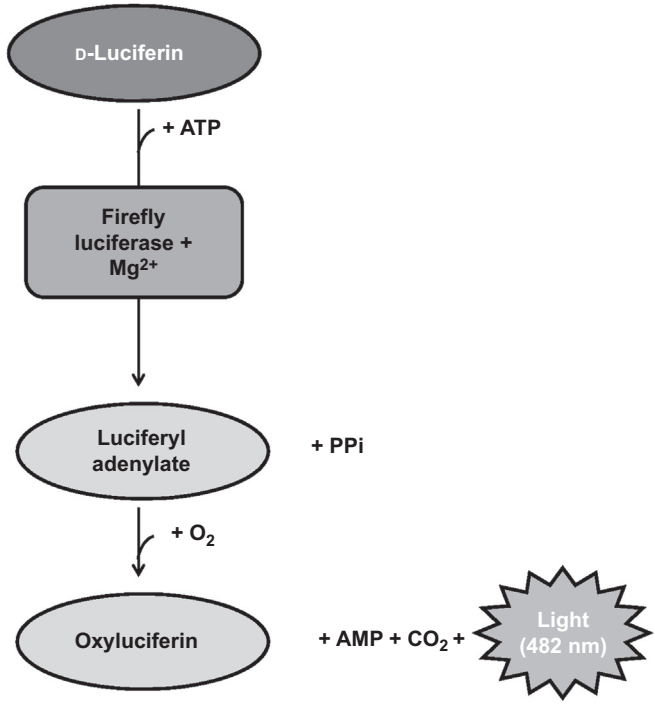

Figure 14.1 ATP Luminescence reaction with firefly luciferase.

It is also possible to use a method with horseradish peroxidase and NAD(P) and/or NAD(P)H. The result of the reaction is the production of H_2O_2 (hydrogen peroxide), which can be detected with the addition of luminol. The luminol reacts with H_2O_2, resulting in bioluminescence (Anand, 2004).

14.2.2 Test systems

14.2.2.1 ATP tests

There are a number of ATP systems currently available, including UltraSnap (Hygiena), PocketSwab Plus (Charm Sciences), Hy-Lite (VWR) and Clean-Trace (3M). The UltraSnap system contains a premoistened swab in a tube and the reaction reagent in a cap on the top. A certain area of surface is wiped over with the swab. Once the cap is closed the reagent moves down to the swab and the tube is shaken a few times. If there is ATP present on the swab it will react with reagent containing luciferyl adenylate. The result of the positive reaction will be bioluminescence, which can be detected in a luminometer.

14.2.2.2 Oxidoreductase tests

The oxidoreductase test is based on the detection of NAD/NADH and NADP/NADPH.

HY-RiSE (Merck), for example, uses a strip or a card and the result can be directly visible by a colour reaction. The strip is moistened or the surface must already be wet

or some rinse water is added on the strip. Once the strip has been wiped across the test surface, two further reagents are added. After a 5 min incubation in the dark the reaction zone will appear yellow (negative reaction = clean) or pink/purple to bluish (NAD/NADH and/or NADP/NADPH positive = presence of living cells or food debris). The test has been validated and compared with the ATP test and there was no significant difference (Goll, Kratzheller, & Bülte, 2003).

14.3 Direct epifluorescence filter technique

The total viable count is an important parameter in industrial fermentations. Traditional methods for total viable count started with the cultivation method of counting the colonies. Later methylene blue and Ponceau S stain came into use for microscopic examinations, which give a direct count (Kunkee & Neradt, 1974). Today, systems are available with smart filtering and concentration steps and sensitive fluorescence stains, and a result can be obtained in less than 10 min. This method cannot be applied to highly viscous or particulate materials.

It is important not to mix up vitality and viability of yeast cells. The vitality is the condition of the physiological capabilities of the cell, while viability describes if a cell is alive or dead. The viability is reported as a percentage of live cells (so live and dead cells are counted), whereas the vitality gives the status of the metabolic function (Report of Subcommittee, 2003; Van Zandycke, Simal, Gualdoni, & Smart, 2003).

14.3.1 Principle of the method (fluorescence stains, differentiation of live and dead cells)

A homogenized sample is prefiltered through a 12 µm pore filter (Priest & Campbell, 2003) to remove large particles and to avoid blockage in the next filter step (0.2 µm pore black polycarbonate filter) and big numbers of large particles being collected on the filter. The disadvantage is that some organisms do not pass the 5 µm filter and therefore some protocols recommend the use of magnetic particles coated with antibody to fish out the organisms (Boschke, Steingroewer, Ripperger, Klingner, & Bley, 2002). On the 0.2 µm filter the microorganisms are concentrated and remain on the filter. Then the microorganisms are stained with fluorochromes. Acridine orange is used as fluorescence dye in DEFT, which is a nucleic acid intercalator, and stains single-stranded DNA (deoxyribonucleic acids) and RNA (ribonucleic acids) red (650 nm). When acridine orange binds to double-stranded DNA molecules the emitted light is green (526 nm) and so it is possible to differentiate dead and live cells as in dead cells single-stranded nucleic acids are rapidly degraded from active nucleases (Rost, 1995). However, some studies on filtered brewery samples found that differentiation of live and dead cells was not successful, as stained debris was a problem (Barney & Kot, 1992; Kilgour & Day, 1983). A number of other stains are available today as alternatives, for example DAPI, primuline and trypan blue (Table 14.1) (Kregiel & Berlowska, 2009; Life Technologies, 2014; Sigma-Aldrich, 2014a). DAPI, which stains double-stranded DNA in the cell blue (Sigma-Aldrich, 2014a), is currently the standard even though it only colours live cells (Charton, 2006). There are numerous other stains and

Table 14.1 Examples of fluorescence stains from Sigma-Aldrich and Life Technologies

Stain type	Description	Cat. No.	Brand	Optical properties	Application
Damaged cells	Primuline			λ_{ex} 340 nm; λ_{em} 425 nm	Primuline is used for visualization of permeabilized or damaged cells. It binds noncovalently to lipid structures.
	Propidium iodide	P4170	Sigma	λ_{ex} 530 nm; λ_{em} 625 nm	Fluorescent stain for nucleic acids. Cell membrane integrity excludes propidium iodide from staining viable and apoptotic cells. Propidium iodide may be used in flow cytometry to evaluate cell viability when used with other dyes that stain viable cells or cells that are early in the apoptosis process. Propidium iodide is useful for staining dead cells.
	SYTOX® blue nucleic acid cell stain	S11348	Molecular probes	λ_{ex} 470 nm; λ_{em} 480 nm	SYTOX® blue nucleic acid stain is an excellent blue-fluorescent nuclear and chromosome counterstain that is impermeant to live cells, making it a useful indicator of dead cells within a population.
	SYTOX® green nucleic acid stain	S7020	Molecular probes	λ_{ex} 504 nm; λ_{em} 523 nm	SYTOX® green nucleic acid stain is an excellent green-fluorescent nuclear and chromosome counterstain that is impermeant to live cells, making it a useful indicator of dead cells within a population.
	SYTOX® orange nucleic acid stain	S11368	Molecular probes	λ_{ex} 532 nm; λ_{em} 547 nm	SYTOX orange dye stains nucleic acids in cells with compromised membranes. This stain is useful as an indicator of cell death.
	SYTOX® red dead cell stain	S34859	Molecular probes	λ_{ex} 640 nm; λ_{em} 658 nm	SYTOX® red dead cell stain is a simple and quantitative single-step dead cell indicator.
	Trypan blue solution	93595	Fluka	λ_{ex} 488 nm; λ_{em} 675 nm	Trypan blue is a blue acid dye that contains two azo chromophores. It is a large, hydrophilic, tetrasulfonated dye. Trypan blue solution may be used in trypan blue-based cytotoxitiy and proliferation assays. It is a vital stain that is not absorbed by healthy viable cells. When cells are damaged or dead, trypan blue can enter the cell, allowing dead cells to be counted. When trypan blue binds to proteins the resulting complex emits red fluorescence. The method is sometimes referred to as the dye exclusion method.

Continued

Table 14.1 Continued

Stain type	Description	Cat. No.	Brand	Optical properties	Application
Viable & damaged cells	Acridine orange solution	A9231	Sigma	λ_{ex} 500 nm; λ_{em} 526 nm (bound to DNA); λ_{ex} 460 nm; λ_{em} 650 nm (bound to RNA)	DNA intercalating dye. Suitable for quantitative analysis. Differentially stains double-stranded and single-stranded nucleic acids.
	FUN® 1 cell stain	F-7030	Molecular probes	λ_{ex} 480 nm; λ_{em} 560–610 nm (live cells); 510–560 nm (dead cells)	The FUN® 1 stain passively diffuses into a variety of cell types and initially stains the cytoplasm with a diffusely distributed green fluorescence. However, in several common species of yeast and fungi, subsequent processing of the dye by live cells results in the formation of distinct vacuolar structures with compact forms that exhibit a striking red fluorescence, accompanied by a reduction in the green cytoplasmic fluorescence. Formation of the intravacuolar structures requires both plasma membrane integrity and metabolic capability. Dead cells fluoresce bright yellow-green, with no discernable red structures.
Viable cells	Bisbenzimide H 33258	14530	Sigma	λ_{ex} 338 nm; λ_{em} 505 nm (pH 7.0); λ_{ex} 355 nm; λ_{em} 465 nm in TE buffer; DNA	Useful reagent for cytogenetic studies; for the fluorescent staining of DNA in cells; it is membrane-permeable and selectively binds to adenine–thymine regions in the minor groove of B-form DNA with a high binding constant, making it a useful stain for DNA, chromosomes and nuclei. The properties of this dye make it useful in several applications, such as sorting living cells based on DNA content. The methodology of the assay is based on Hoechst 33258 binding to DNA and is sensitive for a thousand cells.

	Calcofluor white stain	18909	Fluka	λ_{ex} 355 nm; λ_{em} 433 nm; 0.1 M phosphate pH 7.0	A fluorescent stain for rapid detection of yeasts. Calcofluor white is a nonspecific fluorochrom that binds to cellucose and chitin in cell walls.
	DAPI	32670	Sigma	λ_{ex} 374 nm; λ_{em} 461 nm in 10 mM Tris, 1 mM EDTA, pH 8.0 (DAPI–DNA complex)	DAPI is several times more sensitive than ethidium bromide for staining DNA in agarose gels. It may be used for photofootprinting of DNA to detect annealed probes in blotting applications by specifically visualizing the double-stranded complex and to study the changes in DNA and analyse DNA content during apoptosis using flow cytometry. Cell permeable fluorescent minor groove-binding probe for DNA. Binds to the minor groove of double-stranded DNA (preferentially to AT-rich DNA), forming a stable complex which fluoresces approximately 20 times greater than DAPI alone.
	Ethidium bromide solution	46067	Sigma	λ_{ex} 480 nm; λ_{em} 620 nm in H_2O; λ_{ex} 518 nm; λ_{em} 605 nm (bound to DNA); λ_{ex} 530 nm; λ_{em} 600 nm in 50 mM phosphate buffer pH 7.0 (upon binding to DNA)	The fluorescence of EtBr increases 21-fold upon binding to double-stranded RNA and 25-fold on binding double-stranded DNA so that destaining the background is not necessary with a low stain concentration (10 µg/mL). Ethidium bromide has been used in a number of fluorimetric assays for nucleic acids. It has been shown to bind to single-stranded DNA (although not as strongly) and triple-stranded DNA.

Continued

Table 14.1 Continued

Stain type	Description	Cat. No.	Brand	Optical properties	Application
	Hoechst 33258 solution	94403	Sigma	λ_{ex} 355 nm; λ_{em} 465 nm in TE buffer; DNA	These bisbenzimide dyes are blue fluorescence dyes used to stain dsDNA.
	Hoechst 33342	14533	Sigma	λ_{ex} 355 nm; λ_{em} 465 nm in TE buffer; DNA	
	Hoechst 34580	63493	Sigma	λ_{ex} 357 nm; λ_{em} 490 ± 10 nm in H_2O (free dye)	
	Nancy-520	01494	Sigma	λ_{ex} 520 nm; λ_{em} 554 nm in TE buffer; DNA	Nancy-520 is a fluorescent stain for dsDNA with higher sensitivity than ethidium bromide and an easy, fast and robust staining procedure. It can be used to determine dsDNA concentrations in solution, with a linear range between 0 and 2 µg/mL of DNA.
	SYBR® green I	S-7563	Invitrogen	λ_{ex} 497 nm; λ_{em} 520 nm (DNA–dye complex)	SYBR® green I is an asymmetrical cyanine dye used as a nucleic acid stain.

possible combinations of stains for marking different cell organelles and to differentiate live and dead cells.

Following staining, the membrane filter must be examined under a fluorescence microscope and live cells counted to give the total viable count. It is possible to manually perform the counting of the live/dead cells, but for lower numbers of cells and big filters the chance of errors increases. A semiautomated image analyser or an automated computer-assisted microscopic scanning system could be an interesting option. Sorcerer Image Analysis System (Perceptive Instruments) is an example for semiautomatic enumeration by image analysis; the fluorescing cells are viewed in real time by a high sensitivity CCD video camera and viable and nonviable cells readily distinguished by virtue of contrast differences. A completely automated DEFT instrument called COBRA has a high sample throughput, improved reproducibility and lower count limits (Pettipher, Watts, Langford, & Kroll, 1992; Priest & Campbell, 2003, p. 284). Today it would also be an alternative to use flow cytometry where the automatization is easy and the instrument can easily differentiate between debris and yeast and also between live and dead cells (Breeuwer, Drocourt, Rombouts, & Abee, 1994; Bruetschy, Laurent, & Jacquet, 1994). It is even possible to distinguish between stressed and nonstressed yeasts (Edwards, Porter, & West, 1997; Prudêncio, Sansonetty, & Côrte-Real, 1998). Additionally, the detection of beer spoilers such as *Zygosaccharomyces*, *Dekkera* (*Brettanomyces*) and *Lactobacillus* is possible (Bouix, Grabowski, Charpentier, Leveau, & Duteurtre, 1999; Donhauser, Eger, Hubl, Schmidt, & Winnewisser, 1993; Jespersen, Lassen, & Jakobsen, 1993). The principle of flow cytometry is also based on fluorescence staining or labelling. The cells are brought in a fluid stream passing a thin capillary where the fluorescence molecules are excited by a laser and the emission is detected. The laser is also used to count the particles and determine the size. All data are collected and software analyses the data to generate a report with the result of live/dead cells or detection of beer spoilers. A number of other parameters can also be determined (Brown & Wittwer, 2000).

14.4 Antibody-direct epifluorescent filter technique

A drawback of the direct epifluorescent filter technique (DEFT) is the inability to detect specific pathogens. The use of special dyes allows only a universal distinction between viable and nonviable cells (Bamforth, 2006), but an extension of the method by the specific detection of microbes is a great advantage. It can help to discover the origin of contaminations during the brewing process (Dodd, Stewart, & Waites, 1990) and enables distinction between different lactic acid bacteria, such as several spoiling species of *Lactobacillus*, and those innocuous or necessary for brewing (Priest & Stewart, 2006, pp. 607–627). The antibody-DEFT (Ab-DEFT) combining membrane filtration and pathogen-specific fluorescent antibodies can enumerate spoilage bacteria in food and beverages but with the loss of the evaluation of viability (Tortorello & Gendel, 1993). As demonstrated for *Listeria monocytogenes* and *Escherichia coli* in beef, milk and apple juice, Ab-DEFT correlates well with conventional plate counts and MPN procedures but could be performed in less than 1 h (Tortorello & Gendel,

1993; Tortorello, Reineke, & Stewart, 1997; Tortorello & Stewart, 1994). A detection limit was reported of approximately 16 cells per gram for ground beef (Raybourne & Tortorello, 2003; Tortorello & Stewart, 1994). A preceding enrichment of the cells, which allows cells to proliferate initially, could significantly improve the sensitivity of the Ab-DEFT method and result in a detection limit of 0.1 CFU/g and only 10 h assay duration (Raybourne & Tortorello, 2003; Restaino, Castillo, Stewart, & Tortorello, 1996). In summary, the Ab-DEFT represents a very rapid and sensitive method.

14.5 Oligonucleotide-direct epifluorescent filter technique

The oligonucleotide-direct epifluorescent filter technique (Oligo-DEFT) represents a further, more sensitive (Uyttendaele & Debevere, 2006) and specific detection method for the direct enumeration of microorganisms. It has been demonstrated for *E. coli* in water, beverages and sprouts that the oligo-DEFT method can achieve a detection limit of 1 CFU/mL (Tortorello & Reineke, 2000). Fluorescent-labelled oligonucleotides complementary to 16S rRNA were combined with DEFT. Because of the high abundance in cells, the ribosomal RNA represents an optimal target for fluorescence microscopy analysis. After the membrane filtration followed by a short 2 h hybridization step, it is possible to distinguish between either species or groups of microorganisms (Raybourne & Tortorello, 2003).

14.6 *In situ* hybridization detection systems

Hybridization is the process by which two complementary strands of nucleic acid bind together by hydrogen bonds to form a single double-stranded complex. By adjusting the temperature and buffers the most energetically preferred complex is built and this special technique used in laboratories is called annealing. The temperature needed for annealing depends on the number of complementary bases, respectively the number of hydrogen bonds which are formed. *In situ* hybridization is used for the detection of specific sequences by using a labelled complementary DNA or RNA strand, called the probe. Since the first *in situ* hybridization experiments in 1969 (Gall & Pardue, 1969), many variations of the method have been developed. Results include improved sensitivity and specificity and also different ways of detection and working procedures. The best known *in situ* hybridization procedures are fluorescent probes to detect DNA sequences, also called FISH (fluorescence *in situ* hybridization) (O'Connor, 2008). Modern methods using RNA as the target nucleotide and new techniques with sandwich hybridization and detection systems with chromogenic reaction or a biochip (electric measurements) are gaining popularity (Femino, Fay, Fogarty, & Singer, 1998; Pioch et al., 2008; Raj, van den Bogaard, Rifkin, van Oudenaarden, & Tyagi, 2008; Rautio et al., 2003). Radioactive labels have been used, but because of stability and safety issues they are no longer employed (Rudkin & Stollar, 1977). Compared to

PCR the *in situ* system has no problem with inhibitory effects from the beer matrix as no polymerase enzyme is needed.

14.6.1 Probes

The range of probes is wide. The success of this technology relies on finding probes which are highly specific and have an excellent hybridization rate. Specificity is achieved by targeting conserved or unique rRNA sequences. A probe is normally composed of an oligonucleotide with about 20 nucleotides (Kempf, Trebesius, & Autenrieth, 2000), but some tests are carried out with peptide nucleic acid, which can have some advantages as the molecule is more stable, specific and sensitive (Almeida, Azevedo, Fernandes, Keevil, & Vieira, 2010). For pathogens and beer spoiling organisms, 16S rRNA, 23S rRNA or respectively the corresponding DNA are usually selected as the target (Almeida et al., 2010; Frischer, Floriani, & Nierzwicki-Bauer, 1996; Fuchs, Syutsubo, Ludwig, & Amann, 2001).

There are a number of different probes available, including detection probes, which are labelled with a fluorescence marker or, for example, digoxigenin for a further linkage with an antibody–enzyme complex and then a later colorimetric reaction with a chromogenic substrate (such as nitro blue tetrazolium for alkaline phophatase) (Helentjaris & McCreery, 1996; Kempf et al., 2000), Capture probes are used to bind the target sequence (RNA or DNA) to a plate or another surface. In most cases the probes are labelled with biotin to react with avidin, which is coated on a plate (Riley, Marshall, & Coleman, 1986).

14.6.2 rRNA as detection target (application, theory to RNA, RNase, live and dead cells, probes, sample preparation, comparison to PCR)

For the detection of bacteria and other organisms it is interesting using rRNA as a detection target as RNA in normal cases only exists in living cells and also in numerous copies (up to several thousands of ribosomes per bacteria (Kaczanowska & Rydén-Aulin, 2007) and for yeast even more, close to 200,000 (Warner, 1999)). PCR methods, for example, also detect DNA of dead cells as double-stranded DNA is quite stable while the single-stranded RNA is decomposed within a few hours from nucleases. Because of the numerous ribosomes in a bacteria or yeast cell there is no need to do a PCR and a direct detection of bacteria or yeasts is possible.

14.6.3 FISH (fluorescent in situ hybridization system)

There are three classical FISH detection kits available on the market called VIT®-beer (Vermicon), which detects the beer spoiling organisms. The protocol of the method takes no more than 3 h and can be directly used for isolates or for beer samples after an enrichment step. One kit detects all members of beer-spoilage lactic acid bacteria (red fluorescence) and additional specific *Lactobacillus brevis* (red and green fluorescence), the most prominent beer spoiling organism. With another kit it is possible to

detect *Megasphaera cerevisiae* and *Pectinatus* spp., two obligate beer spoiling organisms. For the detection of obligate and potentially fermentative spoilage yeasts in beer and beer-based drinks another kit is provided.

The principles of these classical FISH kits are quite easy to understand but the procedure takes a while, because several incubation periods are required. The target for the detection probes is the rRNA of the spoiling organisms. First the cells are fixed on a slide and then the cell membrane has to be made permeable with an enzyme mix for Gram-positive organisms. A drop of reagent containing the fluorescent marked probes is added which can penetrate into the cell. During the incubation at 46 °C the hybridization of rRNA and the probes is performed. Then the slide is washed and afterwards the slides are examined under the fluorescence microscope as fluorescent glowing cells (Thelen, Beimfohr, Bohak, & Back, 2001; Vermicon, 2014).

14.6.4 HybriScan® (a system with colour reaction and in a microplate format)

This relatively new quantitative and qualitative method is a so-called sandwich hybridization. The HybriScan® system (Sigma-Aldrich/Scanbec) is based on the detection of rRNA via hybridization events and specific capture and detection probes. The sandwich hybridization is very sensitive, detecting attomoles of the respective target rRNA molecules. First of all the cell walls are destroyed enzymatically and then the rRNA is centrifuged down, resuspended and used in the assay. The method is highly specific as it uses two probes for the hybridization: capture probes, which are used to immobilize the bacteria on the microplate (streptavidin coated), and detection probes, which are used for the detection reaction. The capture probe is biotin-labelled while the detection probe is digoxigenin-labelled. After the hybridization at 50 °C the probes and the targets are fixed on the microplate. To the detection probe a horseradish peroxidase is linked by building an anti-DIG-horseradish peroxidase Fab fragment. Then a washing step follows and the bound complex is visualized by horseradish peroxidase substrate TMB (3,3′,5,5′-tetramethylbenzidine). The photometric data are measured at 450 nm and compared with standard solutions, respectively their calibration curve. The measured data and the calibration curve can be used for the calculation of CFU (colony forming units) if no enrichment step was used, but it is also possible just to detect, for example, the beer spoiling organisms (Figure 14.2).

The HybriScan®***D*** Beer kit detects all beer spoiling bacteria of the genera *Lactobacillus*, *Pediococcus*, *Pectinatus* and *Megasphaera*. The sensitivity is 1–10 CFU/L after 24–30 h pre-enrichment in NBB broth, or isolates can directly be used.

The HybriScan®***D*** Yeast kit is used for the detection of yeasts in filterable, nonalcoholic drinks. The specificity covers yeasts including the genera *Zygosaccharomyces*, *Saccharomyces*, *Candida*, *Dekkera*, *Torulaspora* and *Pichia*. For direct detection and quantification at least 500 CFU/mL is recommended; after an enrichment step detection of 1–10 CFU/L is possible.

The test can be performed in 2–2.5 h and as it is in a micro titre plate it is quite economical and can be automated. The work flow is very similar to the ELISA test (Sigma-Aldrich, 2014b; Taskila, Tuomola, Kronlöf, & Neubauer, 2010).

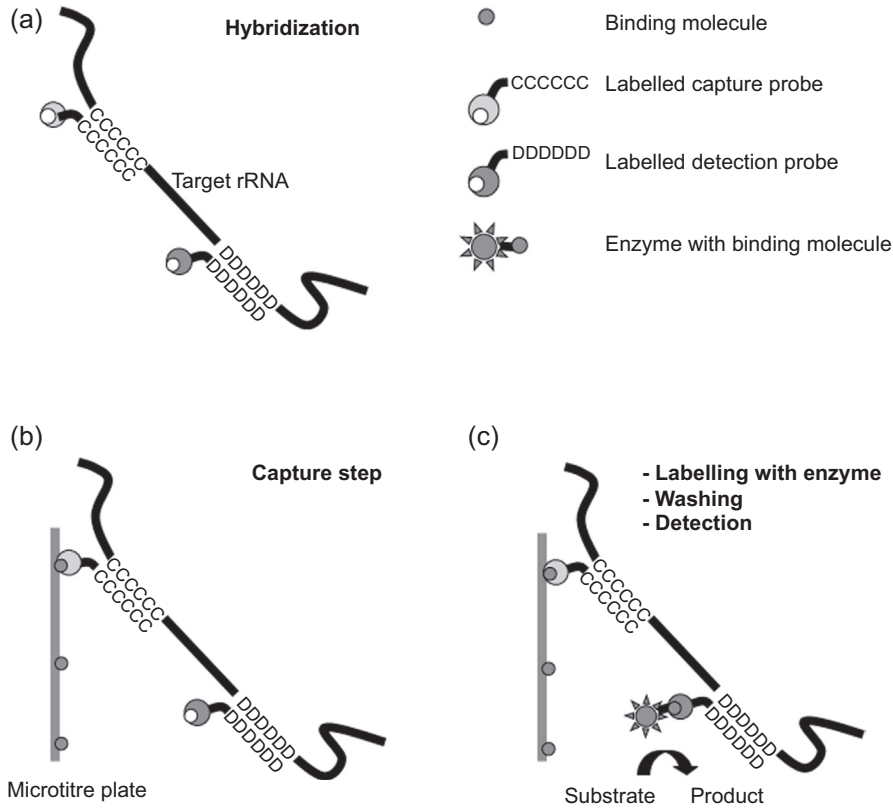

Figure 14.2 Principle of the sandwich hybridization assay.

14.7 Polymerase chain reaction

The polymerase chain reaction (PCR) is a process used to make a large copy number of a specific DNA fragment from genetic material (DNA) in a relatively short time. In 1993, the American Biochemist Kary Mullis was awarded the Nobel Prize for the development of the PCR (Malmström & Andersson, 2013). The new PCR process constituted a major breakthrough because it solved the problem of how to produce multiple copies of any particular piece of DNA using a relatively simple, economical and reliable procedure. Today, all DNA-containing target samples can be analysed by PCR. The applications range from forensic samples, fossil, archaeology and analysis of metabolic pathways to the identification of plants and animals, trace research, as well as identification and classification of microorganisms (Hutzler, Schuster, & Stettner, 2008). The identification and phylogenetic classification of bacteria is carried out today by analysis of the rrn operon, especially the 16S rDNA gene and the 16S–23S spacer region. Thus it has been the most widely used target for developing PCR tests for beer-spoilage bacteria in various taxonomic ranks (Juvonen, 2009, p. 273).

14.7.1 Basic principle

A few basic components are needed to perform a PCR. First, two specific oligonucleotides (15–25 nt), called primers, are needed. They are derived from both strands of the target sequence, thus they determine the size and specificity of the resulting PCR product. The other components are a thermo-stable DNA polymerase, deoxyribonucleotides and a defined reaction buffer, which contains magnesium ions as cofactor and estimates the optimal reaction conditions for the polymerization of DNA (Saiki et al., 1988).

PCR is a cycle reaction and is carried out in a thermocycler. Each cycle consists of three steps. In the first step, denaturation, the initial DNA and primers are heated at 95 °C and denatured into single strands. In the second step, called annealing, the primers hybridize to their opposite sequence at both DNA strands. The annealing temperature is usually 3–5 °C below the melting temperature of the primer. If the selected annealing temperature chosen is too low the primers may also bind at positions which are not 100% complementary and thus lead to nonspecific products. If the temperature is too high the primers do not bind or bind incompletely and no product, or only a small amount, is formed.

During the third step, the elongation, the new DNA strands are synthesized from the 5′-end to the 3′-end by the polymerase. The elongation temperature depends on the working optimum of the DNA polymerase (68–72 °C). The new DNA fragment, which results from the steps 1 to 3 is further multiplied in the following cycles. Ideally, each newly emerging DNA segment is duplicated in 20–50 cycles (Saiki, 1990). Since the original description of PCR as a method to amplify DNA a number of variations of the technologies have been described (Figure 14.3).

14.7.2 Endpoint PCR

Based on the PCR product detection, PCR can be categorized into endpoint PCR and time-point, called real-time PCR. In endpoint PCR the ready PCR product is visualized by agarose or polyacrylamide gel electrophoresis followed by staining with fluorescent dyes, for instance ethidium bromide or SYBR Green I.

Several primer sets have also been designed for the group specific detection of lactic acid bacteria in brewery samples, in wine, in food or in the gut (Heilig et al., 2002; Lopez et al., 2003; Neeley, Phister, & Mills, 2005; Renouf et al., 2006; Stewart & Dowhanick, 1996; Walter et al., 2001). Endpoint PCR may be carried out in 6–7 h (Juvonen, Koivula, & Haikara, 2008) (Tables 14.2 and 14.3). The presence of *L. brevis*, *Lactoballicus lindneri* and *Pediococcus damnosus* in beer samples (50 mL) was detected after 30–40 h incubation in NBB-C broth (Bischoff, Bohak, Back, & Leibhard, 2001). To reliably achieve the detection limit of one cell per sample, the beer samples were precultivated in SMMP medium for 3–7 days. To minimize PCR inhibition, the SMMP enrichments were refreshed in a 1:1 mixture of beer and PYF broth prior to DNA isolation. DNA was isolated from 0.1–1 mL of liquid samples using a commercial DNA extraction kit (InstaGene Matrix kit BioRad, Hercules, CA, USA), which is suitable for DNA extraction from Gram-positive and Gram-negative beer spoilers (Juvonen et al., 1999, 2008).

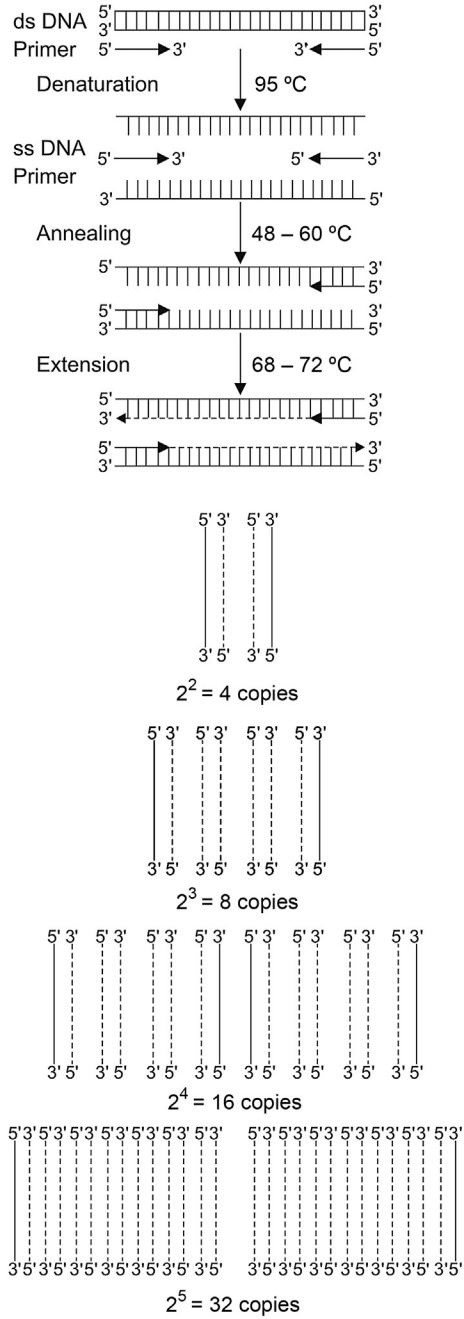

Figure 14.3 Basic principle of a polymerase chain reaction.

Table 14.2 **DNA amplification techniques for other beer-spoilage bacteria**

Target organism	Target gene	Product	Technique	Application	PrePCR processing	Assay sensitivity and time	Comments	References
Beer-spoilage lactobacilli	*horA*	342	EP-PCR	Pure culture	Enzymatic lysis, CTAB and chloroform extractions, acetate and alcohol precipitations	1×10^5 CFU/mL	Good correlation with beer-spoilage ability	Sami, Yamashita, Kadokura, Yoda, and Yamasaki (1997)
Eubacteria 16S–23S spacer	Varies	EP-PCR and RFLP	Beer (30–40 h enrichment), centrifugation, Chelex-100 and proteinase K, Triton X-100, heating	1×10^3 CFU/50 mL	30–40 h enrichment, centrifugation, Chelex-100 and proteinase K, Triton X-100, heating	1×10^3 CFU/50 mL 1 CFU in bottle (enrichment)	Identification by 9 RFLP analysis	Bischoff et al. (2001)
Obesumbacterium proteus biotype II	16S rRNA	435	EP-PCR	Yeast and slurry	Centrifugation ($2500 \times g$), Promega DNA extraction kit	1×10^4 cells/mL 1×10^7 cells/mL		Maugueret and Walker (2002)
Obesumbacterium proteus biotype I	16S rRNA	422 481	EP-PCR and qPCR	Beer yeast slurry	Filtration through PCM, membrane dissolution in chloroform, cell recovery in water, heating, centrifugation, heating	2×10^2–2×10^3 CFU 100/mL 2×10^3–2×10^4 CFU per 2×10^8 yeast cells		Koivula et al. (2006)
Beer-spoilage LAB	*horB*, *horC*		qPCR	Pure culture	As in Report of subcommittee (2003)	5×10^1 CFU/mL	49/51 spoilers had *horB*, *horC*	Suzuki, Iijima, Ozaki, and Yamashita (2005)

Beer-spoilage LAB	*horA*	543	qPCR	Pure culture	As in Report of subcommittee (2003)	1×10^2 CFU/mL		Suzuki, Sami, Iijima, Ozaki, and Yamashita (2006)
Beer-spoilage LAB	*horA*	198	qPCR	Pure culture	As in Report of subcommittee (2003)	1×10^2–2×10^2 CFU 100/mL		Haakensen et al. (2007)
Beer-spoilage LAB	*hitA* *horA* *horC* *ORF5* 16S rRNA	179 210 98 117 148	Multiplex EP-PCR	Pure culture	As in Prienst and Campbell (2003)	No data	Only *horA* predicts beer-spoilage ability	Haakensen, Schubert, et al. (2008)
Firmicutes	16S rRNA		Multiplex qPCR	Beer	Filtration through Durapore enrichment in MRS, DNA isolation using Puregene	5×10^1–1×10^2 CFU/100 mL (*P. damnosus*) 3–10 CFU/341 mL (with enrichment)	Multiplexed with universal eubacterial primers	Haakensen, Dobson, et al. (2008)
Lactobacillus brevis *Lactobacillus casei* *Lactobacillus coryniformis* *Lactobacillus lindneri* *Lactobacillus paracollinoides* *Lactobacillus plantarum*	16S rRNA	861 854 453 850 729 490	Multiplex EP-PCR	Pure culture		1×10^3 CFU/mL	Multiplexed with universal eubacterial primers	Asano et al. (2008)

Symbols: NaOH, sodium hydroxide; qPCR, real-time PCR; SDS, sodium dodecyl sulphate (Juvonen, 2009, p. 273).

Table 14.3 **DNA amplification techniques for *Sporomusa* sub-branch beer-spoilage bacteria**

Target organism	Target gene	Product	Technique	Application	PrePCR processing	Assay sensitivity and time	Comments	References
Pectinatus	16S rRNA	815	EP-PCR	Beer	Filtration through PCM, enzymatic lysis, phenol-chloroform extraction, ethanol precipitation	10 h: 2×10^3 CFU/100 mL		Satokari, Juvonen, von Wright, and Haikara (1997)
Megasphaera cerevisiae *Pectinatus* DSM 20764 *Pectinatus cerevisiiphilus* *Pectinatus frisingensis*	16S rRNA	385 393 443 74	EP-PCR	Pure culture	No data	No data		Sakamoto, Funahashi, Yamashita, and Masakazu (1997)
P. cerevisiiphilus *P. frisingensis*	16S rRNA and 23 S spacer region	600 1000 1200	EP-PCR	Pure culture	Enzymatic lysis, CTAB treatment, phenol–chloroform extraction, acetate	No data		Motoyama and Ogata (2000b)
Pectinatus	16S rRNA	NA	LAMP	Pure culture	Prepman Ultra sample preparation reagent	No data	Detection by real-time turbidimeter	Tsuchiya et al. (2007)
P. cerevisiiphilus *P. frisingensis*	16S rRNA and 23 S spacer region	621 701 + 883	Multiplex EP-PCR	Pure culture	Prepman Ultra sample preparation reagent	No data	Artificial positive control DNA	Asano et al. (2008)

M. cerevisiae *Pediococcus claussenii* *Pediococcus damnosus* *Pediococcus inopinatus*	**16S rRNA**	452 462 566 566	Multiplex EP-PCR	Pure culture	Prepman Ultra sample preparation reagent	1×10^2 CFU/mL	Artificial positive control DNA	Asano et al. (2008)
P. cerevisiiphilus *Pectinatus haikarae* *P. frisingensis*	16S rRNA and 16S–23S spacer	621 508 701 + 993	EP-PCR	Pure culture	Prepman Ultra sample preparation reagent	No data	Modified from Kunkee and Neradt (1974)	Iijima, Asano, Suzuki, Ogata, and Kitagawa (2008)
M. cerevisiae *Megasphaera paucivorans/ Megasphaera sueciensis*	16S rRNA	452 155	Multiplex EP-PCR	Pure culture	Prepman Ultra sample preparation reagent	No data	Modified from Kunkee and Neradt (1974)	Iijima et al. (2008)
Pectinatus, Megasphaera, Selenomonas *Zymophilus*	16S rRNA	342	EP-PCR qPCR	Pure culture Spiked beer Real beer samples		EP-PCR 6–7 h, real-time PCR 2–3 h 100–10^3 CFU per 25 mL of beer		Juvonen et al. (2008)

Symbols: NA, not applicable; EP-PCR, endpoint PCR with agarose gel electrophoresis; LAMP, loop-mediated isothermal amplification; PCM, polycarbonate membrane; CTAB, cetyltrimethylammonium bromide.

14.7.3 Real-time PCR

The development and application of fluorescent dyes, which are incorporated into the PCR product, and fluorochromes for labelling of oligonucleotids opened the possibility for real-time monitoring of the product formation cycle by cycle. The fluorescence signal increases in proportion to the amount of amplicon. Dyes, such as ethidium bromide and SYBR Green I, that intercalate into the DNA are the simplest way to follow at real time the increase of amplicon. The disadvantage of this method is that distinguishing between different PCR products is not possible.

The other possibility for monitoring of PCR product formation offers the application of fluorescent labelled probes, which emit their signal with the incorporation into the PCR product. Using Förster resonance energy transfer (FRET) probes, the FRET between a donor and an acceptor molecule is exploited. The donor fluorochrome is stimulated by a light source and transfers energy to the acceptor fluorochrome that emits a fluorescent signal, which is detected. Therefore two additional oligonucleotides have to be designed, which contain the adjacent donor and acceptor fluorophore. The FRET signal of the acceptor increases only with the incorporation of the probes into the PCR product. Then the distance between the donor and acceptor only amounts to 1–10 Å. This method provides a high specificity but is very expensive.

The FRET principle is applied in different labelled probe systems. TaqMan probes belong to the dual-labelled probes at 5′-end a quencher and at 3′-end a fluorescence molecule. The quencher inhibits the fluorescence signal. During the PCR Taq-polymerase synthesizes the DNA strands from 5′-end to the 3′-end and the labelled probe is incorporated into the PCR product. In addition to the polymerization activity the Taq-polymerase contains a 5′-3′-exonuclease activity to hydrolyse the quencher from the opposite DNA strand. Thus, the fluorophore and the quencher remove from each other and the rising fluorescence signal can be measured.

Another opportunity of the FRET principle is the application of molecular beacon probes. In such a probe the sequences 5′-end and 3′-end form a stem loop, which is labelled with a reporter fluorophore and quencher. With incorporation of the probe into amplicon, the stem loop opens, the distance between the reporter and the quencher increases and the reporter molecule emits the fluorescence signal that is measured. The possibility to visualize the rise of amplicon, in contrast to the endpoint PCR, was a welcome progress. This expanded the role of PCR from that of a pure research tool to that of a versatile technology, permitting the development of routine diagnostic applications for the high- and low-throughput clinical microbiology laboratory (Mackay, 2004).

For the quantification of the initial DNA by real-time PCR a reference gene is usually included in the measurement to perform a relative amount comparison (relative quantification). In the first phase of the product amplification the template amount is limited, because the probability that the template, primer and polymerase meet is suboptimal. When enough amplicon is present, the assay's exponential progress can be monitored as the rate of amplification enters a linear phase (LP). The beginning of the exponential phase, where the fluorescence significantly increases above the background fluorescence, is called the Ct value (cycle threshold) or the Cp (crossing point) value used to describe the cycle. As primers and enzyme become limiting, and product formation has an inhibitory effect to the PCR and is overly competitive to oligoprobe hybridization

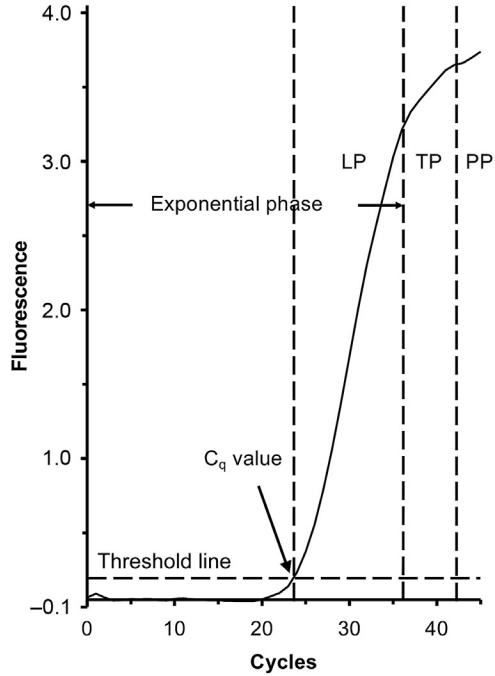

Figure 14.4 Kinetic analysis of a PCR reaction. PCR product amount is measured by fluorescence. If enough amplicon is present the rate of amplification and the increase of fluorescence is linear (LP). Under limiting conditions, product formation is in the transition phase (TP) and eventually reaching a plateau phase (PP) where there is little or no increase in fluorescence.

accumulate, the reaction slows, entering a transition phase (TP) and eventually reaching a plateau phase (PP) where there is little or no increase in fluorescence. The relative quantification is calculated by comparison with the amplification signal of an internal amplification standard (reference gene) over the Ct value (Figure 14.4).

An absolute quantification is more demanding and states the exact number of nucleic acid targets in the sample with respect to a particular unit. Absolute quantification may be necessary when there is a lack of sequential specimens to demonstrate a relative change in microbial load or when no suitably standardized reference reagent is available (Mackay, 2004).

The efficiency (E) of a PCR assay is calculated by the gradient (m) of a standard curve. For that purpose cDNA dilutions (e.g. 100%, 10%, 1%, 0.1%) are used as templates for the graphical structure. A linear regression line through the curve has the gradient $-m$ (when plotted with increasing DNA concentration):

$$E = 10^{-1/m} - 1 \tag{14.1}$$

A gradient of $-3.32m$ would thus mean an efficiency of 1 (100%) indicating a doubling of the amplicon per cycle, a gradient of −3.58 and an efficiency of 0.9 (90%). The formula provides meaningful values that are 100% smaller then gradient value

–3.32 (Higuchi, Fockler, Dollinger, & Watson, 1993). The efficacy of PCR is determined by its efficiency, fidelity and specificity, which are in turn influenced by many factors including target length and sequence and primer design.

14.7.4 Automation

Today PCR and the quantification of PCR product are performed by software of the Lightcycler®. The first real-time PCR instrument was launched in 1996. A large selection of systems is currently available (Espy et al., 2006). They are normally composed of a fluorescence measuring thermocycler, a computer and software for operation and data analysis. A LightCycler® was the first instrument based on rapid cycle PCR. It has the capability to run 30 cycles in 10–15 min (Wittwer et al., 1997). Options for multichannel analyzer (MCA) and 3–4 fluorescence channels are standard features in modern instruments. The configuration with 384-well blocks essentially enables a low-density array setup. Nanoplate systems accommodate up to 3072 reactions in a device with the size of a standard microscope slide (Brenan, Roberts, & Hurley, 2009). Downscaling of a PCR thermocycler on a microchip shortened the run time to a few minutes (Juvonen, 2009, p. 273; Pipper, Zhang, Neuzil, & Hsieh, 2008).

The aim of real-time PCR was to detect and identify exactly the spoiling microorganisms and reduce the analysis time, as compared to traditional cultivation methods. Molecular biological detection only works in combination with a quick and easy nucleic acid extraction method and the precultivation method in order to achieve the detection limit of the PCR. The detection limit for a PCR should lie in a range from 1 to 10 viable cells per sample, which can contain 10^6–10^9 yeast cells. Using a Lightcycler system the total procedure takes 1–2 days, including precultivation and DNA extraction (Kiehne, Grönewald, Chevalier, 2005).

In a brewery approximately 25–30 samples are analysed every day in a beer-screening test by real-time PCR for the detection of *Lactobacilli*, *Pectinatus*, *Megasphaera* and *Pediococci* (Hutzler et al., 2008).

Another platform for automation is the GeneDisc® Rapid Microbiology System (Pall System, 2012). This system consists of two components: the DNA extractor and the Gene Disc Plate. The GeneDisc DNA Extractor is used to prepare samples for analysis, based on four simple steps (filtration, sonication, heating and DNA purification). The Gene Disc Plate is a two-part molded device with the same diameter as a DVD and enables the detection of a range of microorganisms simultaneously within the same sample DNA extract. The Cycler performs gene amplification in the plate, and each plate can be used to test either six, nine or 12 samples in parallel. When all subunits are in use, the Cycler can analyse up to 96 samples in an hour. All known beer spoiling bacteria species and genera can be analysed by the system.

14.7.5 Primer design

The specificity of the PCR depends on the quality of the primers. The sequences of primers are derived from the comparison of target sequences from various beer contaminating bacteria. These sequences are available in databases such as the

Ribosomal Database Project (http://rdp.cme.msu.edu/) or the Basic Local Alignment Search Tool (BLAST). These sequences have to be compared with each other by creating a sequence alignment. The alignment shows common and different sequence regions between the individual beer spoiling bacteria. Common regions are applied to the design of group specific primers. Species specific primers are derived from different regions. The sequences should comprise 20–25 nt in order to obtain primers with a melting temperature of 50–60 °C. The melting temperature of the primers can be predicted according to the formula by Marmur and Doty (1962):

$$\mathrm{Tm} = 4 \times \mathrm{GC} + 2 \times \mathrm{AT} \tag{14.2}$$

Today, primers can also be designed by computer programmes, such as Primer-BLAST (Ye et al., 2012). The potential primer sequences are tested in the database, which includes all known sequences, on their specificity.

14.7.6 Multiplex PCR

A number of modifications of the original PCR basic reaction conditions and technique have been developed to enhance the efficacy. In a multiplex PCR different primer pairs are used to amplify one or more genes in one reaction as well as one gene from different marker organisms. It is made more complicated by the development time, since the designed primers have to be adjusted with respect to the melting temperature, and the sequences have to be compared among each other to prevent dimerization of the primers. These interactions would reduce the sensitivity of the multiplex PCR and must therefore be excluded (Devlin, 2010, p. 269). Beer-spoilage lactic acid bacteria, *Pectinatus* spp. and *Megasphaera* spp. can be detected by multiplex PCR (Asano et al., 2008; Haakensen et al., 2007; Haakensen, Dobson, Deneer, & Ziola, 2008; Haakensen, Schubert, & Ziola, 2008).

14.7.7 Nested PCR

Nested PCR is well suited when only very small amounts of the target DNA are present in comparison to the total sample amount of DNA. Then two PCR are performed consecutively. The formula provides PCR the target gene is amplified beside unwanted sequence regions as a result of nonspecific binding of the primer. The resulting amplicon is used as a template for a second round of PCR with other primers that bind within the first target region and generate a product with very high specificity. Since the DNA region of choice is amplified a second time, it produces sufficient DNA for further procedures (Busch, 2010, p. 113). Maugueret and Walker (2002) and Koivula, Juvonen, Haikara, and Suihko (2006) developed a nested PCR based on primers, which were developed by the 16S rDNA for the detection of *Obesumbacterium proteus* Biotype I and II in beer, wort and yeast slurry. The detection limit varied in the individual matrices between 10^2 and 10^7cells/sample.

14.7.8 Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) is a one-step amplification reaction that amplifies a target DNA sequence with high sensitivity and specificity under isothermal conditions (about 65 °C). The reaction takes place in three steps: the initial step, a cycling amplification step and an elongation step by a DNA polymerase with strand displacement activity. For the amplification a set of two outer and two inner primers are required, which are derived from six regions of the target sequence. It provides high amplification efficiency, with DNA being amplified 10^9–10^{10} times in 15–60 min. The increase reaction product can be monitored by turbity measurement (Mori & Notomi, 2009). The method can also be combined with an RT-PCR. It should be able to amplify a few target copies and be less sensitive to nontarget DNA than PCR. A LAMP-based application for the identification of *L. brevis*, *L. lindneri*, *P. damnosus* and *Pectinatus* from isolated colonies in 1.5 h has been developed (Tsuchiya et al., 2007). The advantage of this technology is that significant investments in equipment are unnecessary.

14.7.9 RT-PCR

Besides the use of DNA as a template for PCR, it is possible to convert RNA by a reverse transcriptase into complementary DNA (cDNA). This RT-PCR followed by PCR or real-time PCR is a powerful technique for the qualitative and quantitative detection of messenger RNA. Bergsveinson, Pittet, and Ziola (2012) investigated the expression level of *horA* and *horC* in *L. brevis* and *Pediococcus clausenii* during growth in beer. A deoxyribonuclease (DNase) pretreatment has been shown to be very effective in eliminating DNA contamination when applied prior to RT-PCR analysis, resolving one of the concerns related to this technique in quantification of ribosomal RNA or prerRNA in living cells.

14.7.10 Differentiation between viable and nonviable cells

A possibility for differentiating between viable and nonviable cells uses ethidium bromide monoazide (EMA), a DNA binding dye that is used for the differentiation of living and dead cells in flow cytometry and PCR. Dead cells have membrane damage; EMA penetrates and binds covalently to the bacterial DNA. This binding inhibits the amplification of the bound DNA so that the polymerase is sterically hindered (Wang & Levin, 2005).

Weber, Sahm, Polen, Wendisch, and Antranikian (2008) published an RT-PCR in combination with an oligonucleotide array for the detection and identification of viable beer-spoilage bacteria. In this study a set of primers for the detection of viable bacteria was designed to target the intergenic spacer regions (ISR) between 16S and 23S rRNA. These results suggest that rRNA content is stable and does not necessarily correlate with growth of bacteria. On the other hand, pre-rRNA is a suitable marker of growing bacterial strains. Therefore, RT-PCR targeting the ISR rRNA is a very effective method for detecting growing bacterial cells. Unfortunately it lacks the evidence that the ISR is also in real beer samples at detectable levels.

14.8 MALDI-TOF mass spectroscopy

MALDI-TOF is a mass spectroscopy method and is an interesting method for the identification of microorganism species. At least 10^3 to 10^6 cells are needed for a determination. The ground principle of MALDI is to look at the mass spectrum, which depends on the protein profile of the cells, and the so-called fingerprints are unique for each microbial species. After the cultivation on an agar plate a colony can be picked and the crude cells or the extracted proteins are spotted on a special slide and covered with a α-cyano-4-hydroxycinnamic acid saturated solution (matrix). Then the drop is dried for a few minutes. Extraction is done by suspending the colony in 80% ethanol followed by a 2 min centrifugation. The pellet is resuspended in 70% formic acid and the same volume of acetonitrile and again centrifuged for 2 minutes. One microlitre of the supernatant is then pipetted to the target and covered with the α-cyano-4-hydroxycinnamic acid saturated solution. When the small droplets are dried the target plate is put into the MALDI where the laser (impulse of 1–5 ns) shots the dried droplets which results in positive loaded proteins fly towards the electrode. Based on the weight and the electrical charge they fly faster or slower and with the detector the time of flight is measured and then converted into a mass (Claydon, Davey, Edwards-Jones, & Gordon, 1996; Holland et al., 1996; Krishnamurthy, Ross, & Rajamani, 1996; Lay, 2001). With a simple sample preparation the profile is measured and a database checked for reference spectrums and makes a ranking of the hits. The identification is very efficient (about 15 min plus the cultivation) but the machines are still very expensive and the access to the reference database is needed. Newer research studies demonstrated that it is possible to differentiate *L. brevis* strains based on their spoilage potential (Kern, Vogel, & Behr, 2014) (Figure 14.5).

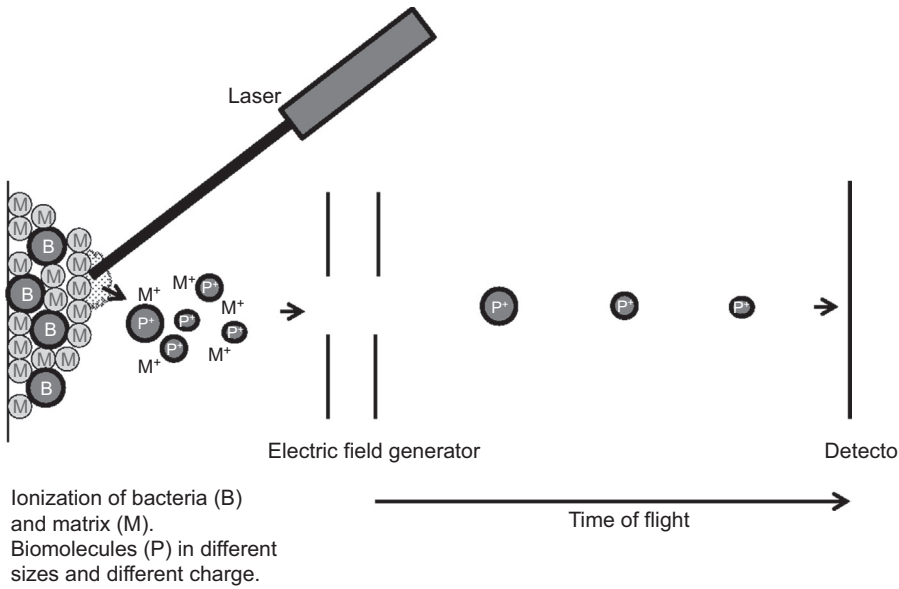

Figure 14.5 Principle of MALDI-TOF mass spectroscopy.

14.9 Conclusions

Modern methods can help breweries save time and increase quality and safety. ATP is established as the standard for hygiene control and is unlikely to be replaced in the near future. DEFT methods may become more automated with flow cytometry, but the investment cost for the machine is still quite high, although the first 'low' cost systems are available on the market. Interesting methods include systems which can analyse all species together in one step (e.g. multiplex PCR or HybriScan®) or tests which can be done within a few minutes (e.g. MALDI-TOF). Additionally, it is beneficial if the system can be automated. For example, the GeneDisc PCR system (Pall System, 2012) can be done with the sample, a disc and a machine, while others have a microplate format which can be used with standard robots (e.g. HybriScan®***D*** beer kit and Tecan robot (Tecan Journal, 1-2014)). In recent years, PCR has also become a second standard and there is now one of the first systems available which can exclude dead cells. The prices have come down for PCR, but it is still expensive and sensitive to matrix. From that perspective, more robust systems such as *in situ* hybridization or MALDI-TOF are very interesting and could also be adopted as future methods. Currently, MALDI-TOF requires a high initial investment for both the machine and a professional database, but it is worth the expense since it is one of the best tools to identify microorganisms as it needs only a pure overnight culture and about 15 min for measurement. Although the modern methods have great advantages over the classic methods, the culture-based methods are still used as they are the only way to guarantee detection of 1 CFU in a sample. All previously mentioned modern systems need at least an enrichment step, but in some cases, this enrichment step can be shortened to between 6 and 8 h (Hayashi et al., 2013; Josefsen, Krause, Hansen, & Hoorfar, 2007).

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Beer packaging: microbiological hazards and considerations

15

R. Hofmann, J. Fischer
VLB Berlin, Berlin, Germany

Beer packaging and, in particular, beer filling may be considered as the process with the highest risk potential for so-called secondary contaminations of the product. This chapter gives an overview on the microbiological hazards that may be present in the filling hall. Certain popular filling systems and container forms show differing hazards and are therefore looked at separately.

With regards to growth conditions in the filling hall, the formation of biofilms as a highly resistant form of microbial cultivation is discussed in detail. Life in a multi-species environment like a biofilm may be a key to understanding how and why even highly specific beer-spoiling microorganisms survive outside of their natural conditions in various parts of the brewery and the packaging hall. With respect to that, possible contamination sources during beer packaging are discussed. Considerations and recommendations for sufficient hygiene and sanitation protocols are given.

15.1 Introduction

Within the processing of beer, the step of filling and packaging can be considered as the last stage at which a contamination of the product is possible. Any further contamination would be due to manipulation of the packaged product or due to a critical malfunction of the packaging material (e.g. loss of sealing property) and shall not be considered further. Generally, the filling hall can be divided into a 'dry section' that includes the palletizing, unpacking, and packing, and into a 'wet section' that usually starts with cleaning/rinsing of the packaging materials and ends with control of the filled container. The possibilities of stabilizing the beer in terms of its microbiological state are subject of Chapter 11 and will not be specifically addressed within this chapter. The literature used for this survey can be found at the end of the chapter.

15.2 Microbiological hazards in the filling hall

Microbiological contamination in the packaging hall can also be referred to as secondary contamination. In contrast to primary contamination during beer production, secondary contamination will usually not be noticeable without specific microbiological analysis methods. The risk potential derives from the type of microorganism that is causing the contamination. Referring to the nomenclature by Back (1994),

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the microorganisms may be divided into several categories from which especially the obligate and the potentially beer-spoiling organisms are crucial for the long-term stability and, therefore, quality of the bottled product.

If microorganisms get into the filled container and are able to reproduce in the specific product, several negative impacts on the beer's quality may arise, for example,

- Increased turbidity
- Formation of floating particles or sedimentation
- Change of flavour (off-flavour)
- Acidification
- Pressure build-up (increasing CO_2 concentration)
- Deformation of the container

Generally, at every stage where the product has contact with any kind of 'new' surface or gets directly exposed to the environment, the risk of contamination is given. In the filling hall, these process stages are limited to the 'wet section'.

This chapter will describe the possible risks according to their sources. In particular, the respective filling and capping equipment as well as the process characteristics are reviewed. The packaging materials and their specific risk potentials are addressed. In addition, the supply with water, air and carbon dioxide together with the microbiological hazards in the direct filler periphery (environment) is listed.

15.2.1 Filling machine

Filling machines are first of all characterized by their container that is to be filled. Furthermore, the number of filling organs, shape of filler (circular or line), container size or filling mechanism can be used for characterization.

The purpose of filling can be described as the filling of the product with as low as possible losses and at the desired level within acceptable tolerances. In addition, the preservation of the products quality has to be secured. Preservation of the product quality includes avoiding oxygen pick-up, loss of carbon dioxide and any contamination.

As beer is a carbonated drink, the filling of beer requires a certain pressure level to avoid degassing and thereby loss of carbonation or foaming during the filling process. The necessary pressure level depends on the CO_2 content of the beer and the product temperature during filling. Filling beer at temperatures higher than room temperature is not practice relevant; usually beer is filled at cold temperatures. The temperature difference may lead to water condensation on the container wall.

Within this chapter, the reference filling system is the most common circular glass bottle filling. Particular differences and characteristics of filling containers other than glass bottles will be discussed in Section 15.2.3.

15.2.1.1 Filler design

A modern filler design directly reflects the possible measures to reduce contamination risks (hygienic design). Some basic requirements shall be mentioned briefly. For more detailed information on hygienic design, please see Chapter 11.

- All surfaces should be suitable for automated CIP and manual cleaning.
- Surfaces with product contact should have an average roughness of ≤1.6 µm (better 0.8).
- Surfaces with product contact should be suitable for sterilization (T ≥ 80 °C).
- Surfaces should be designed to allow easy run off of product residues, cleaning of shards, etc.
- The quality of welding has to be appropriate to the neighbouring surfaces.
- Any kind of niches, gaps or open profiles should be avoided (round, plain surfaces with welded ends preferable).
- Open bores, holes, threads, screws, etc. are to be avoided.
- The floor should be designed in such a way that fluids can easily run off.

Additional measures to minimize the risk of contamination during the filling process will be addressed in Section 15.4.

15.2.1.2 Process steps in the filling machine

Generally, the filling process can be divided into the following steps:

- Transfer of container to the filling valves
- Centreing and pressing on of the container onto the filling valve
- Possible pre-evacuation and rinsing as well as pressurizing with CO_2
- Opening of product inlet
- Filling
- Closing of product inlet
- Resting
- Depressurizing and decoupling of container
- Transfer of filled container to next process step (closing/capping machine)

During the transfer to the filler, the container is usually open. Therefore, the risk is given that microorganisms can enter the container by air or due to aerosol formation. In addition, microorganisms that are attached to construction above the transfer belt (e.g. housing, crossing pipes, etc.) can fall into the open containers. The longer the open containers are exposed to the environment during transfer, the higher the risk of contamination. Especially longer times of stoppage may have negative effects.

During the filling process itself, the container is directly attached to the respective filling valve and can be assumed to be a closed system. Any contamination risk during the filling process arises either from contaminated surfaces within the container and/or product contact or from gas or product flows that are directly involved in the filling process.

The filling is followed by the transfer to the capping or closing equipment where again the container can be considered as highly vulnerable to contamination by spraying water or microorganisms in the direct environment. After the container has been closed, the risk of contamination is limited to technical failures only.

15.2.1.3 Media transport

The product is pumped from the pre-filling process step to the filler through a central media distributor and into its buffer vessel. Usually the product buffer vessel of modern (beer) filling machines has the form of a ring channel. In state-of-the art filling machines,

the ring channel is built as a ring tube to ensure the most suitable cleaning conditions and optimal reduction of mass. The media distributor and the ring channel have to be constructed in such a way that any cross-contamination or mixing of media streams can be avoided. In all modern systems, the whole construction can be included in the CIP system. Nevertheless, dead ends and pockets, valves, broken equipment or rough surfaces may increase the potential of contamination due to the growth of microorganisms.

Here not only the transport pipes for the product have to be considered. The supply of CO_2 and vacuum, as well as the pipes for depressurizing, may carry substantial microorganism loads or get covered with product residues during the filling process. Any product residue always represents a potential risk for growth of beer contaminants. Therefore, it is absolutely necessary to include all media distribution and transfer pipes in the CIP protocols.

15.2.1.4 Filling process

When beer is filled, usually the glass bottles are evacuated after pressing on onto the filling valve. In some cases, an initial rinsing process with inert gas is applied. After evacuation, the container is pressurized with CO_2 to filling pressure. Depending on the filler design, a second or third evacuation—each followed by CO_2 rinsing up to atmospheric pressure—takes place before the container is pressurized to filling pressure. The purpose is to reduce the oxygen level in the bottle.

The filling valves can be designed in several ways. Possible are valves with short, long or without filling tube. The valves can be controlled mechanically, pneumatically or electro-magnetically. The filling level is adjusted either by height (level) filling or by sensor-based control of mass/volume flow.

From the microbiological point of view, filling systems are preferable that enable a ‘dry’ degassing and a strict separation of gas/fluid flows. Back-pressuring into the ring channel should be avoided to decrease the risk of cross-contamination. Valves for degassing or level control that have product contact may supply possible microorganisms with nutrients. Mechanically moving parts may be difficult to clean and may supply spray shadows or niches for microorganism growth and thereby lead to elevated contamination risks.

15.2.1.5 Transfer to closing machine

Before the container is closed, it has to be transferred to the closing machine. During that time, the container is still open to the atmosphere. Usually a very thin high-pressure water jet is applied to induce a minimal over-foaming of the bottle before it is closed. The high-pressure injection has the purpose to displace the air on top of the product surface in the bottle neck and therefore to reduce the oxygen pick-up during filling.

The water injected into the product has to be treated to avoid contamination. Usually the water used for the injection gets membrane filtered ($\leq$0.45 µm) and heated to temperatures of 85–90 °C. The injection nozzle should be implemented in the CIP program. Nevertheless, the presence of a nozzle directly above the open container represents a contamination risk, and the nozzle should regularly be checked for absence of microorganisms.

15.2.2 Closing machine

For beer filled in glass bottles, crown corks are the dominating form of closure. For reasons of clarity, this chapter focuses on the microbiological risks during the closing with crown corks only. Other possible closures for beer bottles include screw caps, swing tops or corks from various materials. Although technological details may differ, the general microbiological risk potentials are similar for all types of closures.

The closing machine is, on one hand, separating the incoming bottles and transferring them to the closing element. On the other hand, the closing machine has to supply the closures. The closing elements apply the closures and close the bottles mechanically. After the closing procedure is finished, the closed containers are transferred to the following process steps (e.g. labelling). It is possible that, after the closing element, a container shower rinses product residues to avoid growth of microorganisms on the outside of the bottle or at edges of the crown cork. Although the growth of microorganisms does not directly represent harm for the product quality, the presence of moulds and other organisms growing on product residues on the outside of the container should be avoided.

The closing elements are mechanically driven systems with several moving parts. Similar to the filling elements, the moving parts and the housing of the moving parts represent microbiological risk potentials. In modern machines, the closing elements are designed to be as 'open' as possible. Thus, the cleaning of the closing elements and the whole machine can be better automated, and inner parts of the closing element may be better implemented in the cleaning procedures. Ideally, the closing element runs grease free. Grease-free elements do not provide possible niches or nutrients to microorganisms and are more cleanable.

Crown corks are transferred magnetically in modern closing machines. Older systems often used pressurized air to transfer the crown corks to the closing element. From a microbiological perspective, the use of pressurized air is not preferable. The use of pressurized air for the crown cork transport leads to possible swirling and aerosol formation. Especially with product residues caused by overfoaming, the risk of contamination is elevated.

Similar to the filling machines, the rules of hygienic design should apply for closing machines as well. For more detailed information on hygienic design, please see Chapter 11.

15.2.3 Packaging material

The packaging has, along with others, the purpose of keeping the beer's quality as good as possible. Of course this purpose includes microbiological stability. Therefore, the packaging material and its surface with product contact has to be free of pathogen or product-harming microorganisms.

Depending on the type of packaging, different possibilities exist to clean the packaging material from any harmful microorganisms. This part of the chapter will deal with these actions, as they represent risks if the packaging material is not properly cleaned. Furthermore, depending on the type of packaging, the filling process might differ from the procedures described in Sections 15.2.1 and 15.2.2. Special attention is paid to possible process differences compared to glass bottles.

15.2.3.1 Nonreturnable bottles

Nonreturnable bottles can be considered clean after their manufacturing. Nevertheless, in terms of product safety, it may be useful to rinse the bottles before they are filled. The rinsing can be done either with ionized air or with water. In addition, the bottles get turned upside down to use gravity.

The purpose of the rinsing process is to remove particles that may have fallen into the bottles during transport or handling on site. From a microbiological perspective, only a low-risk potential for beer arises from nonreturnable bottles. In the special case of aseptic filling, it may be necessary to decontaminate the nonreturnable bottles before filling. Usually hydrogen peroxide or peracetic acid are used for disinfection. Aseptic filling is not necessary for filling beer and beer products.

15.2.3.2 Returnable bottles

Returnable bottles have to be cleaned before they can be refilled. The cleaning has the purpose of removing all particles and fluids from the bottle. Particles and fluids include product residues and grown microorganisms, labels, glue, foils and other material that may be found in the bottles.

After the cleaning process, the bottles shall be clean and bathed all over their surface. All residues or foreign materials shall be removed, and no pathogen or product-harming microorganisms will be found on the bottle surface.

The cleaning of (glass) bottles is done by applying caustic solution with approximately 2% NaOH concentration at a temperature of approximately 80 °C. The bottle passes several baths to heat up and to achieve the necessary time for soaking (up to 8 min). Spraying nozzles are installed as well, to achieve an additional mechanical cleaning effect. The spraying nozzles must regularly be checked for organic and inorganic coating that reduces the effectiveness of the rinsing effect and may lead to contamination instead of cleaning in the worst case.

After the cleaning process is finished, the bottle has to be rinsed with fresh water and is cooled down to approximately room temperature. A temperature difference of more than 15 K between glass temperature and product temperature should be strictly avoided; otherwise the risk of exploding bottles during filling is very high.

The fresh water for the bottle rinsing and cooling is another microbiological risk point. Microbiological control of the fresh water is essential to avoid any re-contamination of the bottle after cleaning. Disinfection with, for example, chlorine dioxide or peracetic acid is used to reduce the risk of microbiological infection in the rinsing zones.

When the cleaned bottles leave the bottle washing machine, they have to be transported to the filling machine. The time and distance for this transport should be kept as short as possible to avoid any contamination with microorganism from air flow or installation above the transport belt.

15.2.3.3 Cans

The filling of cans is performed in a manner similar to the filling of glass bottles. Nevertheless, the evacuation cannot take place because the can axial pressure resistance does not allow the application of a vacuum. Therefore, only purging with CO_2 or

another inert gas can be used. The fairly high surface-to-volume ratio at the can finish creates difficulties in terms of oxygen pick-up. From a microbiological perspective, the same risks apply as for glass bottles.

15.2.3.4 Plastic bottles

When plastic bottles are filled, the bottles themselves are usually produced on site. Polyethylene terephthalate (PET) bottles are blown from preforms. The preforms can either be purchased or produced on site as well.

During the blow molding process, the PET preforms are treated with pressure up to 40 bar and temperatures up to 240 °C. Nevertheless, microorganisms that may be present in the preforms are not totally inactivated, since heat as well as pressure are applied in a dry environment for a comparably short time.

In terms of product safety, it may be useful to rinse the bottles before they are filled. The rinsing can be done either with ionized air or with water. In addition, the bottles are turned upside down to use gravity.

In the special case of aseptic filling, it may be necessary to decontaminate the PET bottles before filling. Usually hydrogen peroxide or peracetic acid is used for disinfection. Aseptic filling is not necessary for filling beer and beer products.

In contrast to cans and glass bottles, the container walls of PET bottles are permeable. Thus, an increased loss of CO_2 and uptake of oxygen may result. Both factors can be considered as attributes that have a significant impact on growth rates of several microorganisms. Recent research has shown that high permeation rates for plastic bottles may lead to accelerated growth of beer spoilage organisms. Contamination with aerobic bacteria that are not able to grow under low oxygen conditions was seen to increase significantly for samples with elevated permeation rates.

In addition, a tunnel pasteurization to decrease the contamination risk with a heat treatment after the filling process is limited by the temperature and pressure sensitivity of the plastic material. Generally, a heat treatment of PET containers filled with highly carbonated beverages cannot be recommended, and may lead to deformation and loss of sealing properties.

15.2.3.5 Kegs

Kegs in this chapter are to be considered reusable stainless steel kegs in their various sizes and forms. Various types of fittings are available and used for different markets. Nevertheless, the same basic considerations and hazards apply for all types of stainless steel kegs.

In the first step, kegs are emptied and purged with water. The cleaning cycle usually consists of a hot caustic cleaning step followed by water steam treatment for sterilization. Prior to filling, the kegs are pre-pressurized with carbon dioxide to minimize oxygen pick-up during filling.

With respect to sufficient cleaning and sanitation of the filling equipment, keg filling is a considerably low-risk procedure for beer filling. The highest microbial risk for kegged beer is usually to be seen at the point of sale (bars, restaurants, etc.) where an unsatisfactory hygienic state of the tapping equipment and low-level trained staff

may be the root causes of a secondary contamination of the beer inside the keg. Microbial infection may grow through poorly cleaned hose connections back into the keg and lead to spoilage of the keg content. Assuring the microbiological quality of draft beer is discussed in Chapter 17.

15.2.3.6 Other containers

The group of special containers for beer packaging covers one-way kegs, wooden barrels and bag-in-box systems.

Due to their physiological structure, one-way kegs usually neither withstand a hot caustic cleaning or a steam disinfection step. Therefore, sterilization on the filling equipment is not possible, which increases the need for a highly hygienic filling process.

In wooden barrels, the natural structure of the wall will enable microorganisms to settle into smallest structural gaps. In addition, modern hygienically designed equipment is most often not suitable for filling of wooden barrels.

Bag-in-box systems and other possibilities to transport larger volumes of noncarbonated liquids are not very common for beer. Later carbonation at the point of sales or when transferred to the next production step (filling in bottles, cans, etc.) will be necessary. The absence of carbon dioxide increases the susceptibility of the product and therefore enhances the risk of contamination, especially with microorganisms that, because of their low tolerance of carbon dioxide, would not be considered as beer spoilage organisms.

15.2.3.7 Closures

All types of closures for small beer packages have direct product content and have to be kept free of possible beer spoilage organisms. In most cases, the closures are transported from the manufacturer as bulk material in large boxes and sealed in plastic bags. Within the dry environment, no microbial growth is possible, except if, due to careless transport or storage conditions, the cardboard and plastic bag material gets physically damaged and moist.

Further risks of the closing procedure are described in the Section 15.2.2. In case of approaches toward ultraclean or aseptic filling, the closures may be disinfected before being transferred to the closing machine.

15.2.3.8 Other packaging aids

Other packaging aids (e.g. labels, glue, shrink films, carton boxes, trays, crates) are not be considered microbial hazards. Nevertheless, a certain standard of hygiene is necessary to deliver an appropriate product appearance to the consumer. Beer in packaging that has a dirty or even microbially spoiled look will not be accepted, although the packaged beer may be of best quality.

15.2.4 Water

Within the filling area, several uses of water are necessary. During cleaning and sanitation steps, water is the medium to transport the active ingredients to the surfaces where

they are to be acting. All product lines and parts that have direct product content have to be purged with fresh water after cleaning to avoid any carry-over into the product. During product changes, water is used to push out the old product before the new product is running. In addition, a possible high-pressure injection directly brings in hot water to create a controlled overfoaming. All these processes carry a certain risk of microbial product contamination, since the used water will have more or less direct product contact.

Due to the direct impact on the microbial state of the product, any process water that is used in the filling area has to be under strict microbial control. Next to a certain water treatment in terms of its mineral and other substance content, the microbial state should be regularly monitored. To reduce the microbial load of process (and product) water, several methods are available that shall not be further discussed here (e.g. ultraviolet treatment, microfiltration, chlorine dioxide use, ozone use and many others).

To ensure safe use of the process water after treatment, the water supply pipes have to be part of the regular cleaning regimen. Spray nozzles as well as the high-pressure injection nozzle often show mineral clogging over time and therefore have to be cleaned regularly. Otherwise the growth of biofilms may be supported, with the effect that microbes are spread over clean surfaces when fresh water is sprayed at the end of the cleaning cycle or may directly enter the container at the high-pressure injection nozzle.

15.2.5 Air

In the filling area, air is used mainly to run pressurized air valves. Next to needs of the valve function itself, the air should be sterile filtered so as not to bring any kind of microorganisms into the filler system. Although the microorganisms will not be able to reproduce in the air itself, the risk is high that, via the pressurized air channels, single cells may get to an environment that enables their growth. For example, in pressure valves of the filling tube, product residues appear during the filling cycles, which may provide the necessary nutrients to start biofilm growth.

15.2.6 Carbon dioxide

Carbon dioxide is used in beer filling to purge the containers. The main purpose is to reduce the air or oxygen content in the package before filling. For can filling, carbon dioxide is additionally used to purge the surface under the lid during closing.

The used carbon dioxide gas has direct product contact and may thereby be a direct source of product contamination. The hygienic state of the carbon dioxide gas has to be of highest quality. Especially when carbon dioxide from fermentation is reused, a carry-over of single cells may occur. Usually the technical needs of the carbon dioxide (purity) demand a certain washing and purification step that may be considered as a stop barrier for the carry-over of microorganisms. Nevertheless, all carbon dioxide pipes should be regularly cleaned to avoid any build-up of biofilms. Especially in the filling and closing machine itself, the formation of aerosols as well as back-pressure may lead to product contamination of the gas supply pipes.

15.2.7 Environment

The environment plays a very important part in terms of microbiological hazards in the packaging hall. Often the source for growth of microorganisms is the direct environment of the filling machine. All kinds of microorganisms may be brought into the direct environment of the filling machine. The transport may, for example, be supported by air flows caused by the fast-moving filling machine, natural air flows in the packaging hall, transport belts, or fork lifter, or by operators.

Ideally, the air pressure in the packaging hall should be adjusted to a minimal overpressure. As a consequence, the natural air flow in the packaging hall will always be directed to the outside instead of bringing in possible contaminants with air from outside the packaging hall. Furthermore, the design of the packaging hall should avoid (open) doors or windows close to the filling machine. Ceilings, walls and floors are to be designed in a hygienically appropriate way. Hygienic design is not limited to machines and pipes with product contact but can be applied for the complete packaging hall.

Within the packaging hall, ideally the returned containers (not cleaned) should be strictly separated from cleaned containers and the filling area (wet part of the packaging hall). Any possibility for cross-contaminations should be minimized.

Empty as well as filled containers have to be transported to and from the filler. Usually belt transportation systems are used. These transportation systems may bring in microorganisms and therefore have to be disinfected constantly. The transport systems should be checked regularly for their hygienic status.

When the packaging process is running, the machines have to be operated and supported with the necessary packaging materials or packaging aids. Operators as well as, for example, fork lifters are moving close to the filling area and may bring in microorganisms from outside the building or another section of the production area. To avoid or minimize contamination risks, an appropriate hygiene protocol should be followed when acting in the packaging hall.

15.3 Biofilm growth in the packaging hall

In modern microbiology, the biofilm is often referred to as the natural environment microorganisms. Next to the natural environment, technical equipment also may provide conditions that enable biofilm formation. In fact, many filling devices for beer and other beverages show unwanted growth of biofilms. Points of high risk for the development and growth of biofilms can be found especially at the filling and the closing devices. Furthermore, the transport belts, bottle washing machine or inspectors can be subject to biofilm growth.

Biofilms usually consist of water, microorganisms and extracellular polymeric substances (EPS). These EPS react with water to build-up hydrogels. The hydrogels form a slime that coats the microorganisms inside. Within that coating nutrients, metabolites, or further substances may be present. The EPS consist of polysaccharides, proteins, lipids and nucleic acids.

The build-up of a biofilm can be divided into several steps. In the first phase, a so-called conditioning film is evolving. The conditioning film is formed by irreversible adsorption of organic macromolecules (e.g. proteins, polysaccharides) on suitable surfaces (interfaces). On these conditioning films, microorganisms may settle. At the beginning of the biofilm growth, species that show strong adhesion abilities and the possibility to excrete EPS predominate. In the next step, the primary organisms allow the attachment of secondary microorganisms by co-adhesion.

As soon as the first microorganisms have completely attached to the respective surface, the phase of microbial growth begins. The phase of microbial growth is dominated by the multiplication of the primary microorganisms. In addition, new microorganisms originating from the closed environment may attach to the growing biofilm. A general characteristic for the phase of microbial growth during biofilm development is the start or initiation of release of EPS [2]. Prerequisites for the growth as well as the EPS production are the availability of nutrients and water. The evolving biofilm is characterized by a three-dimensional structure.

Therefore, the following prerequisites for biofilm growth can be summarized:

- Surface (interface)
- Sufficient water availability
- Nutrients
- Microorganisms.

The filling equipment and its environment provide suitable interfaces as well as microorganisms. When product (substrate) is filled in the filling equipment, the necessary water and nutrients are provided as well. For technical reasons (see Section 15.2.1), a certain spreading of beer residues during the beer filling process can hardly be avoided. Moving parts and residual moisture lead to the formation of aerosols (fine dispersed liquids in the air). The high turning speed of modern filling equipment additionally creates airflows and turbulences that bring the aerosols to practically all surfaces within the filling machine and, therefore, to the suitable interfaces for biofilm development.

Next to the product, residues in returned bottles, grease or lubricants can also serve as substrate for biofilm growth when transferred to suitable surfaces. The mentioned fluids may also serve for the build-up of a conditioning film.

The agglomeration in biofilms provides advantages over single-species colonies. A hydrogel matrix not only provides a certain protection from external stress, but it may also keep water and nutrients that may be degraded by exo-enzymes and metabolized. Several different species of microorganisms may be present in biofilms and create synergies. The respiration of aerobic microorganisms may produce spots of anaerobic conditions within a biofilm structure where anaerobic microorganisms are now enabled to survive and reproduce. Cells of microorganisms are able to communicate via signal molecules (quorum sensing), and genetic information may be exchanged (horizontal gene transfer). The biofilm structure is always in progress and change. Thereby, a permanent adaptation to the environmental conditions is possible.

The presence of biofilms automatically leads to higher risks of product contamination throughout the complete production process. Single cells or whole biofilm

fragments may be transferred to other production areas as well. All moving equipment (belt conveyor, fork lifters, etc.), operators, spraying of fluids or airflow may spread the microorganisms and biofilms within the production area. Especially the filling area has a high risk potential for secondary contamination. As mentioned before, the rotation of the equipment, together with high humidity and eventually elevated temperatures, add up to produce very good conditions for aerosol formation and biofilm development. The risk of cells to enter and contaminate the open containers is high, and contamination reduction measures after filling are limited or unwanted.

Next to the risk of contamination, the potential of microbial influenced corrosion (MIC) must be mentioned. Microorganisms that are organized in biofilms may be able to excrete substances that have corrosive properties. Parameters such as pH, redox potential, or oxygen concentration may shift and enable the corrosion and damaging of the surface material. Due to the presence of certain microorganisms, for example, *Gallionella*, *Nitrosomas* or sulfate-reducing organisms, the electro-chemical potential may shift significantly. Additional acid production may lead to the corrosion of metals, mineral-based substances or coatings. Assumptions relate up to 20% of corrosion damage on metals or other materials to MIC.

The association of microorganisms in the form of biofilms provides a better protection against environmental stress (e.g. high temperatures, ultraviolet radiation, changes in pH or chemicals) than do single cells. Due to the synergetic organization of different species of microorganisms, the EPS excretion, combined with horizontal gene transfer, may produce mature biofilms with a high resistance to cleaning agents and disinfection measures.

Although there has been a certain gain of knowledge on biofilms and their formation, the diagnosis and targeted counter measures are not very common in practice. The main reasons may be found in the high diversity of biofilm compositions and structures. Different organisms, interfaces and substrate-related aspects lead to differentiated and very specific biofilm structures. Depending on the environmental conditions, the same organisms may build-up a significantly different and unique biofilm structure.

The environmental conditions may vary substantially within a filling machine. Product specifications and changes, cleaning intervals, cleaning procedures and detergents are influencing factors. The design of the filler defines possible weak spots, such as spray shadows, niches, etc. In addition, weather conditions, seasonal changes, operator hygiene and geographic details influence the growth and structure of microorganisms organized in complex structures such as biofilms.

A recent research project targeted the brewery-specific identification of microorganisms found in local biofilm structures. As the main inhabitants of the fully grown biofilms that were analyzed, various wild yeasts were detected. In single cases, also lactic acid– or acetic acid–producing bacteria were detected.

Subsequent research focused further on the role of nonacid-producing microorganisms during the development of biofilms. As primary organisms, strains of *Acetinobacter* and *Pseudomonas* were detected. The development of biofilms at surfaces with indirect product contact may be related to all kinds of ubiquitously existing microorganisms that are slime producing. In particular, Pseudomonas and

Enterobacteria are mentioned. In addition, the yeasts *Rhodotorula glutinis* and *Cryptococcus albidus*, as well as the molds *Geotrichum candidum* and *Aureobasidium pullulans*, are named.

15.4 Minimization of risks

15.4.1 General

Minimization of risk may be achieved by two different approaches. On one hand, the product stability or susceptibility may be increased or decreased. Processing a less susceptible product does mean reacting on a possibly inadequate level of hygiene to avoid the consequences. Increased levels of toxic or antimicrobial ingredients (e.g. ethanol, carbon dioxide, hop bitter acids) may be a possibility, or the use of food-grade preservatives such as dimethyldicarbonate or derivates of sorbate and benzoate.

On the other hand, measures may be taken to avoid contamination in the first place. These measures include hygienic design of the equipment in use and its periphery. In addition, a suitable cleaning and sanitation protocol has to be in place, accompanied by appropriate training of the respective operators. Furthermore, a regular control of the hygienic status is a useful tool to track possible weak points within the cleaning and sanitation protocols and to further improve these protocols.

15.4.2 Cleaning and sanitation

Biofilms are usually treated with biocides. However, the use of disinfectants (biocides) has several weaknesses. Even if the disinfection measures successfully inactivated the organisms, the biomass does not leave the system and may serve as nutrient for other microorganisms. The development of a new biofilm structure may result. Furthermore, the disinfectant or the kind of disinfection treatment has to pass through the hydrogel matrix without reacting with the matrix. If reactions take place, the result may be lesser disinfection properties of the reaction products or even the supply of additional nutrient to the microorganisms that are organized within the hydrogel matrix (biofilm).

Therefore, an intense mechanical cleaning success to bring out the organic material is as important as the disinfection or inactivation of microorganisms. Due to the design of the equipment, not all parts and places within the machines can easily be reached and cleaned. Efforts in engineering machines with the best possible hygienic design (Chapter 11) are a possible solution. Nevertheless, biofilms become visible to the human eye only after the structures reach certain size and cell density. Thus, surfaces that seem visibly to be clean are not necessarily free of contaminants and growing biofilms.

There is no general solution available for treating biofilm formation in the filling process; the individual conditions in a particular filling department must always be considered. No cleaning and sanitizing strategy is available that would fit each filling machine with its individual risk potentials. The cleaning and disinfection procedures

have to be tailored to the (local) demands of the existing equipment. Ideally this would include a deep knowledge of the local microbial flora.

Within the beer and beverage sector, it is the manufacturer's responsibility to use best practice techniques to ensure a hygienically acceptable product quality. Surfaces, especially when regularly in indirect or direct contact with product, should be easily cleanable and suitable for disinfection. The best way to ensure an appropriate state of hygiene in the production facility is by scheduled controls of surfaces, equipment and product as well as by sufficient safety measures before products are released to the market.

15.4.3 Ultraclean filling

The trend toward beer-mix beverages, low-alcohol and alcohol-free beers is a worldwide driver of product innovations in the brewery sector. The low contents of natural preservatives such as ethanol or hop acids and the increased amounts of (fermentable) sugars have led to a higher microbial susceptibility of these products. If tunnel pasteurization is avoided or is not possible (with plastic packaging), the filling process should occur under highly controlled hygiene conditions.

The so-called ultraclean filling technology was designed to handle the respective containers under more secure hygiene conditions than standard filler designs would allow. The filler is kept in housing, and sterile filtered air is used to achieve a constant overpressure within the filling compartment. Sanitizing of containers and closures is also part of the approach to reduce the microbial risks of filling. In comparison to aseptic filling, the targeted cell count reduction is lower than the five logarithmic steps that are common for aseptic filling. A reduction of target organisms by three logarithmic steps is common for ultraclean fillers.

Aseptic filling is not common for beer or similar fermented beverages with elevated carbonation and lower levels than pH 5.

15.5 Future trends

Trends, for the near future, of filling and packaging may be seen in two directions. On one hand, the markets often demand new, innovative products to accompany established brands. In terms of the filling process, the number of single stock-keeping units will increase, and more product or container change will challenge the packaging process and its efficiency.

On the other hand, the productivity of filling lines will undergo constant improvement with better automated processes (cleaning, product change-over, etc.) and fewer or shorter downtimes.

Hygienic design implementation, combined with high throughput rates, have been the most important improvements over the past years. The biggest challenges for the coming years will be to become even more flexible and to further reduce energy and detergent consumption for a more sustainable process.

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Assuring the microbiological quality of draught beer

16

D.E. Quain
International Centre for Brewing Science, School of Biosciences, University of Nottingham, Loughborough, Leicestershire, UK

16.1 Introduction

The need to assure draught beer quality is not new. In 1912, in the Journal of the Institute of Brewing, Mr. G.R. Seton noted that 'it is not possible to find a subject fraught with greater importance to the brewing trade than cellar management'. He further observed (Seton, 1912) that 'the national beverage, as it is served over the counter of many of the public houses in England today, has not the flavour and appearance commensurate with the care bestowed upon its manufacture in the brewery, a fact that often leads the public into the mistaken notion that the beers of today are inferior to those of our forefathers'. Obviously not a shrinking violet, Seton then hit home with 'it (is) difficult to understand why at the most critical point in its passage from the brewery to the consumer, viz., the public house, beer is allowed to be treated under conditions which are in direct antithesis to those strictly enforced in the brewery'.

So, some 102 years on, with draught beer now being (predominantly) a keg rather than cask offering, Mr. Seton's remarks still regrettably hold true. Perhaps, to quote Ecclesiastes, 'what has been will be again, what has been done will be done again; there is nothing new under the sun'. Whilst a long recognised issue, draught beer hygiene has received only sporadic attention over the years with publications in the 1950s on cask beer (Hemmons, 1954; Wiles, 1950) and, with the transition to keg, the comparative golden age in the 1970s and 1980s from the British School of Malting and Brewing (Casson, 1982, 1985; Harper, Hough, & Young, 1980; Hough et al., 1976). In the last 20 years or so, there have been occasional communications including a number from Germany (e.g. Ilberg, Schwill-Miedaner, & Sommer, 1995) although regrettably (for me) not in English. Inevitably the focus is on aspects of hygiene such as the impact of line composition (Thomas & Whitham, 1996), application of ATP bioluminescence to validate cleaning (Orive i Camprubi, 1996; Storgårds & Haikara, 1996), use of technology to extend line-cleaning frequency (Price, 2002), hygienic design, installation, and standards (Jurado, 2003), and possible the role of enzymes in treatment precleaning (Walker, Fourgialakis, Cerezo, & Livens, 2007). Finally 'dispense' has been covered, albeit with different emphasis, in two big books on brewing (Boulton & Quain, 2001; Briggs, Boulton, Brookes, & Stevens, 2004).

16.1.1 Global beer market

The worldwide beer market is increasingly contradictory. The Statistical Handbook from the British Beer & Pub Association (BBPA) (Sheen, 2013) has long been the

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'go to' source of drinks industry data. This shows that since 2000, beer production in Europe has either declined (e.g. UK, Germany), has been flat (Italy), or has shown reasonable growth (Belgium, Spain). Similarly in North America, the market is static (Canada) or in decline (USA). In Africa there has been reasonable (South Africa) or appreciable (Nigeria) growth. In Australia, beer production between 2000 and 2011 has been flat. However, things are very different in the so-called BRIC countries of Brazil, Russia, India and China, where growth has been substantial and, in the case of China, extraordinary. Indeed, in 2000, China was slightly shy of the USA at 221 million hectolitres (hL) but by 2011 has romped away, more than doubling production to 490 million hL. This partly contributes to the pleasing statistic that global beer production has increased from 1391 million hL in 2000 to 1929 million hL in 2011.

16.1.2 Draught beer market—size of the cake

'Beer racked into kegs and served on draught is generally considered to be an optimal method to showcase the brewers' art' (Grossman, 2012). Given this, it is ironic that, in terms of packaging format, draught beer is the poor relation to bottle and can. The above BBPA Handbook reports 'draught sales' in 2011 for 25 of 42 leading countries. These 25 countries produce some 709 million hL of beer (37% of the global total) of which 18% (130 million hL) is draught. As noted in the Handbook, 'for some countries it is very difficult to obtain all necessary data' and consequently there is no sense of the draught contribution from 17 countries that include the aforementioned BRIC and other significant countries (e.g. South Africa, Ukraine, and Mexico). Accordingly, even if the draught contribution is only 1% or 2% (or more), the true total global draught volume is realistically way greater than the above 130 million hL. Indeed, over time, these figures may become more transparent, as in some countries draught beer is seen by consumers as being more aspirational than the small pack offering.

The top 10 countries cut by draught volume are detailed in Table 16.1 The proportion of the mix as draught beer varies widely, with the major players being Ireland

Table 16.1 Top 10 draught beer countries

Country	Production (000 hL)	Imports - exports	Universe (000 hL)	Draught (%)	Draught (000 hL)
USA	225,540	26,862	252,402	10	25,240
UK	45,694	2332	48,026	48	23,052
Germany	95,545	–7889	87,656	15	13,148
Spain	33,573	2151	35,724	28	10,003
Japan	54,470	72	54,542	18	9818
Czech Republic	18,181	–2598	15,583	42	6545
Ireland	8514	–1224	7290	61	4447
Australia	17,420	442	17,862	22	3930
France	15,910	2786	18,696	18	3365
The Netherlands	23,644	–11,503	12,141	26	3157

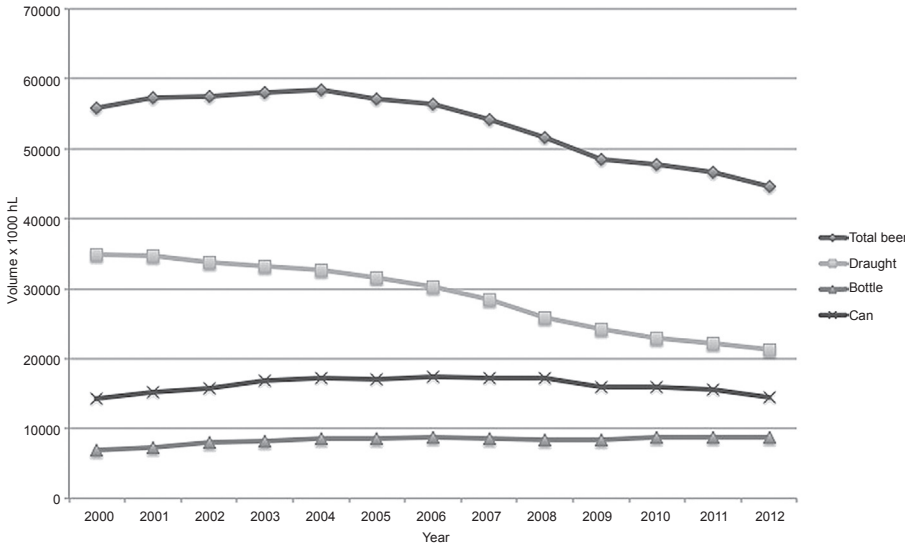

Figure 16.1 UK beer market 2000–2012.

(61%), the United Kingdom (48%) and the Czech Republic (42%). For a wide variety of reasons (see PEST analysis in Quain, 2007), the UK market is in decline in terms of both total volume and the proportion of draught beer (which uniquely includes the 'cask' category). Indeed, between 2000 and 2012, the decline in draught beer (13.5 million hL) outperformed the total decline (11.4 million hL) in the UK beer market. As shown in Figure 16.1, draught beer has declined year on year, whereas small pack volumes over this period have either been flat (can) or, in the case of bottle, grown. Further analysis (Figure 16.2) shows that both draught lager and draught ale have lost more than 5 million hL, although this is more damaging for ale, as this accounts for some 65% of the volume in 2000, whereas with lager the loss is 29%. Stout has also been less robust, losing some 40%, with cask losing 30%. The different rates of decline are surprisingly linear (Table 16.2), which encourages extrapolation. Indeed, should the current trends continue, then the draught beer volumes will fall to 15 million hl around 2017. This would have a dramatic impact on keg ale, with its share falling from 14.4% of 21.5 million hL in 2012 to an estimated 3.6% of 15 million hL in 2017. Both stout and cask would increase slightly, with lager winning out with its market share moving to 71% (Table 16.2).

16.2 Draught beer quality

Cost and quality are indelibly linked. In the case of draught beer, poor or 'so-so' quality coupled with high purchase price are two of many drivers for consumers to switch from the on-trade to drinking beer in small pack at home. Regrettably, quality issues are all too common with draught beer, which, in the consumer's eyes, reflects badly

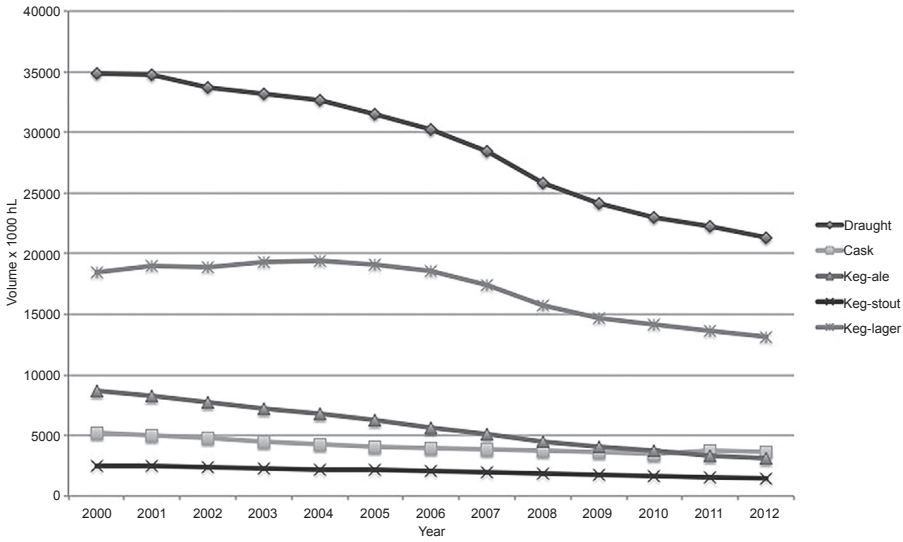

Figure 16.2 UK draught beer market 2000–2012.

Table 16.2 Draught beer category mix, 2012–2017 (estimated)

	2012	2017	Linear regression (2000–2012)
Keg lager	61.6%	70.9%	$Y = 0.494x + 3741$ $R^2 = 0.9250$
Keg ale	14.4%	3.6%	$Y = 0.3724x - 5047$ $R^2 = 0.9611$
Keg stout	6.9%	7.3%	$Y = 0.1019x + 1231$ $R^2 = 0.9789$
Cask	17.3%	18.3%	$Y = 0.0679x + 69$ $R^2 = 0.7823$

on the brand or the account. Although mostly subliminal, beer quality is assessed by consumers' 'eyes, nose and throat' (Bamforth, 1998), although consumers are increasingly adept at picking up things that are not (in their experience) right! This may include 'temperature', particularly as brand owners and retailers talk-up 'cold' dispense. In the case of flavour and aroma, this may not hit the heights of 'diacetyl', 'phenolic' and 'lactic' but may well spot 'vinegar' or simply be described as 'off'. Appearance, though, is a little more straightforward, especially if the beer is either fobbing or flat. Although perhaps not strictly in scope, beer in the wrong branded glass is also a fault and undermines the 'quality' offer. In terms of clarity, issues are invariably more obvious in lager and ales, in which haze readily confirms a problem. That said, clarity is not a good measure of compromised quality *ex* dispense with

Table 16.3 Draught beer dispense—simple versus complex

Variable	Simple	Complex
Container	Keg (20 L)	Primarily keg (20–100 L) but resurgence in large volume (unpasteurised) 'tank' beer (2.5–10 hL)
Keg cooling	Keg cooler	Cellar (12 °C) or cold room (4–6 °C); chilled supply chain can be an option
Line	Single	Many (4–14) bundled together in an insulated 'python'
Line age	New	Up to 10 years or more
Line length/ID	1–4 m/ID ≈ 6 mm	5–120 m ID ≈ 6 mm; require a FOB detector
Line cooling	None	Wide bore lines circulate cold water or glycol python—*ex* icebank or glycol remote cooler/chiller in cellar/cold room
Cooling at the point of dispense	None	'Extra cold' products require trim cooling via underbar icebank flash coolers or 'pod' heat exchangers. Trace cooling an option in fonts
Line cleaning	None—line is disposable	Required regularly—recommended frequency varies (reflects storage and dispense temperature). Performed in-house or via third-party service providers

wheat beers, dark ales, stouts, porters and— in the growing 'craft' category—unfined and unfiltered beers.

Whatever the packaging format, a combination of hygienic practices and processes ensures that, on leaving the brewery, beer is fit for purpose and its quality assured. Accordingly it is a reasonable deduction that any quality defects in a glass of draught beer are a consequence of the 'dispense' process from container to tap. However, at its simplest, beer dispense (e.g. directly from a cask or a home 5 L mini-kegs) is both straight forward and without complexity. However, this is not the norm for beer dispense, which is increasingly complex and can be performed over substantial distances from container to tap. This and the need for end-to-end cooling, together with the trend for installing more taps than are commercially sustainable, have resulted in draught beer dispense in the UK and elsewhere being overly complicated. Table 16.3 details the void between a simple, commercial dispense system for low-volume accounts (<50 hL p.a.) against the many variables involved in standard accounts (large and small) in countries where draught beer is a significant contributor to the beer market. In terms of complexity, Table 16.3 can be even further developed to include an overlay of hardware detail that includes numerous snap-in connectors, flow restrictors, a FOB (foam on beer, see 16.4.4) detector, a tap spout (which may or may not be removable), and beer line, which (parking age!) could be stainless steel or mid-density polypropylene either 'as is' or lined with nylon or other finishes.

16.3 Microbiology of draught beer

As noted above, keg beer leaving the brewery is fit for purpose in terms of the distribution chain and subsequent retail. Microbiologically such beer is 'commercially sterile' in that the microbial loading is very low or barely detectable (e.g. <1 colony/L) and accordingly will not grow to any noticeable level during the product shelf life or beyond. However, dispensed beer is not commercially sterile and contains a diverse mixture of yeast and bacteria that are derived from the dispense system. The loading in beer *ex* dispense varies widely, reflecting system hygiene but also the subtleties of sampling and testing. As a rule of thumb, data from commercial accounts suggest good-quality beer to typically contain around 1000 colonies (or 'colony-forming units') per millilitre of beer. Loadings can, of course, be much lower (<100/mL) or substantially higher (>10,000/mL) (Boulton & Quain, 2001; Quain, 2012; Storgårds & Haikara, 1996). A survey in Germany (Ilberg et al., 1995) found that the total count exceeded 10^3/mL in 81% of tested accounts, with an alarming 10^5–10^6/mL being observed.

Given the worldwide scale of draught beer and the spotlight on food safety, it is surprising that there is only one set of standards that covers drinks dispense. The DIN (Deutsches Institut für Normung) is the International Organisation for Standardisation (ISO) body for Germany and is responsible for DIN 6650 ('Dispense Systems for Draught Beverages'), published in seven parts, the headlines of which have reported elsewhere (Jurado, 2003). Specifically, part six of the standard (Deutsches Institut für Normung, 2006) covers 'requirements for cleaning and disinfection', whilst generic for draught beverages (e.g. beer, wine, water, carbonates, etc.) provides a guideline for microbial loading. Here 'a typical guideline value for a positive result with respect to microbial contamination would be 1000 colony-forming units per millilitre (cfu/mL), a value of more than 50,000 cfu/mL being considered unacceptable. If the count is 10,000 or higher, cleaning is necessary'.

Although relatively inhospitable to microorganisms, beer is vulnerable to spoilage by a selection of bacteria and yeasts. With changes in technology and product composition, microbial suspects come and go, but the usual suspects (Bokulich & Bamforth, 2013) remain Gram-positive bacteria (*Lactobacillus* and *Pediococcus*), Gram-negative acetic acid bacteria (*Acetobacter, Gluconobacter*) and wild yeasts (*Saccharomyces, Brettanomyces* and less so *Pichia, Candida*). Specifically, there have been relatively few publications (mostly from the UK) on the microflora found in draught beer. Broadly, over a 50-year period, Table 16.4 (yeast) and Table 16.5 (bacteria) confirm the general picture of contaminants against a backdrop of evolving methods of microbial identification, market decline and switch from cask to keg packaging with the associated reduction in the availability of oxygen.

In terms of damage to product quality, archetypal indicators of draught beer microbiological spoilage include turbidity/haze, acidification (lactic and acetic acids), phenolic aromas (medicinal, 'barnyard'), diacetyl/butterscotch and super attenuation. Other less common markers include 'eggy' sulphury aromas (hydrogen sulphide), fruity characters (esters and higher alcohols) and 'sweaty socks' (short-chain fatty acids). It is ironic that unless these are characteristic of the (special) beer type or style, such indicators will (almost) never be found by consumers in bottled or canned beer

Table 16.4 Yeast genera identified in draught beer 1950–2012

	Wiles (1950)	Hemmons (1954)	Hough et al. (1976)	Harper et al. (1980)	Casson (1985)	Quain (2012)
Yeast genus						
Brettanomyces			✓	✓	✓	✓
Candida	✓	✓				
Debaromyces			✓	✓		
Hansenula			✓	✓	✓	
Kloeckera	✓	✓	✓	✓	✓	
Pichia	✓	✓	✓	✓	✓	
Rhodotorula		✓			✓	
Saccharomyces	✓	✓	✓	✓	✓	✓
Torulopsis		✓	✓	✓	✓	

Table 16.5 Bacterial genera identified in draught beer 1965–2013

	Ault (1965)	Hough et al. (1976)	Harper et al. (1980)	Casson (1985)	Quain (2012)	Bokulich and Bamforth (2013)
Bacterial genus						
Acetobacter	✓	✓	✓	✓		✓
Gluconobacter	✓		✓			✓
Lactobacillus	✓			✓	✓	
Obesumbacterium		✓	✓	✓		
Pediococcus					✓	
Zymonomas		✓	✓	✓		

but will experience them periodically in draught beer. It is this simple point that should drive a step change in attitude to the protection of draught beer quality.

Less well lauded is the potential impact of dispense on the concentration in draught beer of biogenic amines, which are natural compounds that are widespread in foods and beverages. The likes of tyramine and histamine are derived from the decarboxylation of the amino acids histidine and tyrosine by lactic acid bacteria and wild yeasts. Whilst levels in beer are typically not a concern, ingestion of high levels of biogenic amines can be associated with headaches, heart palpitations, and other allergy-like responses (Loret, Deloyer, & Dandrifosse, 2005). More directly alarming are reports of two individuals being treated with the antidepressant (a MO inhibitor) phenelzine sulphate who were hospitalised with hypertensive crisis on consuming a glass of draught beer in Ontario, Canada (Shulman, Tailor, Walker, & Gardner, 1997; Tailor, Shulman, Walker, Moss, & Gardner, 1994). As biogenic amines are detoxified

by monoamine oxidase (MO) in the gut, patients treated with phenelzine (which inhibits MO) are vulnerable to overconsumption of tyramine, etc. A survey of 98 beers showed that four of the 49 'tap' beers had elevated levels of tyramine (26–112 mg/L), whereas the remaining beers *ex* tap together with the 49 bottled or canned beers contained <10 mg/L. With the brand associated with hypertensive events having the highest concentration of tyramine, the working hypothesis (Shulman et al., 1997) was that 'the cause of high tyramine levels in tap beer is the contamination of the lines from keg to the tap with bacteria capable of converting tyrosine to tyramine'.

With one exception, the above observations have seemingly triggered little in the way of follow-up. One report (Diel, Herwald, Borck, & Diel, 2009), however, reiterates the higher average loading of histamine in commercial draught beers than bottled. Tellingly, this work connects the dispense system hygiene directly with histamine concentration such that 'mechanically cleaning of the tap and the storage devices reduces histamine concentration up to 35% and combined mechanical and chemical hygienic prevention (by) 93%' (Diel et al., 2009).

16.3.1 Biofilms

In the real world, microorganisms in aqueous environments exist in complex communities of diverse microorganisms that attach to surfaces, creating a multi-layered, heterogeneous, multicellular organism or biofilm. The usual rules of phenotypic and genotypic behaviour gleaned from the pampered world of pure cultures in the laboratory simply do not apply in the real world of biofilms. Driven by pressing commercial issues such as chronic medical infections and industrial biofouling, there has been an explosion of worldwide research activity to better understand and ultimately manage (or better still, control or eliminate) biofilm attachment and growth. Biofilm formation involves five steps of reversible attachment, irreversible attachment, microcolony formation, maturation and dispersion. Furthermore, there is cell-to-cell communication (quorum sensing) within genera, which triggers a collective response to environmental stimulus across the population. Biofilms protect themselves against the wider world by laying down an outer slime layer (extracellular polymeric substance) consisting of glycoprotein. Additionally, microorganisms in biofilms are markedly more resistant to antibiotics, disinfectants, ultraviolet (UV) light and other antimicrobials. Nutrients and metabolic byproducts circulate through the biofilm, which over time becomes thicker and more established. In addition biofilms disperse and establish new sites of attachment via the seeding of free-floating planktonic microorganisms or from the flow-related shedding or sloughing of biofilm 'towers' or 'mushrooms' into multicellular fragments. It is generally recognised that quantitatively the loading of microorganisms in a biofilm outnumbers many-fold the planktonic loading. Consequentially measurement of loading in the aqueous phase (e.g. dispensed beer) is likely to be the tip of the microbial iceberg.

Although it is beyond the scope of this chapter to hone in further on the fascinating details of biofilms, there are scores of available review articles. A good place to start is a (relatively thin) book, The Biofilm Primer (Costerton, 2007), by the 'grandfather' of biofilm microbiology, William 'Bill' Costerton. Closer to home and from a brewing perspective, there are much (shorter) reviews by Quain and Storgårds (2009), Livens

and Pawlowsky (2009) and Mamvura, Iyuke, Cluett, and Paterson (2011). In passing, the fascinating subject of cell-to-cell communication has been demonstrated in bacteria isolated from brewery process biofilms (Priha, Juvonen, Tapani, & Storgårds, 2011).

16.3.2 Biofilms in draught beer dispense

The impact of poor hygienic practices on dispense systems is hard to miss. Visible signs of microbiological colonisation are apparent in FOB detectors, tap orifice plates/diffuser, and those parts of the line can be examined outside the python bundle. Although regular line cleaning is the key player in the armoury of hygiene assurance, dispense systems are innately unhygienic such that post cleaning they recontaminate over time (and require cleaning once more).

Whilst the formation of biofilms is the obvious consequence of poor hygiene, there is little in the public domain about them in the context of dispensed beer. Arguably the only studies that quantify attached microorganisms in dispense lines are Thomas and Whitham (1996) in a study on-line composition, Fielding, Hall, and Peters (2007) on the use of ozone as a line cleaner, and Walker et al. (2007), who evaluated the use of enzymes as line cleaning pretreatment.

Hitherto unpublished work (Quain, 2012) focussed on the impact of line cleaning on the attached sessile and planktonic microorganisms in a beer dispense rig mimicking a commercial system in complexity, hardware, throughput, and length. In outline, the system was infected with a microbial soup (sourced from trade samples *ex* dispense), allowed to stand and then cleaned using a proprietary caustic cleaner. The attached and free microbial loading was monitored before and after cleaning using standard methods for beer microbiology together with biofilm washing and recovery from short segments of beer line.

Figure 16.3 clearly shows the impact of line cleaning and the subsequent recontamination of the line and beer. It is noteworthy that cleaning does not completely clean the system, as both anaerobic and aerobic microorganisms are found on both the surface and in the beer immediately after cleaning. For clarity, the data are the average of the results from two lines, sampled after the dispense system 'void volume' was dispensed and, during recontamination, flushed weekly with a total of 25 L of beer (phased to reflect weekly trading pattern). Although an extreme demonstration, the final loading of attached anaerobes and aerobes was *ca*. 2.2×10^6 cfu/cm^2 and 1×10^6 cfu/mL in the beer.

16.3.3 Sources of contamination

On installation, dispense lines are rarely cleaned and therefore commence working life in an unhygienic state. That said, new installations are not the norm, and beer dispense lines can be in place for a decade or more. Accordingly the primary and ongoing source of external contamination is from either end of the line, that is, the tap or keg coupler. Both are subject to unhygienic practices that result in microbial contamination, particularly at the tap end where the ambient temperature is more supportive of growth. A further route, although quantitatively less significant, is the continual seeding during dispense from 'commercially sterile' beer containing an inevitably low

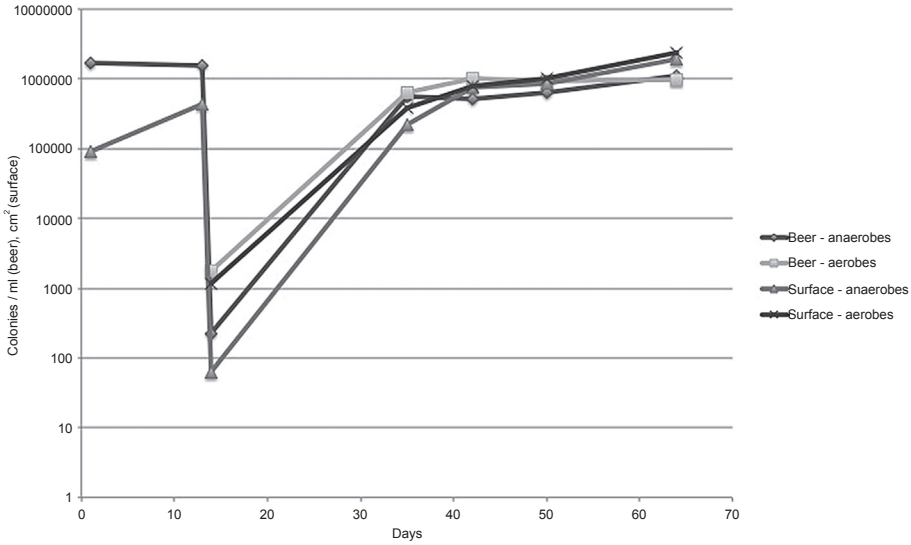

Figure 16.3 Pre/post line cleaning—impact on attached and free microorganisms (aerobes and anaerobes).

level of background microorganisms. This is of course more significant from unfiltered beers such as wheat beers and, to a lesser extent, cask beers.

16.4 Managing the microbiological risk

There is little debate that line cleaning is the key parameter in the assurance of draught beer quality. Whilst the tap and the keg coupler are clearly contributors to dispense system hygiene, they receive comparatively little attention, and what there is (i.e. spout cleaning), can exacerbate the problem.

16.4.1 Line cleaning

On the face of it, line cleaning is a straightforward process involving the regular use of proprietary (usually) caustic-based cleaners to remove biofilms from surfaces. Inevitably and regrettably, it is not that simple; rather, too many tunes can be played, with consequent damage to system hygiene and beer quality.

Of all the variables, line-cleaning frequency is the most damming. Whilst there is no universal rule of thumb, different markets (Table 16.6) recommend the best practice for line cleaning ranging from 7days (UK) to 21–28 days (Canada). This reflects temperature, typically that of the supply chain, container storage and beer dispense. Whatever the recommendation, long-term issues arise when best practice frequencies are relaxed because of concerns over time, beer losses, and word of mouth assurances ‘that it will be fine’. Then, to make matters even worse, in the absence of any significant consumer complaint, line-cleaning frequency drifts further out and becomes the

Table 16.6 Line cleaning best practice by market

Market	Frequency	Storage (°C)	Dispense (°C)	Reference
UK	7 days	11–13	2–12	Profit through quality (2009)
USA	14 days	3		Brewers Association -Draught beer quality manual (2011)
Canada	21–28 days	1–5	1–5	Draught – technical guidance on dispensing (2007)
Australia	7 days	0–10	−0.5–3	Draught beer dispense systems installation guidelines (2009)
Spain	>28 days	Ambient	2–4	—
Ireland	21 days	7–9	2–6	—

new norm. Indeed, it is estimated that in the UK anywhere between 10% and 80% of accounts fail to meet best practice standards. This is despite insight from a high street retailer that shows that accounts grow volume (and profit) when cleaning every 7 days and only marginally every 14 days (Quain, 2007).

Building on this, other variables that get in the way of effective line cleaning include cheap, cheerful, and nonoptimised cleaning solutions (with compromises on strength, surfactants to reduce surface tension, sequesterants, and chelators to bind calcium, etc.), water (cold or hand hot, hard versus soft), and, most importantly, whether the process is essentially static or involves movement as mechanical action.

Operationally the efficacy of manual line cleaning is strongly influenced by knowledge, skill, and understanding. If frequency and other 'corners are cut', the hygiene of the lines and consequently beer quality will be compromised. An effective route around this is the use of line cleaning service providers, which is increasingly common in the UK and a standard approach in Europe.

Although there are a number of best practice manuals around the world (Table 16.6), by far the most exhaustive guide to the assurance of draught beer quality and dispense hygiene is the Brewers Association 'Draught Beer Quality Manual' (2011) with additional resources at http://www.draughtquality.org. Although obviously USA-centric, this manual, through great attention to detail, contains a host of best practice recommendations that should, and do, translate to other markets. For example, quarterly line cleaning with phosphoric acid to remove oxalate and scale, cleaning solutions at 20–43 °C, and use of an electric recirculating pump for caustic (and acid) cleaning as the preferred method for nearly all systems.

Of course line cleaning mirrors cleaning in place (CiP) processes in breweries in particular and the food industry in general. For successful cleaning, four parameters (Boulton & Quain, 2001) are required—time, temperature, chemical action, and mechanical action. Although debatable, basic line cleaning broadly delivers on time, temperature, and chemical action but at its simplest fails to include any significant mechanical action. Indeed in the UK, line cleaning typically consists of four steps: (i) chasing beer out of the line by flushing with water, (ii) filling with cleaning detergent, (iii) allowing to stand for 30 min and moving (pulling a pint or two) halfway, and

(iv) flushing with water before replacing with beer. Given this, it seems a reasonable conclusion to draw that the introduction of mechanical action through recirculation would, at a stroke, improve the efficacy of the process, particularly with regard to the removal of biofilm and penetration into nooks and crannies and difficult-to-clean places. Additionally, mechanical action would be expected to add further value in the removal of dead microorganisms from surfaces, thereby reducing favourable sites for fresh colonisation and biofilm development.

For many, line cleaning is a chore performed reluctantly at the end of a long trading session. Accordingly there have been a number of innovations looking to improve the process such as the automation of line cleaning (16.5.1) or technologies that are claimed to slow biofilm growth and enable the frequency of cleaning to be reduced (16.5.3). Furthermore the long-held view (noted above) that line cleaning results in costly beer losses through flushing has been increasingly challenged, through management or technology, so that beer in the line is dispensed from the line before cleaning.

16.4.2 Taps and spouts

The tap/faucet is rife for contamination through a variety of routes, including human interaction and handling, air and the general environment. Furthermore, taps are not necessarily hygienically designed, and the inclusion of orifice plates, restrictors or diffusers, sparklers and flow straighteners adds sites for microbial colonisation. Accordingly, as shown in previous studies, the tap is a comparatively rich source of contamination (Harper et al., 1980; Hough et al., 1976; Orive i Camprubi, 1996; Storgårds & Haikara, 1996).

Not surprisingly, the management of this hygiene issue varies depending on the market. In the UK, tap spouts or nozzles fall into two categories: removable (either plastic or stainless steel) or a one piece as part of the tap, which are cleaned *in situ*. Removable spouts, which are either 'straight through' or containing diffusers and straighteners, are cleaned via a peculiar daily ritual. At the end of a day's trading all removable nozzles (and any internal plasticware) are soaked in (usually) carbonated water overnight. The next morning they are rinsed and returned (hopefully) to the mother tap. Given that they are effectively soaked in beery water overnight at bar temperatures, it should be no great surprise that this process effectively exacerbates, not minimises, contamination. With this in mind, it is easier to appreciate why the tap is one of the primary contenders for dispense system contamination.

The many practitioners of the soda water steep anecdotally believe that this approach has antimicrobial properties, which improves the hygiene of nozzles and orifice plates, sparklers, etc. Unpublished work (Board, 2010) based on accounts in Edinburgh confirms that this approach does not add value and makes things worse. Indeed a more effective approach (confirmed by Board, 2010) that is gaining traction from particularly regional Brewers in the UK is soaking the spouts in hot water (from the coffee machine) followed by air-drying. That said, the best practice recommendation in the UK (Long, 2003) is that 'beer dispense nozzles should be soaked in food-grade cleaner after each session'.

In the USA, a very different approach is recommended to assure the hygiene of the tap. Here the Brewers Association (2011) takes a very different stance and, as best practice recommended, as part of the 14-day cleaning cycle 'all faucets should be completely disassembled and cleaned'. Intuitively this will add (hygienic) value and should be a best practice aspiration in all markets for draught beer.

16.4.3 Keg coupler

As a potential 'seat' of dispense contamination, the keg coupler gets a less bad press than the tap (Harper et al., 1980; Hough et al., 1976; Storgårds & Haikara, 1996). However, the intimate connection between keg and coupler suggests that this interface is worthy of greater attention, especially as the cellar environment is not usually a place where hygienic best practice is found. Here, on changing containers, keg couplers are likely to be placed on the floor and other surfaces prior to being reconnected. In reality, and although a good idea, treatment of the keg head with antimicrobial sprays or wipes before connection is regrettably very unlikely.

Inevitably the Brewers Association (2011) has this covered. The recommendation here is that every 14 days that 'all keg couplers or tapping devices should be scrubbed clean' and every 3 months that keg couplers should completely disassembled and hand cleaned. That said, although the logic is undeniable, it would be very interesting to get a sense of real-world take-up in accounts in the USA on the practicalities of this, together with tap disassembly and cleaning.

16.4.4 FOB detector

A newer and arguably major contender in the dispense hygiene stakes is the FOB detector, which is present worldwide in all reasonably complex dispense systems. The 'foam on beer' detector's role is to minimise the risk of foam entering the beer dispense line when the container is empty or is being changed. The mechanism is essentially a float control, which in the absence of beer falls and blocks the beer inlet. On connecting a new container the fob detector is bled hygienically to drain and then filled with beer enabling dispense to recommence. As to naming, in addition to 'foam on beer', FOB detectors are also variously known as cellarbuoy, beer saver, FOB-stop, 'froth on beer', 'fobbing pot' or, less exotically, beer monitor.

Anecdotal evidence together with personal observation suggests that FOB detectors are a potent reservoir of system contamination (see for example Figure 16.4). Although FOB detectors are typically located in the cellar or storage area at temperatures (Table 16.6) from cold (12 °C) to very cold (3 °C), they provide an early visual indication of dispense system contamination. Although a generalisation, this is most likely a reflection of the poor cleanability of FOB detectors (especially the upper surfaces) during a manual cleaning without any recirculation or mechanical action.

Remedial action is straightforward and as recommended by the Brewers Association (2011) 'draught beer quality manual' such that every 14 days 'all FOB-stop devices should be cleaned in line, and cleaning solution vented out of the top' and every three months 'all FOB-stop devices should be completely disassembled and hand-cleaned'.

Figure 16.4 Contaminated FOB detector.

16.5 Innovation

Arguably the simplest and most effective 'innovation' to assure dispense hygiene and beer quality is to wholeheartedly apply best practice principles as exemplified in the Brewers Association (2011) manual. Such an innovation would not be exciting or indeed glamorous, but if applied correctly, universally and sustainably, it would achieve a step change (or more) in hygiene and quality. Although there will always be a cohort of enlightened on-trade/on-premise individuals and companies who 'get it' and do buy into best practice, regrettably the majority simply will not. Irrespective of the market and its trading complexities, changing the mindset and culture is a long, uphill struggle involving education, training and commitment.

Whilst the churn of bar staff and misplaced focus on profit (at the expense of quality) will always challenge improvement, there are encouraging signs that the take-up of education and training is on the up! In terms of self-help there are a number of best practice manuals (e.g. Brewers Association) on beer dispense that cover generic and local practices (Table 16.6). Similarly with training, there are short and long, face-to-face or online, opportunities to step change knowledge and competency (see reference to 'Suppliers of dispense training and education'). Accordingly, the more enlightened accounts and retail chains have 'cellar champions' who lead and train colleagues on hygienic practices and other means to assure draught beer quality.

In the last decade or so, there has been a significant amount of investment in dispense innovation through technology above and below the bar (McCrorie, 2014; Quain, 2006). Much has focused on the cold dispense platform with innovations in cooling technology and consumer communication, but perhaps more effort and diversity has been expended in developments that can contribute to beer quality. Many of these are outlined below. Whilst laudable and welcomed, many 'quality' innovations focus on the bottom line but frequently lack independent testing. Accordingly, claims on performance and benefit are from the manufacturer and lack independent validation.

16.5.1 Line cleaning—automation

One of the smarter innovations in dispense technology was the introduction of the disposable line with small-volume integrated systems. Regrettably, though, this is not an option for the vast majority of accounts worldwide, in which regular line cleaning is a routine 'fact of life'. As outlined above (16.4.1), manual line cleaning is, for many, a chore, although on the face of it, automation of line cleaning should be a winner! Potent arguments about reducing wastage, saving time, enhancing health and safety, and improving beer quality through more effective line cleaning (with mechanical action) should be a door opener. However, although this is tried and tested technology from a number of established suppliers, automated line cleaning has yet to take off in the UK. Indeed, Buttrick (2006) estimated that about 4% of UK accounts have adopted this technology, and concluded that there was any one of a number of factors preventing significant take-up in the on-trade. However, he noted that if barriers to entry (inevitably understanding and education) could be lifted, then 'automated line cleaning equipment could be as common as glass cleaning machines are now'.

Whilst cost and (comparative) complexity of integrated automated systems may have hindered take-up, the use of portable line-cleaning approaches has found increasing application, especially by line-cleaning service providers. As with plumbed-in automatic systems, portable approaches also achieve potentially better hygiene, through the added involvement of mechanical cleaning through turbulence and liquid flow.

16.5.2 Line-cleaning solutions

A cleaning solution designed to clean beer lines will be fit for purpose only when it is used at the appropriate frequency. Relaxing the frequency of line-cleaning will result in a greater cleaning challenge, and consequently cleaning may or may not be as successful as is expected or required. Attempts to make up infrequent cleaning by extending line-cleaning time or using at a higher concentration is poor practice. Such 'pickling' or 'bottoming out' both damages and ages the surface of the line (Casson, 1985; Walker et al., 2007) and provides more places for microorganisms to hide and colonise.

Innovation in line-cleaning solutions is a fertile area in which, on a regular basis, another formulation comes along which is claimed to deliver better cleaning capability. However any independent evidence to support such claims is typically non-existent. Furthermore it is surprising how 'tunes' are played with the composition of line-cleaning solutions, especially with regard to claims for the concentration of components. Bizarre

claims for products that are 'caustic free' merely replace sodium hydroxide with potassium hydroxide. Surprisingly, given the risk of flavor active taints (chlorophenol, chloramines), sodium hypochlorite remains a popular inclusion in alkaline cleaners in the UK. However in the USA, the Brewers Association (2011) manual is unequivocal in saying 'never use solutions that contain any amount of chlorine for line cleaning'.

As ever with successful innovation, a clear benefit typically makes a difference, and enhances take-up and acceptability. Although not necessarily new, a new category of line cleaner with colour indicator technology adds real benefit to the user. These cleaners change colour in response to oxidation of organics (*aka* biofilm) (see McCrorie, 2012). This provides a 'control loop' of sorts such that if the cleaning solution is no longer violet or purple but 'discoloured' or yellow/green, this indicates that it is oxidised. On return to the parent colour, the cleaner has done its work and is no longer removing biofilm. Furthermore, the (lack of) colour is useful in determining when the cleaning agent has been fully flushed from the dispense system.

More innovative but less successful innovations in line cleaning include novel approaches such as the use of ozone, chlorine dioxide and peracetic acid. Although these are well-accepted approaches in (brewery) CiP disinfection, none have made an impact on dispense line cleaning, although, pleasingly, ozone has at least been evaluated under controlled conditions (Fielding et al., 2007).

16.5.3 Line cleaning—extending the frequency

In terms of novel innovation, in the last 10 or so years there have been a number of devices that typically are installed in the cellar and wrapped around a short length of line usually leaving the keg. These emit a constant or varying sonic signal which is believed to delay the rate of biofilm build up in the line and, because the line stays cleaner for longer and recontaminates more slowly, it can be cleaned less frequently without risk to quality. These do not clean the line but inhibit or slow down the growth of contaminants. The sell-in is about reducing the number of cleans and consequently saving time, effort, and money. Whilst prone to a plethora of quasi-scientific descriptions about radio frequency and electromagnetic fields, these technologies seem to have stuck inasmuch as the suppliers are still in business some 10–15 years later.

There has been some attempt to validate the performance of some of these devices (Godfray, 2005; Price, 2002), although both of these publications are comparatively slight with little corroborating data. However, a hitherto unpublished study (Quain, 2008), independently evaluated a 'magnetic field' technology in a protracted and detailed paired trade trial of a number of products in three accounts over 16 weeks with and without the technology. The conclusion from this study, based on microbiological and beer analytical data, was that the frequency of line cleaning could be decreased from 1 to 4 weeks without threat to product quality.

So there is some tentative evidence that one of the 'sonic' approaches does indeed disrupt the microbiology of commercial dispense systems and delivers the opportunity to relax line-cleaning frequency. As ever, with such work, it poses more questions and hopefully provokes other technology providers to take the brave step of independent testing.

Biofilm research is, of course, focused on larger issues than draught beer dispense, notably the twin challenges of chronic medical infections and industrial biofouling. From this there can be related and transferable leanings that may underpin and support some of the approaches described here. Of possible relevance are insights into the 'electrocidal effect' (Del Pozo, Rouse, Mandrekar, Steckelberg, & Patel, 2009) or 'antimicrobial fields' (Giladi et al., 2008, 2010) in biofilms. In the former, long-term exposure (up to 7 days) to low-intensity direct current from 20 to 2000 microamperes reduced the bacterial biofilm load *in vitro*. Building on this, the use of low-intensity alternating electric fields of high frequencies (antimicrobial fields) has an inhibitory effect on the growth of pathogenic bacteria, including *Staphylococcus aureus* and *Pseudomonas aeruginosa in vitro* (Giladi et al., 2010) and *in vivo* (Giladi et al., 2008) with mice with a *P. aeruginosa* lung infection.

Less exotic but easier to sell-in and comprehend is enhanced end-to-end cooling in which the product is kept unambiguously cold, which then allows the frequency of line cleaning to be confidently decreased to 4 weeks without threat to product quality (see 'SmartDispense' in McCrorie, 2014). Importantly this 'cold approach' includes the FOB detector, which is considered to be an important source of microbial recontamination (see Section 16.4.4). Keeping the FOB cold together with re-engineering its hygienic design and performance are important 'game changing' additions in the assurance of total system hygiene.

16.5.4 Line composition

At 20 or so metres long, the beer line provides a substantial surface area for microbial colonisation (Table 16.3). Accordingly, the composition of the line has become an increasingly fertile ground for innovation directed at preventing the attachment and growth of biofilms. This is not new with Casson (1985) and Thomas and Whitham (1996) evaluating the adhesion of microorganisms to different plastics. These studies have informed developments in beer dispense line tubing which, these days, are dominated by medium-density polyethylene (MDP) and, increasingly, nylon-lined MDP multilayer barrier tubing. The sell-in here is that MDP is more susceptible to biofilm attachment than nylon. Other innovations include (for one manufacturer) the inclusion of a 'specially selected antimicrobial in the inner nylon layer' and for another 'a coating which releases silver ions gradually, protecting the inner surface of the tubing where microorganism growth is likely'. More recently, a new generation of barrier layer lines has been introduced which are significantly less permeable to gases and consequently reduce the egress of carbon dioxide or the ingress of oxygen. These lines are also marketed in reducing the growth of yeast and bacterial biofilms. Such developments are likely to continue, prompted by acceleration of interest in materials research and surface engineering to control adhesion and biofilm formation (Gu & Ren, 2014).

16.5.5 Data logging

Like automatic line cleaning, data logging technologies would be anticipated to add real value in terms of understanding key success parameters and diagnostics such as

volume, flow rate, throughput, temperature, and, to the case in point, line cleaning. Again the technology is available, successful, and increasingly sophisticated in providing real-time insight. Many large pub groups use data logging to reconcile delivered stock against dispensed volume of beer. Data logging has also found application in the assurance of dispense temperature and to minimise associated volume losses through fobbing. The technology clearly also lends itself to troubleshooting and, in response to throughput, identifying the 'hot spot' on the bar and, where appropriate, rationalising the number of branded fonts. Clearly wonderful, insightful stuff! However, the benefits of this technology have been diluted by a mindset that data logging is a 'spy in the bar' with a special focus on reconciliation of sales and sourcing of products. This, allied with controversy over accuracy of volume data and ensuing disputes between retailers and tenants, has ensured that the focus on benefit to hygiene and product quality has been well and truly lost.

16.5.6 ATP bioluminescence

The use of real-time hygiene testing with ATP bioluminescence has truly made a difference in the assurance of cleaning operations in the brewing and wider food industry (Boulton & Quain, 2001). Its application to validate line cleaning has been a mixed success (Orive i Camprubi, 1996; Storgårds & Haikara, 1996) and although used seemingly regularly in dispense development work, it has never really taken off as a routine in-trade test. This reflects two major issues that need to be resolved with a view to using ATP bioluminescence in line-cleaning validation and product testing *ex* tap: namely, improving sensitivity and detecting lower levels of ATP and, more importantly, robustly compensating for the high background levels of ATP in beer.

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Part Four

Impact of microbiology on sensory quality

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Impact of yeast and bacteria on beer appearance and flavour

17

S.-Q. Liu
National University of Singapore, Singapore; and National University of Singapore (Suzhou) Research Institute, Suzhou, Jiangsu, China

17.1 Introduction

Beer is a product of microbial transformation of cereal-based substrates, predominantly yeast and barley malt. Microorganisms are inevitably associated with the whole beer production process, from barley grains, germination, malting, wort preparation, fermentation, post-fermentation processing, to the finished product (beer) (Van Nierop, Rautenbauch, Axcell, & Cantrell, 2006). As a result, microorganisms are expected to have an impact on beer quality in a positive or negative manner at different stages of the brewing process. This chapter focuses on the impact of yeast and bacteria associated with beer fermentation and spoilage on beer appearance and flavour. In this context, beer appearance refers to haze, turbidity, sedimentation and foam; flavour refers to taste and aroma (and compounds that cause these sensations). Off-flavour (off-taste, off-odour) is also covered where appropriate.

This chapter is divided into five sections including an introduction (Section 17.1), impact of yeast on beer appearance (Section 17.2), impact of yeast on beer flavour (Section 17.3), impact of bacteria on beer appearance and flavour (Section 17.4), and future trends (Section 17.5). The emphasis is on the impact of yeast on beer appearance and flavour because more information is available on this topic. Although most of the discussions are centred on the impact of main brewing yeast strains (*Saccharomyces cerevisiae* and *Saccharomyces pastorianus*) on barley malt–based beers (ale and lager, respectively) (Lodolo, Kock, Axcell, & Brooks, 2008; Stewart, Hill, & Russell, 2013), references are also made to other yeasts involved in brewing specialty beers derived from both barley malt and other cereal malts. Lactic acid bacteria (LAB) are the focus of discussion with regard to bacterial impact on beer appearance and flavour due to their relatively common occurrences in beers (Menz et al., 2010; Sakamoto & Konings, 2003; Suzuki, 2011; Suzuki, Asano, Iijima, & Kitamoto, 2008).

17.2 Impact of yeast on beer appearance

Growth of yeasts in finished beers obviously brings about turbidity changes and cell sedimentation that are undesirable for most beer types. Furthermore, yeast metabolism during fermentation can affect haze formation, foam stability and head retention,

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as well as colour of beers in a negative manner subsequently. Beer haze consists mainly of proteins, polyphenols, and polysaccharides (largely glucans). Proteins and β-glucans from autolysed yeasts can contribute to haze formation and increased turbidity (Steiner, Becker, & Gastl, 2010). Brewing yeasts have a significant impact on foam formation, stability, and head retention as reviewed by Blasco, Vinas, and Villa (2011), and a summary of this review is provided below.

During the fermentation process, brewing yeasts produce ethanol and carbon dioxide: the former destabilises foam, whereas the latter facilitates foam generation. Mannoproteins derived from yeast cell walls not only help minimise haze formation but also stabilise foam by adhering to the gas–liquid interface of foam bubbles (i.e. the bioemulsification effect of yeast mannoproteins). Overfoaming during fermentation can lead to loss of foam-active substances such as proteins and thus is detrimental for beer head formation and retention. Yeasts under stress conditions secrete protease A that degrades malt proteins involved in forming and stabilising beer foam. In addition, autolysed yeasts can release β-glucanases that hydrolyse β-glucans, resulting in viscosity reduction and liquid drainage from the foam. Recently, a novel foam-negative protein, thioredoxin, which has an adverse effect on foam stability, has also been identified in finished beer using proteomics, presumably as a result of yeast proteolysis upon autolysis (Wu, Rogers, & Clarke, 2012).

Besides the effects of proteins, protease A and β-glucanases associated with yeast autolysis and stress, lipids and longer-chain fatty acids released from autolysed yeasts damage beer foam by promoting the coalescence of foam bubbles through a film-bridging mechanism (Bravi, Perretti, Buzzini, Sera, & Fantozzi, 2009). In addition, brewing yeasts are known to produce SO_2 during fermentation (Baert, De Clippeleer, Hughes, De Cooman, & Aerts, 2012; Duan, Roddick, Higgins, & Rogers, 2004; Samp, 2012), and excess SO_2 can have a bleaching effect on beer colour, although the extent of such a bleaching effect is still not clear and merits further investigation.

17.3 Impact of yeast on beer flavour

Beer is a product of transformation of the wort and hops by yeasts under brewing conditions. Although the hops added principally contribute to the bitter taste and aroma to a certain extent, it is the yeast used in brewing that makes a significant impact on beer flavour, especially aroma, through yeast autolysis, catabolism of sugars, assimilable nitrogen, organic acids, and other substances, then generation of acids, alcohols, aldehydes, esters, ketones, volatile phenolic compounds, terpenoids, and volatile sulphur compounds (VSCs).

17.3.1 Impact of yeast on beer taste

Brewing yeasts contribute positively to beer mouth-feel by producing carbon dioxide and foam bubbles. The impact of yeasts on beer taste varies with the yeasts involved in beer fermentation. In the spontaneous fermentation of acidic beers such as lambics, the non–*Saccharomyces brettanomyces* yeasts can ferment malto-oligosaccharides that

are unfermentable and left behind by the conventional *S. cerevisiae* brewing yeasts (Verachtert & Derdelinckx, 2014), and this is expected to negatively affect mouth-feel and result in thinner beers. If a lactic acid–producing yeast such as *Lachancea thermotolerans* (now *Kluyveromyces thermotolerans*) (Gobbi et al., 2013) is involved in or used in beer production, the resultant beer would have a higher acidity and a sour taste.

Glycerol, another product of yeast fermentation in the glycolytic pathway, is also found at various levels in different beer styles (Klopper, Angelino, Tuning, & Vermeire, 1986). Glycerol production can be heightened in high-gravity wort fermentation due to osmotic stress that results in redox imbalance and metabolic shift. This alcohol has a sweet taste and is viscous, and thus at excessive levels it can affect beer taste and mouth-feel.

Yeasts are known to undergo autolysis during fermentation and beer aging; as a result, some substances such as amino acids (e.g. glutamic acid, Glu), nucleotides (e.g. guanosine monophosphate (GMP) and inosine monophosphate (IMP) and glutathione (GSH) are released that may affect beer taste directly or indirectly. Glu, GMP and IMP are common savoury or umami flavour enhancers used in the food industry. Limited evidence suggests that nucleotides could have an effect on wine taste (Charpentier et al., 2005). However, it remains to be seen whether these nucleotides and Glu can affect beer taste. On the other hand, GSH is reported to induce the so-called kokumi flavour (mouth-feel), which is enhanced by Glu and IMP (Maruyama, Yasuda, Kuroda, & Eto, 2012; Ueda, Yonemitsu, Tsubuku, Sakaguchi, & Miyajima, 1997). Possibly GSH, Glu and nucleotides can have a synergistic effect on beer taste, and further study is warranted.

17.3.2 Impact of yeast on beer aroma

Beer contains numerous volatile compounds, depending on malt type, hop variety, beer type, adjunct, yeast strain, brewing and process conditions and maturation (Angelino, 1991), just to name a few. However, not all volatile compounds are aroma-active, and many do not contribute to beer aroma. In fact, only a small number of volatiles in beer are aroma-active (Fritsch & Schieberle, 2005; Langos, Granvogl, & Schieberle, 2013). Furthermore, some of the beer volatiles (both aroma-active and aroma-inactive) are not of yeast origin. In addition, among the aroma-active volatiles, some impart positive aromas to beer such as fruity, floral, and fragrant flavour notes, whereas others may cause off-odours such as green, butter-like, sulphury, and phenolic. In this section, the impact of yeasts on beer aroma compound formation is discussed with regard to their potential positive and negative influences on beer aroma, covering the following classes of odourants: alcohols, aldehydes, acids, esters, ketones, volatile phenolic compounds, terpenoids and VSCs.

17.3.2.1 Alcohols

Besides ethanol, higher alcohols (also known as fusel alcohols or fusel oils) are the major alcohols that impart sensory properties to beer, including n-propanol, isobutanol, active amyl alcohol, isoamyl alcohol and 2-phenylethyl alcohol (2-phenylethanol)

(Angelino, 1991). Ethanol not only gives off an alcoholic odour but also acts as a carrier of other odour-active volatile compounds. The higher alcohols impart a range of organoleptic attributes such as alcoholic, fruity, pungent, solvent-like and rose-like or floral, depending on the concentration and type of alcohol. The aroma importance of high alcohols extends to other facets of beer flavour by serving as ester precursors (elaborated below). The biogenesis of higher alcohols in beer in relation to yeast metabolism has been recently reviewed by Pires, Teixeira, Branyik, and Vicente (2014), and a summary is given below. For detailed pathways of higher alcohols formation by brewing yeasts, readers are referred to this review article.

Ethanol is produced by yeasts through the glycolytic pathways, with pyruvate being the key intermediate compound, which is then decarboxylated into acetaldehyde, followed by reduction to ethanol. Higher alcohols, on the other hand, can be biosynthesised by yeasts from sugars and selected amino acids (typically branched-chain and aromatic amino acids) via the anabolic pathway and Ehrlich pathway, respectively. In the anabolic pathway, α-keto acids are generated from carbohydrates via de novo biosynthesis of amino acids, whereas in the Ehrlich pathway, α-keto acids are formed from amino acid breakdown by way of transamination. The α-keto acids are then decarboxylated with the formation of aldehydes, which are subsequently reduced to higher alcohols. In the Ehrlich pathway, the type of higher alcohols produced is determined by the type of amino acids present, commonly threonine (n-propanol), valine (isobutanol), leucine (isoamyl alcohol), isoleucine (active amyl alcohol) and phenylalanine (2-phenylethyl alcohol). Indeed, the addition of valine, leucine, isoleucine, lysine, histidine and proline increased the formation of higher alcohols (Lei et al., 2013; Procopio, Krausea, Hofmann, & Beckera, 2013). Tyrosol and tryptophol can also be produced from tyrosine and tryptophan through the Ehrlich pathway, respectively; however, at the levels normally found in beer, tyrosol and tryptophol are not expected to affect beer flavour (Li, Yang, Hao, Shan, & Dong, 2008).

17.3.2.2 Aldehydes

There are a number of aldehydes present in beer such as acetaldehyde, hexanal, (*E*)-2-nonenal, furfural, 2-methylpropanal (isobutanal), 2-methylbutanal (amyl aldehyde), 3-methylbutanal (isoamyl aldehyde), 3-methylthiopropanal (methional), 2-phenylacetaldehyde and benzaldehyde (Angelino, 1991; Baert et al., 2012). These aldehydes affect beer flavour by imparting organoleptic notes ranging from green apple–like (acetaldehyde), malty (branched-chain aldehydes), worty (methional), flowery (2-phenylacetaldehyde) and almond- or cherry-like (benzaldehyde), which are concentration-dependent. Naturally, not all aldehydes are of yeast origin. The chemical and biological origins of aldehydes in beer have been thoroughly discussed in a review by Baert et al. (2012). The yeast origin of aldehydes in beer is described briefly below with reference to this review article and other recent reports.

Acetaldehyde is a by-product of alcoholic fermentation by brewing yeasts. It is a precursor to ethanol in the glycolytic pathway, and small amounts of acetaldehyde are excreted under normal physiological conditions and excess levels are produced under abnormal physiological conditions such as in high-gravity fermentation. Small

amounts of branched-chain aldehydes and 2-phenylacetaldehyde can be excreted by brewing yeasts during fermentation from the catabolism of respective amino acids by way of the Ehrlich pathway mentioned above. Although the contribution of aldehydes to the final beer in this fashion may be quantitatively rather limited due to the aldehydes being the direct precursors to the corresponding alcohols (most are reduced to the alcohols), even low levels of aldehydes may have an impact on beer aroma individually, additively, or synergistically, given their very low odour detection thresholds.

Some aldehydes formed either biologically during wort fermentation and/or chemically during beer aging cause aged or stale beer aroma (beer staling), for instance, cardboard aroma ((*E*)-2-nonenal), cooked potato-like (methional) and honey-like (2-phenylacetaldehyde). Yeasts are known to reduce the aldehydes in the wort to their corresponding alcohols during fermentation. The aged beer aroma can be decreased considerably by taking advantage of the reducing activity of the yeasts through bottle re-fermentation (also known as bottle conditioning) such that beer flavour is refreshed (Saison et al., 2010).

17.3.2.3 Acids

The acids in beer consist of inorganic acids (mainly phosphoric acid) and organic acids (nonvolatile and volatile acids); together they contribute to the total acidity of beer. The nonvolatile acids include, but not restricted to, malic, citric, pyruvic, α-ketoglutaric, succinic and lactic acids (Rodrigues et al., 2010). The volatile acids mainly comprise acetic (C2), butyric (C4), caproic (C6), caprylic (C8), capric (C10) and lauric (C12) (Angelino, 1991; Horak, Culik, Jurkova, Cejka, & Kellner, 2008). Other minor but relatively potent volatile acids found in beer are 3-methylbutanoic (cheesy, sweaty) and 2-phenylacetic acids (honey-like, sweet) (Angelino, 1991). Whereas the nonvolatile acids contribute to the sour taste of beers, the volatile acids of C2 to C12 exert a significant effect on the sour and/or caprylic odour of beer if present in high concentrations (Clapperton, 1978). Some of the acids originate from the wort, whereas others are derived from yeast autolysis and metabolism.

Brewing yeasts are known to produce both nonvolatile and volatile acids during fermentation and beer aging. The nonvolatile acids produced by the yeasts are pyruvic, α-ketoglutaric, succinic and lactic acids, although usually in small quantities, whereas most of the volatile acids are formed by the yeasts. Yeast autolysis also produces the longer-chain fatty acid due to membrane lipid breakdown, especially the unsaturated fatty acids, which, upon oxidation, would affect beer flavour adversely. Quantitatively the most significant odour-active volatile acids derived from yeast metabolism are acetic, caprylic, capric and lauric acids (Amata & Germain, 1990; Clapperton, 1978). Production of nonvolatile and volatile acids by yeasts is predominantly associated with glycolysis (e.g. C2), the TCA cycle (e.g. the nonvolatile organic acids), amino acid metabolism (e.g. branch-chained and aromatic acids) and fatty acid metabolism (e.g. C4–C18 fatty acids).

17.3.2.4 Esters

Esters of short-chain and branched-chain fatty acids, which are most aroma-active, are arguably the most important volatile compounds in beer. They have a positive impact

on the overall beer flavour, especially aroma, but excessive levels of esters can lead to overly fruity, fermented off-flavour. Esters found in beer can be categorised into two main groups: acetate esters (typically ethyl acetate, isoamyl acetate and 2-phenylethyl acetate) and ester esters of medium-chain fatty acids (mainly ethyl hexanoate and ethyl octanoate) (Angelino, 1991). Other quantitatively minor but organoleptically important esters include active amyl acetate, isobutyl acetate, ethyl butyrate, ethyl decanoate and ethyl dodecanoate (Angelino, 1991); all of which may affect beer aroma, collectively in synergy and/or additively. In general, esters impart fruity flavour notes with sensory descriptions ranging from fruity and solvent-like (ethyl acetate), banana- and pear-like (isoamyl acetate), rose- and honey-like (2-phenylethyl acetate), or apple-like and sweet (ethyl hexanoate and ethyl octanoate).

Esters can be synthesised chemically or biologically in beer. Brewing yeasts are undoubtedly the principal ester producers in beer fermentation. Esters and their formation mechanisms in brewing *S. cerevisiae* yeast strains have attracted much research attention and have been reviewed several times (Peddie, 1990; Pires et al., 2014; Saerens, Delvaux, Verstrepen, & Thevelein, 2010; Verstrepen et al., 2003). Therefore, it is beyond the scope of this section to elaborate the details of ester biosynthesis, and interested readers are referred to these review articles for further information. However, a summary of ester biosynthesis based on these reviews are provided below.

Ester formation is associated with yeast growth in the early phase of fermentation. Acetate esters are produced via the reaction between an alcohol and acetyl Co-A, which is catalysed by the enzyme alcohol acetyl transferases (ATF1 and ATF2). Ethanol, branched-chain alcohols and 2-phenylethanol are the common moieties of acetate esters. Ethyl esters of medium-chain fatty acids are formed through the reaction between ethanol and respective fatty acyl Co-A, which is catalysed by the enzyme alcohol acyl transferases. *Saccharomyces cerevisiae* strains also produce esterases that hydrolyse esters, and thus the final concentration of esters in beers is the net balance between ester synthesis and hydrolysis. Strains of brewing yeasts produce predominantly ethyl esters of fatty acids, particularly ethyl octanoate, with relatively little formation of acetate esters. Ester production in beer is regulated by a number of factors such as yeast strain, temperature, hydrostatic pressure, wort composition, sugar type and concentration, type and amount of yeast-assimilable nitrogen, aeration, and unsaturated fatty acids (Hiralal, Olaniran, & Pillay, 2014; Pires et al., 2014).

17.3.2.5 Ketones

Beer does not seem to contain many ketones. Among the few ketones found in beer, such as 2-nonanone, β-damascenone, β-ionone, 2,3-butanedione (diacetyl) and 2,3-pentanedione (Angelino, 1991), the vicinal diketones diacetyl and 2,3-pentanedione are the most important ketones with respect to their impact on beer flavour (Krogerus & Gibson, 2013a). Diacetyl is a very potent, volatile odour-active compound with a butter- or butterscotch-like odour, causing a flavour defect in most beer styles, especially lager-style beers. On the other hand, 2,3-pentanedione has a toffee-like aroma but is not as potent as diacetyl and can also bring off-flavour in beer. The vicinal diketones are generally undesirable but may constitute a special feature in some beer styles such as some English ales (Yeo & Liu, 2014).

Brewer's yeasts are the indirect producers of the vicinal diketones in beer in the sense that they produce the precursors to the diketones during metabolism. The formation pathways of diacetyl and 2,3-pentanedione, as well as their control, have recently been reviewed by Krogerus and Gibson (2013a). Briefly, their precursors α-acetolactate and α-acetohydroxybutyrate (both are the respective intermediates in the biosynthesis of valine and isoleucine) are secreted out of the cells during yeast growth and metabolism, respectively, due to certain rate-limiting steps in the biosynthetic pathways. The α-acetohydroxy acids α-acetolactate and α-acetohydroxybutyrate in the fermenting wort are then nonenzymatically and oxidatively decarboxylated into diacetyl and 2,3-pentanedione, respectively.

Yeast cells also have the ability to reduce the vicinal diketones to acetoin, 2,3-butanediol and 2,3-pentanediol, which have much higher odour detection thresholds and which are the biological basis of the industrial practice of 'diacetyl rest' to decrease or even remove the vicinal diketones. Vicinal diketones can be controlled by regulating process conditions, wort composition, fermentation technique and yeast strain improvement or modification (Krogerus & Gibson, 2013a). Restriction of oxygen ingress and addition of selected amino acids such as branched-chain amino acids (e.g. valine) are effective measures of reducing vicinal diketone production in beer (Krogerus & Gibson, 2013b).

17.3.2.6 Volatile phenolic compounds

A number of odour-active volatile phenolic compounds are present in different beers, including guaiacol, 4-vinylsyringol, 4-vinylguaiacol, 4-vinylphenol, 4-ethylguaiacol, 4-ethylphenol, eugenol and vanillin (Vanbeneden, Gils, Delvaux, & Delvaux, 2008). Most volatile phenols impart various odour notes including clove-like, spicy, smoky, medicinal or phenolic (collectively known as 'phenolic odour' or 'phenolic off-flavour'). Phenolic flavour is undesirable in most beer types, but is regarded as an attribute or even as essential in certain specialty beers such as lambic and wheat beers.

Only some of the odour-active volatile phenols are produced by yeasts during beer fermentation, mainly 4-vinylguaiacol, 4-vinylphenol, 4-ethylguaiacol and 4-ethylphenol. The substrates for these volatile phenols are phenolic acids, particularly ferulic and *p*-coumaric acids, which are most abundant in wort and beer. Volatile phenols are generated from the enzymatic decarboxylation of the respective phenolic acids, ferulic acid and *p*-coumaric acid, with the production of corresponding 4-vinylguaiacol and 4-vinylphenol, which may or may not be reduced to 4-ethylguaiacol and 4-ethylphenol, respectively, depending on the yeasts (Vanbeneden, et al., 2008).

Apparently *S. cerevisiae* yeast strains are able to carry out only the decarboxylation step (Cogher et al., 2004; Tchobanov et al., 2008; Vanbeneden, et al., 2008), which may explain the frequent occurrences of 4-vinylguaiacol and 4-vinylphenol, rather than 4-ethylguaiacol and 4-ethylphenol, in most beer types such as ale and lager. On the other hand, some yeasts of *Brettanomyces* sp. are capable of carrying out both decarboxylation and reduction steps (Godoy et al., 2009; Tchobanov et al., 2008; Vanbeneden et al., 2008), which may account for the common presence of 4-ethylguaiacol and 4-ethylphenol in specialty beers fermented with *Brettanomyces* yeasts.

Besides the yeast conversion of phenolic acids into flavour-active volatile phenols that may have an adverse impact on beer aroma as discussed above, there is an interest in the contribution of phenolic acids to beer antioxidant activity (Piazzon et al., 2010). Indeed, the use of hydrolytic enzymes (e.g. esterases) to release phenolic acids during mashing has been reported (Szwajgier, 2011). It must be cautioned that boosting the level of phenolic acids such as ferulic acid in the wort may increase the content of volatile phenols in the finished product, and a flavour defect may ensue.

17.3.2.7 Terpenoids

Hops play a crucial role in beer flavour by contributing not only the bitter taste but also the fruity, citrus-like, floral aroma; the latter is ascribed to terpenes and terpenoid compounds in hops (Schonberger & Kostelecky, 2011). The terpenes and terpenoids found in hops and beer include myrcene, limonene, farnescene, humulene, β-caryophyllene, β-citronellol, nerol, α-terpineol, linalool, geraniol, citronellyl acetate, geranyl 2-methylpropanoate and geranyl acetate, etc. (Angelino, 1991; King & Dickinson, 2003). Some of the terpenes are carried over from the hops, whereas others such as terpenoid esters and terpene alcohols are produced or released from other monoterpene alcohols and glycoside precursors during yeast fermentation (Figure 17.1).

Monoterpenes decrease to trace levels during fermentation, either due to degradation by yeasts or binding to yeast cells (King & Dickinson, 2003). More significantly, monoterpene alcohols undergo a series of complex biotransformation by yeasts. As illustrated in Figure 17.1, geraniol (most abundant in wort) is converted into β-citronellol (almost absent in wort), while linalool is produced from both geraniol and nerol; then α-terpineol is produced from nerol and linalool. Most of the monoterpene alcohols can be transformed into esters, especially acetate esters. It is expected that these biotransformations would have an impact on beer aroma, since oxygenated terpenes possess subtle odour differences, for example, rose-like/floral for geraniol, fresh, coriander or lavender for linalool, citrus (lemon or lime) for β-citronellol, floral, fresh, or green for nerol, and lilac for α-terpineol.

It appears that coexistence of geraniol, β-citronellol and excess linalool have an additive or synergistic effect on the total flavour impression by maximising the citrus character (Takoi, Itoga et al., 2010; Takoi, Koie et al., 2010). One strategy to realise the coexistence of the three monoterpene alcohols is to control the degree of yeast

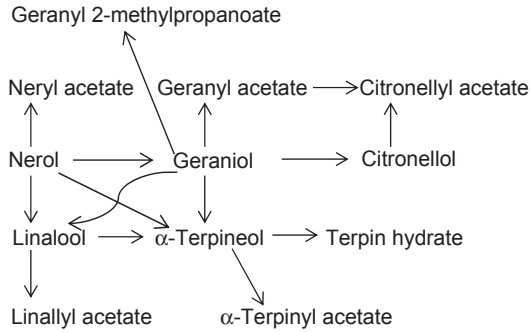

Figure 17.1 Proposed pathways of terpenoid biotransformation by yeasts. Modified after King and Dickinson (2000, 2003).

biotransformation by delaying hop addition so as to retain some geraniol in the finished beer (Takoi et al., 2014). Besides the flavour impact of terpene alcohols, terpenoid acetate esters add more fruitiness. Furthermore, some terpenoids are released from glycosidically bound precursors due to the action of yeast glycosidase activities (Praet, Van Opstaele, Jaskula-Goiris, Aerts, & De cooman, 2012; Takoi, Koie et al., 2010). Thus, biotransformation of oxygenated terpenes would at least partially explain the hoppy aroma differences between the raw hops and the finished beer (plus some other odorous volatiles in the beer).

17.3.2.8 Volatile sulphur compounds

Numerous odour-active VSCs have been detected in beers. Examples of beer VSCs encompass methanethiol, ethanethiol, H_2S, dimethyl sulphide, dimethyl disulphide, methional, methionol, 3-(methylthio)propyl aceate and 2-mercapto-3-methyl-1-butanol (Angelino, 1991; Hill & Smith, 2000). Most VSCs cause off-odours such as rotten egg-like, cabbage-like, onion-like and garlic-like. However, some VSCs have a positive impact on beer flavour by accentuating fruitiness (e.g. 3-mercaptohexanol and 3-mercaptohexyl acetate).

Brewing yeasts contribute to the genesis of some VSCs in two modes: direct production of VSCs and further chemical and/or a combination of chemical and yeast-mediated conversions of these VSCs into other potent odourous VSCs. H_2S is a well-known off-odour volatile produced by yeasts from cysteine or sulphate via the sulphate reduction sequence, especially under nitrogen limitation (Duan et al., 2004; Ogata, 2013; Swiegers & Pretorius, 2007). The content of H_2S in beer usually decreases to trace levels toward the final stage of fermentation, possibly due to re-assimilation or binding by yeast cells (Oka, Hayashi, Matsumoto, & Yanase, 2008). However, H_2S can react with unsaturated aldehydes or ketones to generate several thiols that may be desirable or undesirable for beer flavour (Vermeulen, Lejeune, Tran, & Collin, 2006).

Methional is known to be a key contributor to the worty flavour of alcohol-free beers (Perpete & Collin, 1999). This sulphur-containing aldehyde and methionol are also precursors to dimethyl trisulphide in aged beers (giving off onion- and garlic-like off-odours) (Gijs, Perpete, Timmermans, & Collin, 2000). Metabolism of methionine via the Ehrlich pathway in yeasts leads to the production of a variety of VSCs, including methional, methionol, methionic acid, methionyl acetate and ethyl 3-methylthio-1-propanoate (Figure 17.2). Methional is mostly reduced to methionol, which is the VSC of methionine metabolism by yeasts. There is evidence that trace amounts of the highly potent methional (raw potato-like odour) with a very low detection threshold at a few ppb (0.2–40 ppb) can be secreted out of the yeast cells (Liu & Crow, 2010; Quek, Seow, Ong, & Liu, 2011; Seow, Ong, & Liu, 2010; Tan, Lee, Seow, Ong, & Liu, 2012).

Cysteine and homocysteine are also important precursors of VSCs, as shown in Figure 17.2. A number of thiols can be generated from cysteine, cysteine conjugate and homocysteine transformation during yeast fermentation, including 2-mercaptoethanol, 3-mercaptoethanol, 3-mercaptohexanol,

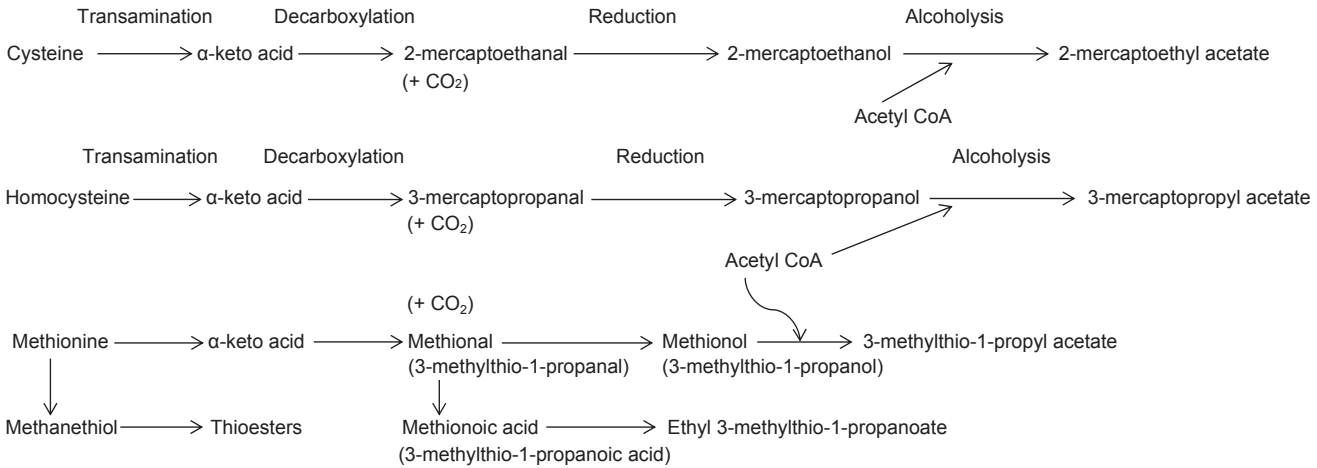

Figure 17.2 Proposed pathways of bioproduction of volatile sulphur compounds from cysteine, homocysteine and methionine by yeasts. Modified after Vermeulen et al. (2006) and Tan et al. (2012).

4-mercapto-4-methyl-2-pentanone and acetate esters such as 3-mercaptohexy acetate (Gros, Peeters, & Collin, 2012; Kishimoto, Morimoto, Kobayashi, Tako, & Wanikawa, 2008; Nizet et al., 2013).

17.4 Impact of bacteria on beer appearance and flavour

Relative to the impact of yeast on beer appearance and flavour as discussed above, information is scarce on the impact of bacteria on beer appearance and flavour. Bacteria mainly cause turbidity, sediments, acidification, off-flavour formation and ropiness (Sakamoto & Konings, 2003; Suzuki, 2011; Suzuki et al., 2008). Suzuki (2011) and Vriesekoop, Krahl, Hucker, and Menz (2012) recently summarised the positive and negative influences of bacteria on flavour and off-flavour in recent reviews, which form the basis of this section. Interested readers are referred to these articles for further details.

On the whole, Gram-positive bacteria are inhibited by the hop constituents and do not grow in beer. However, some LAB (mainly lactobacilli and pediococci) are hop-resistant and can grow in beer. On the other hand, foodborne pathogenic bacteria do not grow or survive in the harsh environment of beer. The main bacteria that can grow in beer and affect beer appearance and flavour are those from the genera of *Lactobacillus*, *Pediococcus*, *Pectinatus* and *Megasphaera*, besides the less frequent bacteria, acetic acid bacteria, some *Enterobacteria,* and *Zymomonas*.

LAB can have both negative and positive impacts on beer quality. The negative impact includes high turbidity, sedimentation, acidification and off-flavour, typically diacetyl production, although diacetyl is not always formed by these bacteria. In contrast, LAB play a beneficial role in the production of acidulated malt, acidified wort and acidic beers (Van Nierop et al., 2006; Verachtert & Derdelinckx, 2014; Vriesekoop et al., 2012). The benefits of biological acidification include lower risk of protein haze formation and microbial contamination (and associated microbial turbidity, sediments, and off-flavour), finer foam bubbles and stable, longer-lasting foam, fresher mouth-feel, smoother bitterness, and fuller and smoother flavour profile, as summed up in the review of Vriesekoop et al. (2012). It should be stressed that strains selected for biological acidification must not produce diacetyl.

Pectinatus can spoil beers, resulting in increased turbidity, a sour taste, and a rotten egg odour due to the production of acetic, propionic, and other acids as well as hydrogen sulphide. *Megasphaera* also increases beer turbidity, together with the production of hydrogen sulphide and a variety of odorous fatty acids including butyric, valeric, and caproic acids that are undesirable for beer aroma. Acetic acid bacteria can bring about off-flavour by producing acetic acid, which imparts a vinegary odour, in the presence of oxygen. Enterobacteria such as *Shimwellia pseudoproteus*, *Citrobacter freundii* and *Rahnella aquatilis* can spoil beer by producing a range of off-odour compounds such as acetoin, diacetyl, 2,3-butanediol, acetaldehyde, lactic acid, dimethyl sulphide, propanol, and/or isobutanol. The maltose-negative *Zymomonas mobilis* can grow in beers supplemented with sucrose and

produce high levels of acetaldehyde, hydrogen sulphide and sorbitol, imparting 'green, sulphuric' characters and sweetness.

17.5 Future trends

Beer has been the subject of intensive biotechnological research in recent years to improve appearance and flavour as well as to add functional and technological benefits. With the advent and maturation of molecular biology over the past few decades, genetic engineering is a typical tool that has been exploited extensively to modify brewing yeast strains for the production of beers with specific functional attributes. Examples include low-alcohol lager beer in which the glycerol level is increased (Nevoigt et al., 2002), low-calorie beer in which the carbohydrate content is decreased (Park et al., 2014; Wang et al., 2010) and less turbid fresh lager beer in which the haze particle size is reduced (Omura, Nakao, Teranishi, & Fujita, 2009). Even though genetic modification still faces consumer resistance, it is conceivable that genetic modification of brewing yeasts will continue in the foreseeable future to advance the brewing process and beer quality.

Recent years have also seen the rising application of self-cloning techniques and mutagenesis in improving brewer's yeast strains for alcohol reduction and flavour enhancement, which is at least partially due to consumer aversion toward genetic engineering. Wang, He, Liu, and Zhang (2008) successfully used self-cloning to construct brewing yeast strains to increase glutathione production and to decrease diacetyl formation, and Iijima and Ogata (2010) constructed a self-cloning brewing yeast strain with enhanced sulphite production and diminished formation of undesirable VSCs including hydrogen sulfide, 3-methyl-2-buten-1-thiol and 2-mercapto-3-methyl-1-butanol. Furthermore, various spontaneous mutants of brewing yeasts are applied to the production of alcohol-free or low-alcohol beers as well as to the making of high-tyrosol sake for health benefits such as an antioxidant (Selecky, Smogrovicova, & Sulo, 2008; Soejima, Tsuge, Yoshimura, Sawada, & Kitagaki, 2012; Strejc, Siristova, Karabin, Almeida e Silva, & Branyik, 2013). Self-cloning and mutagenesis do not involve foreign DNA and are expected to be more acceptable to the public. Therefore, it may be envisaged that more research will be conducted to improve beer appearance and flavour using such techniques in the coming years.

In this 'omics' age, more research is being directed at the genomics, metabolomics, lipidomics and proteomics of brewing yeasts with a view to better understand their impact on beer appearance and flavour such as foam, haze, flocculation and flavour stability (Stewart et al., 2013). An example is the application of proteomics in elucidating the role of proteins of barley malt and yeast origins in beer foam, haze and flavour (Wu et al., 2012). The proteomic approach helped reveal that some cysteine-rich yeast proteins such as thioredoxin can exert a negative effect on foam stability but a positive impact on flavour stability (the latter being due to the higher thiol content acting as antioxidants) (Wu et al., 2012). Thus, it is not difficult to envision that the 'omics' approach will continue to be taken in this field.

Specialty beers are gaining popularity among beer drinkers who seek novelty and/health benefits, such as alcohol-free, low-alcohol, low-calorie and fruity beers (Yeo & Liu, 2014). Acidic beers such as lambics fall into the domain of specialty beers and are naturally fermented with mainly yeasts and LAB (Verachtert & Derdelinckx, 2014; Vriesekoop et al., 2012). An alternative to spontaneous fermentation for producing acidic beers is to use lactic acid-producing yeast such as *L. thermotolerans*, which has been trialled in wine fermentation (Gobbi et al., 2013). Interestingly, a new category of specialty beer, acidic probiotic beer, may emerge in the near future to take advantage of increasingly popular probiotic health cultures (Yeo & Liu, 2004). This potential beer is expected to contain live probiotics and must be unfiltered and unpasteurised. Furthermore, it would be necessary to select probiotic bacteria that are hop-resistant to ensure survival and do not produce diacetyl.

17.6 Further information

Journal of the Institute of Brewing has published a series of reviews (125th Anniversary Reviews) on various aspects of brewing and beer, including bacterial and yeast impact on beer appearance and flavour. The 125th Anniversary Reviews Virtual Issue is recommended for further reading. The Leuven Institute for Beer Research (http://libr.be/) is a source of information on brewing yeast and beer fermentation in relation to impact on appearance and flavour, in addition to the International Centre for Brewing and Distilling (http://www.icbd.hw.ac.uk/).

Information covered in this chapter can also be found in several journals devoted to beer and brewing listed below:

The Journal of the American Society of Brewing Chemists (http://www.asbcnet.org/journal/default.htm)
Journal of the Institute of Brewing
(http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)2050-0416)
Master Brewers Association of the Americas Technical Quarterly.
(http://www.mbaa.com/publications/tq/Pages/default.aspx)
Brewing Science – Monatsschrift für Brauwissenschaft (http://www.brewingscience.de/)
Cerevisia (http://www.journals.elsevier.com/cerevisia)

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Sensory analysis as a tool for beer quality assessment with an emphasis on its use for microbial control in the brewery

18

G. Spedding[1], *T. Aiken*[2]
[1]Brewing and Distilling Analytical Services, LLC., Lexington, KY, USA;
[2]Data Collection Solutions, Lexington, KY, USA

18.1 Introduction

Sensory analysis of beer forms a complex topic, the mechanics of which have been well documented in entire volumes. A single chapter here is not adequate to thoroughly explore the subject. An attempt then is made here to outline some key sensory terms and approaches to investigating, mainly from an organoleptic perspective, key flavors produced by microorganisms that are regarded either as unwanted contaminants, causing beer spoilage, or as desired organisms used to produce distinctive flavor profiles in specialty beers (Part 1). In this regard, we believe this chapter to be unique. The methods outlined will indeed apply to testing other flavor attributes in beer, although the focus is on microbially generated flavor notes that might typically form only a small part of full sensory training in the brewery; that said, references will be provided for those wishing to set up a full sensory evaluation program in the modern brewery environment. Following some definitions on taints and off-flavors, a brief overview of microbial spoilage organisms and the different stages at which they provoke either the damage to flavor quality or desirability for consumption, or provide desirable aroma/flavor properties to unique beer styles, is made. This is followed by an outline of the key properties of the atypical flavor notes that they provide and descriptors to be learned by a trained sensory panel or team. Finally, a brief review of setting up sensory evaluation programs completes the chapter (Part 2). Organisms generating atypical flavor notes in beer are outlined (and covered in more detail elsewhere in this volume, along with other aspects of beer spoilage) and then those flavor notes are described, from a point of understanding how and when they are evaluated, using the senses as related to contamination and beer flavor spoilage issues or to the specific use of bacteria and wild yeast in creating unique flavor profiles.

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18.2 Part 1: microbes, flavors, off-flavors, and taints in brewing

18.2.1 Microbial spoilage overview

Mold, yeast, and bacterial spoilage of beverages can mean different things—overcarbonation and package rupture, hazes, sediments, visibility of colonies in the product, etc. (Boulton & Quain, 2001; Hill, 2009; Romano, Capece, & Jespersen, 2006; Stratford, 2006)—but here we focus on flavor changes involved and the assessment of those changes through sensory evaluation. In addition to desired culture yeast or bacteria in the case of "Brett" (*Brettanomyces*), sour or other beer styles—the brewer making use of unique bacterial strains—a spoilage yeast or bacteria is one with ability to cause spoilage over a short or longer timeframe dependent upon actual growth conditions (Stratford, 2006). Detectable spoilage requires a great number of yeasts (and/or bacteria) in the order of 1×10^5–1×10^6 cells (Stratford, 2006) and it is, therefore, only noted or detected through the continued growth, within a contaminated product, of the yeast or bacterial population. Such strains can be isolated and examined, but, with the generally low threshold of detection for many of the diverse metabolic or autolysis-derived components produced, spoilage may often be determined by sensory means well before the physical detection of deterioration of a product or the actual identification of specific microbial species present.

Most spoilage yeasts and bacteria are nonpathogenic, and the properties of beer ensure that no pathogens can survive the process and in the packaged product (Hill, 2009; Suzuki, 2011; Vaughan, O'Sullivan, & van Sinderen, 2005; Vriesekoop, Krahl, Hucker, & Menz, 2012). However, spoilage can occur early on in the production of beer, and any taint flavors (defined below) will often remain, leading to a "spoiled" product. That said, mycotoxin-forming molds can pose a health risk from tainted raw materials, although again, issues would be more obvious via gushing of beer contents or moldy flavor notes rather than through any illnesses caused by such organisms (Hill, 2009; Vaughan et al., 2005).

Although beer contaminated with spoilage organisms will normally not be harmful, the results of the spoilage may be noticeable by consumers and will be rejected by them. Microbial spoilage of beer is, therefore, defined as growth of the spoilage organisms to a sufficient level as to promote an alteration in that beer perceptible to a consumer and liable to cause dissatisfaction, complaint, or rejection of that beer (Stratford, 2006).

Visual cues such as distorted cans (due to overcarbonation), hazes, or visible yeast colonies, blooms (surface films), or pellicles, etc., can provide clues to spoiled product (Stratford, 2006), as can beer that pours in a viscous–oily way; but it is odor/flavor that we turn our main sensory attention to here.

18.2.2 Off-flavors and off-odors

In general, consumers are not sufficiently aware as to what constitutes an off-flavor or taint (defined below) in beer unless it presents in a very pronounced way. However,

they are becoming more and more educated as to several significant issues such as diacetyl (see more later). Yeast and bacteria produce organoleptically powerful (low threshold detection) metabolic by-products; many are quite volatile, which means that they present both as off-odors and as off-tastes. This chapter deals with those organisms, given the right conditions that can grow and either spoil the beer organoleptically in the main or provide those desirable but atypical flavors in specialty beers. It is to be noted that trained panels can detect flavor issues at levels below which consumers may notice them.

18.2.3 Taints and off-flavors

To follow the approaches to understanding the microbial metabolites that have a sensory impact and to generating sensory training programs (outlined in Part 2), a definition of taints and of off-flavors is in order (Hughes, 2009; Kilcast, 1996; Saxby, 1996). A taint or an off-flavor is caused by the presence of a chemical that imparts a flavor that is unacceptable/unusual (or is atypical) in a food or beverage product. A taint is often defined further as the presence of a substance totally alien to all foods (and may include components imparting atypical flavors or odors from external sources such as air, water, packaging materials, processing lines, etc.).

An off-flavor is defined as arising from a chemical reaction of a naturally occurring component in the food or beverage (or through internal deteriorative changes), giving rise to an atypical compound with an undesirable or unexpected taste. For purposes of microbiologically derived flavors, a metabolic by-product (or perhaps autolytic components) leading to atypical or unwanted flavors or odors in beer would be regarded as an off-flavor rather than a taint. Microbes can, however, metabolize certain compounds derived from disinfectants and sanitizers, for example, to generate undesirable flavors such as chloroanisoles (moldy or musty accents) from the methylation of chlorophenols (in water supplies) or the production, by cyanobacteria, of low threshold–detectable compounds such as geosmin (conveying an earthy, musty, or beetroot-like aroma), which would be undesirable in any beverage. Such flavor notes would be considered as taints rather than off-flavors. Other sources of taints may be from mold-derived flavors carried in to beer production from contaminated raw materials. Generally we will be referring to off-flavors, not taints, through most of this chapter (but see Part 2).

18.2.4 Air, water, and raw materials

The potential for beer spoilage occurs prior to the production of wort (see below). Water supplies and all raw materials, as well as any exposure to air or surfaces that come into contact with wort or beer, may carry wild yeast, molds, and/or bacteria that may cause downstream processing problems. These sources can also be addressed in sensory programs if their key “microbial contamination indicators” are based on sensory perception. Air will also carry a multitude of microorganisms that are taken advantage of for spontaneously fermented beer production.

18.3 The microbiology of "atypical flavor" production in brewing—an overview

18.3.1 Molds

A number of fungi and molds may infect barley and stored malt, and some fungi of the *Fusarium* genera are associated with gushing (the violent spontaneous ejection of beer from containers) (Hill, 2009). This is clearly noted visually, and any beer showing gushing should be examined for its cause, including microbiological contamination. Although molds are not spoilers of wort or beer, their presence in barley may have a negative impact on the quality of the malt, wort, and beer (Vaughan et al., 2005). Observations have been made that mold growth on malt can be responsible for strong off-flavors in beer produced from it. These off-flavors ranged from 'burnt molasses' to 'unclean', 'winey', and 'harsh' (Kneen, 1963, p. 51; cited in Vaughan et al., 2005; the latter terms are too vague today, and such tainted beers need more technical evaluation to clearly define sensory terms). Beer brewed with malt contaminated with *Aspergillus fumigatus* had pronounced roughness (again a vague term Kneen, 1963, p. 51; cited in Vaughan et al., 2005) and a stale flavor (defined stale flavor notes are actually better understood today and covered on the beer flavor wheel discussed in Part 2). Suffice to say, molds can carry through to beer in the form of a range of off-tastes and odors, and raw materials such as barley malt should be taste tested to ensure that they are free of moldy, musty, or earthy taints.

With respect to water supplies algal blooms (often seasonally) may invoke the flavor taint of geosmin (Westerhoff, Rodriguez-Hernandez, Baker, & Sommerfeld, 2005). Many cyanobacteria (blue-green algae) produce intracellular and extracellular metabolites, including taste and odor compounds in water supplies such as 2-methylisoborneol and trans-1,10-dimethyl-trans-9-decalol (geosmin). Occurring in nanogram-per-liter levels (parts per trillion!) in surface water supplies, they are responsible for many taste and odor complaints about the aesthetics of drinking water (Westerhoff et al., 2005). They are unpalatable, imparting earthy/musty/moldy tastes and odors to drinking water, are difficult to remove, and can end up causing taints in products made with such contaminated water sources. As stated in the paragraph on molds above, we regard these notes as taints (defined above) rather than off-flavors and end the main discussion about them here.

18.3.2 Wort and beer—overview

Wort and beer are both perishable liquids prone to microbial attack. While beer provides a less hospitable environment for microbial growth than the initially nonsterile beer wort (Hill, 2009; Suzuki, 2011; Vriesekoop et al., 2012), the solution still retains residual sugars, nitrogenous compounds, minerals, and vitamins that can provide nutrients for bacterial and wild-yeast contaminants to thrive. The pH of beer, normally around 4 to 5, is also favorable for the survival and growth of certain species of bacteria. Overall, however, a limited number of the multitude of known microorganisms are responsible for beer spoilage, comprising a few species of bacteria and

wild yeasts (Ault, 1965; Back, 2005; Boulton & Quain, 2001; Hill, 2009; Linske & Weygandt, 2013; Manzano et al., 2011; Middlekauff, 1995; Priest & Campbell, 1987; Rainbow, 1981; Spedding & Lyons, 2001; Storgårds, 2000; Storgårds, Haikara, & Juvonen, 2006; Suzuki, 2011; Vaughan et al., 2005; Vriesekoop et al., 2012). Molds are not regarded as beer spoilage organisms, as they require oxygen to grow; but they can, as noted above, cause flavor issues through their contamination of raw materials. Many of the details regarding the growth and properties of the organisms responsible for beer spoilage are presented throughout this volume and in the references cited immediately above, so only a brief description is provided below with respect to those producing flavor notes of interest for sensory detection and evaluation purposes.

Descriptions of many key organisms involved in spoilage, together with the metabolites causing detrimental sensory qualities to the beer, are presented briefly below and in Tables 18.1–18.3. The properties of the indicator compounds addressed in Table 18.3 include threshold of detection values, sensory descriptors, and potential changes in sensory qualities imparted to beer via their presence, which then lead to evaluation of the key flavor notes in a sensory program, as discussed in Part 2. With regard to Table 18.3, the typical levels of odor/flavor compounds in beer and threshold values are obtained from various sources (Anderson et al., 2000; Angelino, 1991; Engan, 1981; Kunze, 2010; O'Rourke, 2000; Taylor & Organ, 2009; Spedding, 2013) and are to be regarded as guides only; these values vary by type and style of beer as well as production methods. A threshold value is the concentration at which an aroma or taste can be detected as here in beer or beer wort, with a recognition threshold being a concentration at which a compound can be positively identified. As threshold values in beer and other food items are dependent upon a number of variables, the simple definitions above will have to suffice here. Threshold values may best be determined through the sensory approaches outlined in Part 2 and the references cited in that section.

18.3.3 Beer wort–spoiling bacteria

Wort is particularly susceptible to contamination by bacteria and wild yeasts. Wort provides an ideal nutrient medium for many organisms: enteric bacteria, acetic and lactic acid, and some wild yeast strains (Back, 2005; Hill, 2009). Bacterial species found are often Gram-negative asporogenous rods, and were originally termed "termo bacteria"; they are unable to develop in beer, but the off-flavors produced often carry through to finished beer. Poor attention to wort production leads to spoilage with sewer-like, parsnip, and celery notes (related to dimethyl sulfide [DMS] and other sulfur metabolites). In general termo bacteria may be regarded as mixed populations containing representatives of the Enterobacteriaceae and Pseudomonadaceae (Back, 2005). Predominantly found are species of *Enterobacter, Citrobacter, Hafnia, Klebsiella, Serratia, Pseudomonas*, and *Xanthomonas*. Some of these strains and off-flavor production are described further in Tables 18.1 and 18.3 and elsewhere (Ault, 1965; Back, 2005; Boulton & Quain, 2001; Hill, 2009; Linske & Weygandt, 2013; Manzano et al., 2011; Middlekauff, 1995; Priest & Campbell, 1987; Priest, Cowbourne, & Hough, 1974; Rainbow, 1981; Spedding & Lyons, 2001; Storgårds, 2000; Storgårds et al., 2006; Suzuki, 2011; Vaughan et al., 2005; Vriesekoop et al., 2012). *Enterobacteria* may

Table 18.1 Bacteria associated with beer spoilage with specific reference to typical flavor notes produced and general sensory flavor changes

Group, species, or genera—wort and beer spoiling bacteria (some useful for flavor production in specialty beers)	Spoilage/flavor notes produced
Acetobacter species: *A. aceti, A. hansenii, A. liquefaciens*, *A. pasteurianus*.	Produce acetic acid (vinegar) and ropiness. Aerobes so limited to certain process points can be used to impart notable levels of acid in certain specialty beers.
Enterobacteriaceae ("termo"—original designation to a loose group of wort spoiling bacteria) (see Back, 2005). Includes: *Enterobacter* *Citrobacter* (*Citrobacter freundii*) *Hafnia** *Rahnella* (*Rahnella aqualitis*) *Klebsiella* *Serratia* **Hafnia protea* (formerly *Obseumbacterium proteus*).	Wort: acetate, celery-like, parsnip, phenols, and cooked cabbage and dimethyl sulfide (DMS) notes. Acetaldehyde, diacetyl, isoamyl alcohol (fusel alcohols), and volatile organo-sulfur compounds also reported,[a] and phenolic notes. *Hafnia protea* and *Rahnella aqualitis* can produce excessive amounts of diacetyl and DMS. *Hafnia* contamination leads to a parsnip-like odor. *Hafnea protea* spoils beer and wort by producing acetoin, DMS, isobutanol, lactic acid, propanol, and 2,3-butanediol[b], and phenols[c]. *Citrobacter freundii* and *Rahnella aquatilis* produce various off-notes and aromas; acetaldehyde, acetoin diacetyl, DMS, lactic acid, and 2,3-butanediol. *Parsnip-like refers to early descriptors for wort spoilage flavor. Strong sulfury and vegetal notes indicate spoilage along with turbidity. Sensory evaluation would deal with specific flavor terms such as DMS and with research and training other specific sulfur notes and standards for the other flavors noted above. In general terms are rather vague for wort spoilage flavors*.
Gluconobacter (*Acetomonas*) *G. oxydans*.	Produce acetic acid in beer sometimes giving a cidery note.
Lactobacillus species	Turbidity and souring of beer via lactic and acetic acid formation some strains produce diacetyl and ropiness.

Megasphaera (*M. cerevisiae*)	Leads to turbidity and production of a variety of fatty acids, butyric, caproic, and valeric and isovaleric acids along with acetic acid and H_2S; manure-like aromas also described.
Micrococcus spps.	Hazes and fruity esters.
Pectinatus (*P. cerevisiiphilus*)	Acetic acid, acetoin, propionic acid, succinic acid, and turbidity. Sour and rotten egg aromas due to the H_2S and variety of acids produced. Other sulfur notes—methyl mercaptans (sewer-like notes also possible) and manure-like aromas also described.
Pediococcus species (*P. damnosus* - "Beer sarcina" – early term).	Sours beer: lactic acid and some strains produce diacetyl (esp. *P. damnosus*). Sediments and reduced foam stability may also result. Sarcina sickness referred to major *Pediococcus* infections.
Selenomonas	Acetic, lactic, and propionic acids.
Zymomonas species (*Z. mobilis*)	Causes fruity and sulfidic (H_2S) characters and acetaldehyde (rotten apples) during fermentation. Also turbidity. Higher alcohols and acetic esters, DMS (dimethylsulfide), and dimethyl disulfide also reported[d].
Zymophilus	Acetic, lactic, and propionic acids.

The data in the table represent summaries of information culled from many of the references cited in the text and so for clarity specific points are not referenced in the table itself. Certain details are also included in the text. Other details may also be found elsewhere in this volume (see Storgårds, 2000; Storgårds et al., 2006).
[a]Harrison, Webb, and Martin (1974).
[b]Middlekauff (1995).
[c]Back (2005).
[d]Back (2005) and Dennis and Young (1982).

Table 18.2 Yeast associated with beer spoilage with specific reference to typical flavor notes produced and general sensory flavor changes

Yeast strain—wort and beer spoiling yeast (some useful for flavor production in specialty beers)	Spoilage/flavor notes produced
Brettanomyces (*B. bruxellensis*) Reclassified as *Dekkera*	Imparts typical acetic acid ester aroma (high ethyl acetate fruity-solvent notes). Produce large quantities of acids; lactic and consequently ethyl lactate, and acetic acid. 4-ethylphenol and 4-ethylguaiacol also associated with Brett beers (see text).
Candida	Cloudiness and off-flavors; esters (ethyl acetate), acids (acetic) and phenols (4-vinylguaiacol).
Hansenula (now merged with genus *Pichia*, Kurtzman, 1984, 1986)	Cloudiness and off-flavors (high ethyl acetate ester production; solvent-like odor) (see *Pichia*).
Kloeckera	Produces acid, acetic and lactic, esters (ethyl acetate: fruity odor), and cloudiness in beer.
Pichia (*P. anomala*)	Cloudiness and off-flavors. Volatile phenols (4-vinylguaiacol), ethyl acetate, amyl acetate (higher alcohols). Aerobic: spoilage potential limited to beers stored in the presence of air. However, under suitable conditions, they grow rapidly and often give rise to films on the surface of the beer as well as resulting in the production of hazes and off-flavors.
Saccharomyces (Wild strains)	Phenolic off-flavors and contamination can lead to overcarbonation of beer via overattenuation.

The data in the table represent summaries of information obtained from many of the references cited in the text. For clarity specific points are not generally referenced in the table itself. Certain details are also included in the text.

grow in warm wort, causing unwanted flavors such as hydrogen sulfide, acetaldehyde, vinegar and other acidic flavors, diacetyl, phenolic compounds, and fruity off-flavors from ester formation. These flavors are produced in quantities greater than typically produced by culture yeast during normal fermentations. Culture or pitching yeast may also be a source of both bacteria and wild yeast that can affect beer flavor and stability. As stated above, wort spoilage flavors often persist through the brewing process.

A second group of wort bacteria include *Bacillus* and *Clostridium* species, sporogenic species with high heat–resistant spores that may enter into beer processes via raw materials (malt and hops). These are associated with the production of butyric acid and sulfur compounds (Back, 2005). A third group includes the genera *Lactobacillus*, *Enterococcus*, *Lactococcus*, and *Pediococcus* many producing lactic acid and diacetyl (Back, 2005).

Table 18.3 Characteristic flavor notes found in beer and associated with yeast and bacterial metabolism

Flavor note/indicator compound (generic and specific names)	General flavor descriptors[a]	Typical concentration (ppm) found in beer[a]	Typical threshold mg/L (ppm)[b] (thresholds vary with beer style)	General notes and example associated microorganisms capable of producing the flavor note (see also Tables 18.1 and 18.2)
Acidic (generic; see specific acids)	Sour cream (*sourness and apples); see also acetic and lactic.	30–280	Varies with acid; see individual acids	*Lactobacillus* spps. (see details in Table 18.1) (**Acetomonas*). Microorganisms may produce various acids and give a sour/tart note to beer.
Acetaldehyde (ethanol)	Apples, emulsion paint, grassy (green/bruised apple), avocado, green leaves, melon, and pumpkin.	2–15	5–15	*Acetomonas*/*Gluconobacter*/Zymomonas
Acetoin (3-hydroxy-2-butanone)	Buttery, sweet-buttery, creamy aroma, dairy, milk, fatty. Fruity nuances.	2.9–19.3	Detected at 150 mg/L in aq. ethanol 8–20 in beer	Lactic acid bacteria, *Pediococcus*, and *Megasphaera*
Acetic acid (ethanoic acid) (see acidic)	Sour, vinegar, acidic, acetic.	30–200	130–200	*Acetobacter*
Autolysis of yeast	Yeasty, sulfury, broth/bouillon or meaty-like.	–	–	Notes associated with yeast autolysis also include caproic, caprylic, and other medium chain fatty acids.
Butyric acid (butanoic acid)	Rancid, sharp cheese, baby vomit, pungent/putrid, sour spent grains.	0.6–3.3 (0.5–1.5 ppm more typical in beer?)	2–3	Produced by wort spoiling bacteria and will not volatilize away—carries through to finished beer. Occasionally formed during bacterial spoilage of packaged beer (*Megasphaera* and *Pectinatus*).
Caproic acid (hexanoic) acid	Sour, fatty, sweat, cheese.	1–5.8	2.3?	*Megasphaera, Clostridium* spps.

Continued

Table 18.3 Continued

Flavor note/indicator compound (generic and specific names)	General flavor descriptors[a]	Typical concentration found in beer[a]	Typical threshold mg/L (ppm)[b] (thresholds vary with beer style)	General notes and example associated microorganisms capable of producing the flavor note (see also Tables 18.1 and 18.2)
Caprylic acid (octanoic) acid	Goaty, waxy, fatty, rancid, cheesy, tallow.	2–14.7	4–6	Part of the caprylic flavor (several medium chain fatty acids associated (low levels) with pale lager beers (released during autolysis of yeast)) (Tressl, Bahri, & Kossa, 1980). May be produced by *Brettanomyces* (wet leather, goat-like, wet dog notes associated with caprylic, capric, and caproic acids).
Cheesy (general note)	Old cheese, sweaty, rancid fat, old hops, stale (see isovaleric acid).	–	–	General descriptor associated with fatty acids; see e.g., isovaleric acid.
Diacetyl (2,3-butanedione)	Butter, butterscotch, movie popcorn (toffee—but may be suggestive based on butteriness?), may also give an oily mouthfeel sensation.	0.08–0.6	0.08 (varies with the beer)	*Lactobacillus*/*Pediococcii* Referred to in early days of brewing as "Sarcina sickness." Yeast strains too (culture yeast and brewing process related (Krogerus & Gibson, 2013)—yeast mutations and wild yeast).
Dimethyl sulfide (DMS) (methylsulfanyl-methane)	Sweetcorn/creamed corn, asparagus, parsnip, tomato juice/ketchup, tinned beans, oysters, sea-spray.	0.05–0.15	0.03–0.08	*Hafnea protea* (*Obesumbacterium proteus*); other enteric bacteria and *Zymomonas* (see Table 18.1). Flavor nuances change with concentration.
4-Ethylguaiacol (4-ethyl-2-methoxyphenol)	Phenolic, clove, smoky, ash-like, bacon, smoked bacon/cheese.	Low	Little known in beer/wines	Ethyl phenol and ethyl guaiacol said to be the characteristic odors of *Brettanomyces* spps.

4-Ethylphenol	Band-aid, contaminated with *Brettanomyces*, plastic medicinal, horsey.	0.006–0.02?	Threshold and concentrations in beer not well known	Ethyl phenol and ethyl guaiacol said to be the characteristic odors of *Brettanomyces* spps. (esp. in wine, little research on beers to date) (Licker, Acree, & Henick-Kling, 1998; Romano, Perello, Lonvaud-Funel, Sicard, & de Revel, 2009; Suárez, Suárez-Lepe, Morata, & Calderón, 2007)
Ethyl acetate (most common ester in all beers)	Acetone (nail varnish remover), estery, paint thinner.	10–50	30–50	A typical component of all beer; can be elevated due to microbial contamination (incl. *Brettanomyces*).
Ethyl butyrate (ethyl butanoate)	Tropical fruit, mangoes, canned pineapple.	0.05–0.25	0.4	Common ester in beer but high levels can indicate bacterially contaminated worts. As for all esters, it derives from condensation of an alcohol and an acid (here butyric acid).
Ethyl lactate (ethyl(S)-2-hydroxypropanoate)	Fruity, strawberry.	0.1–0.8	250	High lactic acid could lead to high ethyl lactate levels.
Eugenol (4-allyl-2-methoxyphenol)	See spicy.	Zero–low	0.013? (No reference)	This compound is often used as a standard for spicy-clove phenolic notes but other phenolic compounds such as 4-vinyl guaiacol are more often found in beers at threshold levels.
Geosmin	Earthy, beetroot.	Not normally present	Detected at parts per trillion levels	*Cyanobacteria*. A taint rather than an off-flavor.
Indole (2,3-benzopyrrole)	Farmyard, like pigs on a farm, fecal, coliform, jasmine.	<0.005	0.015	*Coliform* bacteria during early fermentation.
Iso-amyl acetate (3-methylbutyl-acetate) (banana oil; pear essence)	Fruity, banana also pear drops (US: circus peanuts)	0.5–1.5 ppm (higher in wheat beers)	1.4–2	A typical component of certain beers at detectable levels (wheat beers); can be elevated in other beers as an off-flavor due to wild yeast carrying the (phenolic off-flavor gene (POF) (Tressl et al., 1980).

Continued

Table 18.3 Continued

Flavor note/indicator compound (generic and specific names)	General flavor descriptors[a]	Typical concentration found in beer[a]	Typical threshold mg/L (ppm)[b] (thresholds vary with beer style)	General notes and example associated microorganisms capable of producing the flavor note (see also Tables 18.1 and 18.2)
Iso-amyl alcohol (3-methyl-1-butanol)	Fusel oil (higher alcohol), whiskey-like; represents the main higher alcohol known as "fusel oil."	–	Thresholds vary depending on specific fusel alcohol 50–800 ppm.*	Produced by *Brettanomyces* or other organisms in association with Brett beer production. *(Another higher alcohol: n-propanol with 600–800 ppm. threshold may be found in beers at 7–45 ppm depending upon beer style and is produced by certain contaminating microorganisms.)
Isovaleric acid (3-methyl butanoic acid) (may be confused with butyric flavor)	Cheesy, old hop-like, sweaty, sweat socks. Rancid, putrid, stale cheese.	0.1–3.4	0.1–1.5	Usually from old/aged hops. May be produced by *Brettanomyces and Megasphaera* (see Tables 18.1 and 18.2).
Lactic acid (see acidic)	Sour, sour milk, yogurt. (No odor.) Dulls the sensation of beer.	–	170–180	*Lactobacillus/Pediococcus* contamination; also deliberately encouraged in acidification of malt and wort and in sour or "wild-beer" (see text).
Meaty or broth-like (aka yeasty)	Yeast extract, meat extract, broth, old yeast.	–	–	From autolysis of yeast
Medicinal (an older general term)	TCP, antiseptic, phenolic.	–	–	*Enterobacter/Klebsiella* *Associated with chlorophenol taints and defined phenolic compounds*.
Methyl mercaptan (methanethiol)	Rotten cabbage, garlic, sulfurous, eggy.	0.001 Typically very low.	–	*Pectinatus* Mercaptan (ethanethiol) with similar sulfury notes may also be involved with some contaminating organisms.
Musty (general term)	Musty, moldy, earthy.	–	–	More a taint than off-flavor from mold contaminated grains, or water supplies (see also geosmin).

Phenolic (general term)	Herbal, cloves, medicinal.	–	–	Wild yeast. See specific phenolic notes. eugenol/4-vinyl guaiacol, etc.
Propionic acid (propanoic acid)	Acidic, rancid, dairy, nutty flavor, pungent, cheesy, vinegar.	0.5–5	–	*Pectinatus/Clostridium* spps.
Spicy (general term)	Clove, eugenol, nutmeg, allspice.	–	–	Maybe a more general term but could include eugenol and see 4-vinylguaiacol.
Styrene (ethenylbenzene)	Polystyrene, plastic, burning plastic, styrene.	<0.005 Not detectable in normal beer	0.02	Off-flavor produced by contaminant wild yeast during fermentation; or a taint from raw materials/packaging. (Styrene has a mechanism of production similar to those of traditional wheat beer phenolics. Related to the POF phenolic off-flavor gene, it may be found in bottle re-fermented beers if POF and strains are present (Schwarz, 2012)).
Succinic acid (butanedioic acid)	Odor: none, sour, acidic flavor.	–	–	*Pectinatus/Selenomonas/Zymophilus*
Sulfidic (hydrogen sulfide) (sulfur) H_2S	Rotten eggs, sewer drains, mercaptans, onions/garlic.	0.004 (<4 ppb)	0.004	Wild yeast, *Zymomonas*
Valeric acid (pentanoic acid)	Fatty, earthy, putrid acidic, sweaty, cheesy odor, sharp, acidic, milky cheese, slightly fruity.	–	–	*Megasphaera/Brettanomyces/Clostridium* spps.
Vinyl guaiacol (4-vinylguaiacol 4VG)	Spicy, clove, herbal, phenolic.	0.05–0.55	0.3	A typical wheat beer note (Coghe, Benoot, Delvaux, Vanderhaegen, & Delvaux, 2004). Wild yeasts or specialty yeasts.

This table forms a key part of instruction for sensory evaluation programs in general and specifically for flavors associated with atypical fermentation activities of bacteria and or wild yeast. The data in the table represent summaries of information obtained from many of the references cited in the text, product summaries from suppliers of sensory standards (specifically Flavor Activ) and collected notes of the authors over many years and so, for clarity, not all specific points are referenced in the table itself.

[a]Sensory panels will decide upon the desired term for their team but should arrive at a consensus as to how best to describe the flavor notes (suppliers of standards and sensory training such as FlavorActiv can advise here). The American Society of Brewing Chemists (ASBC) Beer Flavor Wheel provides a tool and the ASBC provides technical material as to standards to use, as do several references listed in Part 2 of this article.

[b]See the text for details on approximate concentrations found in beer and threshold values and definitions (consensus values are provided based in part on subjective author opinion and experience working with standards). For an extensive set of threshold data, see Angelino (1991), and for the most extensive list see Engan (1981). A few references not mentioned in the text are included in this table to keep some facts localized.

18.3.4 Beer spoiling bacteria

Beer spoiling bacteria are characterized as microorganisms capable of multiplying in beer, resulting in product deterioration. Gram-positive bacteria are in general inhibited by hop bittering components, but the growth of Gram-negative bacteria is not affected (see Suzuki, in this volume). Gram-negative bacteria are undesirable; they include acetic acid bacteria, *Zymomonas*, and certain members of the *Enterobacteriaceae* (*Rahnella*, *Hafnia*) and *Acidaminococcaceae* (*Pectinatus*, *Megasphaera, Selenomonas*, and *Zymophilus*). Acetic acid and lactic bacteria can grow in stored beer. Minimizing oxygen can assist in keeping acetic acid bacteria from spoiling beer. In packaged beer, spoilers include *Lactobacilli, Pectinatus frisingensis, Pectinatus cerevisiiphilus*, and *Megasphaera cerevisiae*. These organisms can produce foul-odor metabolites such as methyl mercaptan, DMS, and hydrogen sulfide (H_2S), along with turbidity. Microbially contaminated beer may also convey lactic and acetic notes, diacetyl (buttery, butterscotch), liquid manure odor, rotten egg, cooked vegetable, phenolic aromas, fusel alcohols (propanol and isobutanol), and ropiness (see the extended set of references under the wort section above for detailed accounts). For beer at the point of sale, acetic and lactic bacteria, and sometimes coliforms, may plague dispense systems and bar drains, etc. As for wort-spoiling bacteria, a summary of the major beer spoilage organisms is presented in Table 18.1, along with their associated spoilage flavor notes.

18.3.5 Beer spoiling yeast

A spoilage yeast species is one with the ability to cause spoilage and as such, yeasts simply isolated from foods/beverages are not necessarily spoilage yeasts (Stratford, 2006). Yeasts that are not deliberately used in the brewery but that find their way into beer production are designated as wild yeasts and form a diverse group including both non-*Saccharomyces* and *Saccharomyces* species. Naturally, culture yeasts will be present in most beers, and these, through their fermentative metabolic activities, are the source of most of the chemical species found in beer. These flavor notes are described in detail in several works (Anderson et al., 2000; Angelino, 1991; Engan, 1981; Hammond, 1993), many of these flavors being understood by trained panelists. Major types of wild or spoilage yeasts found in the brewery include *Brettanomyces*, *Candida, Debaromyces, Dekkera, Pichia, Hanseniaspora, Kluyveromyces, Pichia, Torulaspora, Williopsis*, and nonbrewing strains of *Saccharomyces*. Many of these are described elsewhere in this volume and have been discussed by others (extensively by Back, 2005; and see Boulton & Quain, 2001; Hill, 2009; Priest & Campbell, 1987; Spedding & Lyons, 2001; Stewart & Russell, 1998). Many of these yeasts compete with culture yeast and can produce copious amounts of metabolites rising to levels above their threshold concentrations, and this leads to their sensory perception as spoilage off-flavors. In specialty beer production, they may be harnessed to generate high concentrations of some of the same flavor notes that are then considered desirable for the special style intended by the brewer (see below). The details as to which of several yeast genera and species cause specific flavor notes are presented in Table 18.2.

18.4 Specialty beer production and processes

In addition to looking at the sensory properties of beer from a microbiological contamination issue, microbes are used selectively, or from spontaneous inoculations, to create some artisanal and interesting highly flavored beer styles; such beers provide unique or unusual complex flavor profiles with their production requiring much attention to detail to ensure safety, wholesomeness, and consistency of product flavor and quality. Some of these beers require blending, which also requires trained individuals to be able to flavor-match each production run. Such being the case with wheat beers, so-called "sour-beers", Lambic beers, and *Brettanomyces* inoculated beer wort ("Brett beers"), for example, and brewers use *Lactobacilli* species in the mash to lower the pH through lactic acid production.

18.4.1 Biological acidification

From a sensory aspect, the deliberate use of microorganisms to attain acidification of malt and ultimately beer wort warrants a few sentences. Acidification of brewing raw materials, mash, and wort can result in beer of a "superior" flavor quality (Kunze, 2010; Spedding, 2012a). The process deals with careful use of *Lactobacillus* strains and lactic acid production (Back, 2005; Kunze, 2010; Spedding, 2012a; Vaughan et al., 2005). Sensory panels might have a role to play in seeing how the effects of biological acidification play on the overall beer flavor profiles resulting from its use.

18.4.2 Specialty beers—wheat beers

Wheat beers utilize raw or malted wheat, rather than malted barley, as their primary raw material base. Fermentation may be spontaneous (natural flora of the brewery, Belgian Lambic beers [see below]) or with a top fermentation yeast strain. South German wheat beers (weissbier) utilize a top fermenting yeast strain that produces a characteristic phenolic flavor (4-vinyl-guaiacol, clove-like; see Table 18.3; Anderson et al., 2000; McMurrough et al., 1996; Stewart & Russell, 1998; Vanbeneden, Gils, Delvaux, & Delvaux, 2008). Certain wild yeasts can produce phenolic off-flavors in nonwheat beer styles and as such would be regarded as contaminants.

18.4.3 Specialty beers—Berliner Weisse

White beer (weissbier) is a special beer that is very pale in color. A lactic fermentation is carried out following the addition of a starter culture resulting in a very low pH: 3.2–3.4. *Lactobacillus brevis*, a top fermenting yeast, and *Brettanomyces bruxellensis* are used in fermentation resulting in pure lactic acid flavor (Anderson et al., 2000; Back, 2005; Vriesekoop et al., 2012). Sensory panels would need to ensure the quality of the pure lactic acid flavor and lack of any contaminant-generated off-flavors in such highly acidic/tart beers.

18.4.4 Specialty beers—sour beers

Craft brewers have experimented with sour beer production, creating some unique styles, many of which are still being categorized. Brewers of such beers need to be careful of contaminating their other traditional (yeast) fermented beers, and both the production of the sour beers and traditional brands need careful monitoring for inappropriate sensory changes and determination as to trueness of desired flavor qualities. Such types of fermentations are more difficult to control, and consistency may vary with each batch. Sensory panels can determine the range over which the flavors can be allowed to vary in such cases, and provide the brewer with instructions on safety concerns and on blending or aging regimens as appropriate. The acid beers of Roeselare (Rodenbach) would belong here as an example of the use of *Lactobacillus* and *Pediococcus* strains for lactic acid fermentation yielding 500–600 ppm lactic acid! *Brettanomyces* species also play a role (Verachtert & Iserentant, 1995).

18.4.5 Specialty beers—Brett beers

Brettanomyces, an organism with a solid history with regard to beer production, presents us with an interesting topic. Although most research into spoilage caused by *Brettanomyces* (*Dekkera*) stains has been done with wine as a substrate, many beers have had a past with respect to this organism, including beers with "English character" (Licker et al., 1998). Belgian beers are also noted for the production by *Brettanomyces* species, of strong fruity, estery-like aromas, and also metabolites that produce the flavor notes known as "horse sweat" (Licker et al., 1998). As a multitude of flavors are associated with "Brett" character, a lot more sensory work will be needed to pin down a definition as to all the nuances of Brett beers. The key flavors are said to be 4-ethylphenol and 4-ethylguaiacol, although octanoic and decanoic acids and acetic, isobutyric, isovaleric, and several other compounds have all been ascribed to "Brett character" (Anderson et al., 2000; Heresztyn, 1986; Suárez et al., 2007; Van Nedervelde & Debourg, 1995). Recently, a new wine-based flavor wheel has been produced by researchers at University of California Davis that focuses just on *Brettanomyces*-related flavors. When released, this flavor wheel will also provide a neat tool for the sensory understanding of Brett beers. The Brettanomyces project in the United States also provides extensive data on the craft production of such beers (www.brettanomycesproject.com/).

18.4.6 Specialty beers—Lambic and gueuze

Lambic and gueuze are Belgian beer styles of high acidity (pH 3.3) and complex flavor. Wort is cooled overnight in shallow open trays and picks up a variety of microorganisms from the air (they are not usually inoculated with added yeast). Fermentation begins with *Enterobacteriaceae* and strains of *Kloeckera apiculata*, which are then overtaken by strains of *Saccharomyces cerevisiae* and *Saccharomyces bayanus*; then, after several months, strains of *Pediococcus cerevisiae* produce a fivefold increase in lactic acid. Finally *Brettanomyces* strains gain a foothold (*B. bruxellensis* and *Brettanomyces lambicus*), which leads to the production of high levels of ethyl acetate and

ethyl lactate and affects other fruity ester formation (Anderson et al., 2000; Spitaels et al., 2014; Verachtert & Iserentant, 1995; Vriesekoop et al., 2012). The *Brettanomyces* species continue to play a role in cask and bottle. Most Lambics are blends, and one blend component may include a beer produced by a mixed population of *Brettanomyces*, lactic, and acetic acid bacteria (Anderson et al., 2000). *Brettanomyces* may produce a host of other components which give beers made with it the very complex sensory profile alluded to above (Suárez et al., 2007).

18.4.7 Specialty beers—wood- and barrel-aged beers

Another new class of beers emerging, as well as an understanding of early traditional wooden and cask-aged beers, will require sensory studies based on the effect of the microflora present in such barrels. This topic involves understanding a unique set of flavor notes derived also from the wood itself. New flavor wheels describing wood- and barrel-aged flavors are available, and a lot of current research into the production of such beers is underway. Sensory programs as described in Part 2 can be initiated to study these unique beer styles.

18.4.8 Pasteurization, dry hopping, and bottle conditioning

Whether or not to pasteurize is a question to be considered for beer preservation, along with sensory evaluation. Most craft brewers will not even consider it. Some brewers prefer not even to filter their beer at all. Some beers are dry hopped, and some bottle-conditioned and as a result many beers today in craft, as in the past with cask-conditioned ales, are "live products" with some desirable and sometimes undesirable organisms in tow that can lead to sensory degradation of the beer's quality if not properly packaged. Flavor changes can occur with pasteurization (heat induced), use of priming yeast during bottle conditioning, continued metabolic activities or with autolysis of yeast over long maturation or aging periods, etc. and these features would also bear upon sensory evaluation to monitor such changes. Two recent cases in point are the production of volatile polyfunctional thiols during bottle refermentation (Nizet et al., 2013) and changes in Belgian beer flavor profiles during bottle conditioning (Dekonink et al., 2013).

18.5 Conclusion—part 1

Part 1 of this paper has presented an overview of microbial spoilage as it relates to the organoleptic senses, some key sensory definitions (taints, off-flavors, thresholds), and flavor note descriptors for many atypical flavors produced by both wild yeast and bacteria. Other chapters in this volume, as well as references cited here, describe in depth the microbiology of the organisms discussed only briefly here. Aspects of sensory evaluation were alluded to above with the flavor notes/descriptors (Table 18.3), providing a lead now into an outline discussion of setting up sensory evaluation programs in Part 2. It seems that specific works have not addressed in detail sensory programs, for

brewers, from a perspective of understanding all the flavors associated with atypical (special beer production) or contaminant microbial fermentation activities. Wine and distilled spirits have been covered in depth, and more research appears to be underway with wine flavor research (e.g., see Kilcast, 2010). The material from Part 1, used in conjunction with the discussions and cited references in Part 2 will hopefully address this imbalance in coverage of an important area for research. Sensory programs can form a powerful approach to understanding of beverages, including beer.

18.6 Part 2: sensory evaluation

18.6.1 Getting started with a sensory program

Although a sensory panel can be a cost-effective tool for quality control, it is often seen as a luxury that only large well-established breweries can afford. Although it is true that a sensory program can represent a significant investment, even a modest program can deliver significant returns in terms of safeguarding the brand and retaining customers. At a minimum, each beer should be approved by a Go/No-Go (aka Ship/Don't Ship) sensory panel prior to packaging and shipping. This team must be made up of individuals well acquainted with the brand profile (see below). A Go/No-Go ballot can be as simple as a piece of paper with the words "Ship" and "Don't Ship" (Figure 18.1). The panelist circles the appropriate response and writes the reasons for negative findings.

A Go/No-Go ballot is the last chance for a brewery to prevent a flawed batch of beer from reaching the market and perhaps negatively affecting the perception of the brand and brewery. This sort of issue can easily result in costly returns/buy-backs. Due to the gravity of the situation, a Go/No-Go panel would ideally be staffed by the best-trained, most proficient sensory panelists available; however, when just starting a program, a grasp of the brand profile, enthusiasm, and an appreciation of the work's importance may have to suffice.

This description of a Go/No-Go panel is an illustration of the basic philosophy behind sensory panels and their mission. The aim is to find problems before they become big problems (see Table 18.4). If a taint can be found in raw ingredients, it is

Figure 18.1 Go—NoGo ballot.

easier and cheaper to fix before brewing. If a problem is tasted in the brewhouse, it is easier and cheaper to fix than once product has shipped. If an issue arises in market, learn and fix the causes so the next batch is better. This process is true for most all quality assurance, programs regardless of the product. The objective is to find and fix problems as early as possible, and not repeat the same mistakes. This philosophy lends itself to every stage of production and can be extended beyond the brewery to product shelf-life evaluation.

18.6.2 Creating an effective sensory team

A reliable sensory program requires commitment from management to budget sufficient resources (both money and time) along with an understanding of the complex nature of establishing and maintaining an ongoing program and evaluating product. Convincing managers and accountants that people stopping work in the middle of the day to taste beer is a good idea is paramount to the success of a panel. How this is accomplished is beyond the scope of this chapter.

When building a program, the first question facing a panel organizer is, Who will be on the team? First the basic qualifications of panelists must be considered. Of course, one should not consider those who do not imbibe or who have an issue with alcohol. A forklift driver might not be the best choice for a panel that meets during his/her work day. The availability of the head brewer might not be conducive to the rigid time schedule that a panel requires. Although it is not specific to breweries, or beer, an in-depth treatment of the subject of organizing a sensory panel has been presented by Kilcast (2010), Stone, Bliebaum, and Thomas (2012) and Meilgaard, Civille, and Carr (2007).

Table 18.4 Features that can (and should) encompass sensory evaluation in the brewhouse

Phase/purpose	Focus	Details in this chapter
All raw materials and the brewing water	Tainted components	See air, water and raw materials, and taints and off-flavors
Wort and beer at all stages of process	Spoilage and off-flavors	See Table 18.3 and Beer and wort—overview
Final ship/do not ship	Batches that do not fit the brand profile	Getting started
Buy-back program that periodically purchases beer from retailers	Assess product longevity by looking at oxidation, nonbiological haze, and biological issues	Shelf life evaluation
Field and customer support	Evaluate all product returned for any reason	Proactive sensory

Suggested areas of sensory focus are listed here.

In the case of a start-up operation, the problem is more often that the pool of employees is too small. In this case, who else should be considered? The short answer is *everyone*. Are there tap room, gift shop, or maintenance staff available who might be able to devote the time and effort needed? Consider local distributers and retailers as a way to augment the team and build brand loyalty. Are there Beer Judge Certification Program (BJCP) certified (US) or other recognized judge program individuals, or home brewers that might be willing to work for little to no remuneration? Is there anyone who has participated or would like to participate in any of the Cicerone Certification Programs? One should not overlook customers who have exhibited above-average beer and tasting knowledge or who have shown special interest in the brewery operation. It is not at all unusual to find a very proficient taster working in the gift shop, mowing the lawn, or working as an intern. By the same token, individuals who are principals in the business and brewers are not always the most gifted. These decisions can be unique to each operation and have no set rules; one just should not limit the search to the brewery.

Once willing participants are on board, the next step is assessing their basic level of sensory acuity to determine aptitude. An effective means of this testing is with the use of aroma bottles, whereby a person sniffs and then describes an aroma instead of actually tasting the flavor in a beer. For a short explanation as to this test's administration, readers are referred to Lawless (2013a). The goal is to screen out those individuals with impairments or who are blind to basic beer elements. For untrained candidates, correctly describing 60% of common beer aromas is generally considered acceptable.

18.6.3 Building a vocabulary

Having selected panelists, the next step is to train them to consistently identify beer attributes and to build a common vocabulary to be used. Specific flavor notes and qualities must be understood and described in technical terms. Vague terms such as "typical of style," smoothness, etc. should be avoided. Defined terms are to be used wherever possible. Tactile sensations—mouthfeel such as warming, cooling, oily, or stinging (carbonation), may be acceptable but should be related to the correct attributes causing such stimuli when possible. A buttery flavor indicative of diacetyl (see above) may also be detected as an oily slickness or increased viscosity on the palette. These sensations show the complexity of defining a food or beer in technical and quantitative terms. The panel will need to use quite technical terms here, such as DMS or geosmin (see above) for, respectively, the less technical but still descriptive terms such as cooked corn/vegetal or musty. It is of little use for a problem to be identified if is not clearly communicated to those in the brewery and laboratory who are responsible for identifying and correcting the underlying production and microbial issues.

A good place to start building the required vocabulary is with the American Society of Brewing Chemists' (ASBC), *Beer Flavor Wheel* (www.asbcnet.org/flavorwheel). The Wheel provides a concise list of beer attributes as well as broad categorization as to odor and taste (Spedding, 2012b). A further breakdown of the Wheel attributes can be found in *A Practical Guide for Beer Quality: Flavor*, by Bamforth (2014). This work relates an attribute's relevance to odor, taste, mouthfeel, warming, and aftertaste, as well as giving accepted descriptors and reference standards for the Wheel's elements.

In recent years, attempts have been made to split the Beer Flavor Wheel down to a set of more manageable-sized tools. New flavor wheels at various levels of professional activity have included ones for wood-aged beers, for example, and recently (as yet unofficially published) a Brettanomyces flavor wheel. When using the *Beer Flavor Wheel*, there may be elements that never come into to play in an operation and other attributes that are missing from the work. A sensory program administrator is free to build his or her own wheel and list of descriptors. The vernacular used in an established brewery that only produces light lagers will differ from that of a start-up brew-pub specializing in American-style barley wines.

18.6.4 Brand profiles

An in-house sensory program should also have a *brand profile* ("aroma-print"/"taste-print") for every beer in a brewery's portfolio. These profiles describe the key attributes for each brand. Beyond the smallest of start-up operations, this guideline for brands should be a formal document created in cooperation with the brewers and the sensory team and perhaps the marketing department. The work is not unlike style guidelines used at competitions such as the World Beer Cup and other international brewing contests. Rather than describing broad styles, however, it is specific to a brewery's brands.

18.6.5 Raw materials

Even as training progresses, it is important to begin conducting regular sensory panel sessions. Every stage of the brewing process can be subjected to sensory testing. Raw materials should be assessed upon delivery and before use. Grain can be tasted raw and/or a small mash made and tasted. Details of the flavors to expect from beer ingredients can be found in Bamforth (2014).

Water should be tested from time to time in the laboratory for common intestinal bacteria as it enters the brewery (Priest, 1990). The sensory crew should smell, observe, and taste the water collected as it enters the building and at various points before and after conditioning. A taint found in the water that way that is not present in samples of the city's supply is a strong indication of plumbing and/or contamination issues.

18.7 Gathering data for sensory evaluation

There are quite a number of different sensory techniques available that need to be evaluated, depending on the questions that the panel is addressing. These methods are described elsewhere (Bamforth, 2014; Kilcast, 2010; Meilgaard et al., 2007; Stone et al., 2012). One method worth pointing out is the full descriptive ballot used for brand evaluation and troubleshooting. In this approach, trained tasters score beer for a range of flavors, aromas, and appearances.

The example shown in Figure 18.2 is of a general ballot. Panelists are asked to rate each attribute on a scale of 0–5 where 0 is imperceptible and 5 is overpowering. The comment section at the bottom allows for explanation of anything perceived or lacking

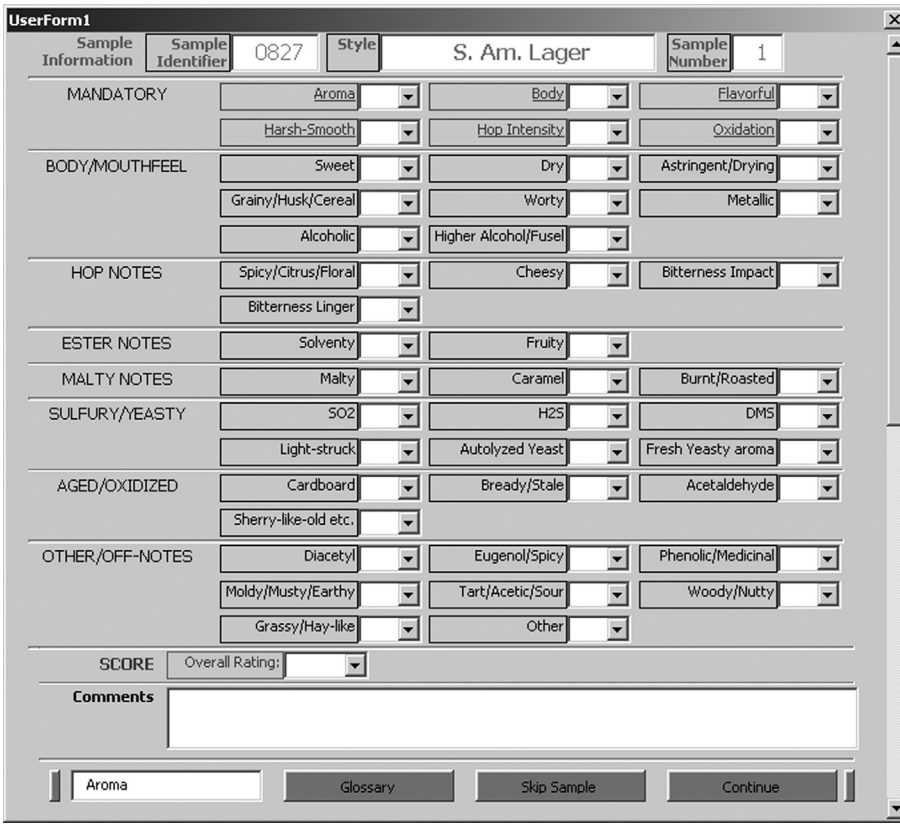

Figure 18.2 General Diagnostic Ballot provided courtesy of Data Collection Solutions.

in the sample that does not fit the format of the ballot. Many of the off-flavor attributes on the ballot can be traced to microbial issues and taints introduced during the brewing, packaging, or handling processes. Of course, the scale mentioned is arbitrary and could just as easily range from −5 to +5, from 0 to 10, or from 1 to 100, or whatever the organization finds most useful. More specific ballots might be created for particular brands, training, or specialized panels.

Although sensory evaluation typically focuses on aroma and taste, all other senses can come into play. Haze and gushing are visual perceptions but can indicate microbial issues. Tactile impressions of the beer on the tongue or the sound of a can or bottle being opened may point to a packaging concern. It will be noticed, however, that the ballot shown in Figure 18.2 does not include visual attributes. It was designed to be used in a setting where a beer's appearance is concealed from the panelists, as visual cues can bias panelists. An interesting discussion of the effects of appearance on flavor perception can be found in Bamforth (2014). If, on the other hand, the sensory panel is a brewery's in-house team, then panelists will be familiar with the brands being assessed, and deviations in appearance are most likely a concern.

18.7.1 Paper or electronic?

The same attributes presented in the Figure 18.2 ballot could be included on a paper ballot. Paper has been used for generations and represents the lowest start-up cost. The issue to consider, however, is time and expense of handling, analyzing, summarizing, and reusing the data recorded. Transcribing data into an analysis tool such as Microsoft Excel is time-consuming and error prone. Strong consideration should be given to a computerized sensory application. The cost of such software will be recovered in the man-hours saved, and the increased usability will lend itself to more frequent sensory sessions and greater productivity.

18.7.2 Presenting sensory panel data

Sensory data may contain many important details that will remain just a collection of results unless they can be presented in a concise, understandable manner. The illustration in Figure 18.3 is a spider chart (aka Radar Profile) showing the results from a descriptive ballot evaluating a brewery's India Pale Ale (IPA) that meets the requirements of the brand profile and is considered True to Brand. (Full statistical approaches can be implemented for data analysis, and discussions can be found in the main references cited above and extensively in Lawless, 2013b and O'Mahoney, 1986.)

18.7.3 Shelf life evaluation

Buy-back programs have two forms. One is when retailers or distributors return product that has not sold and no longer fits its *brand profile*. The other form is proactive programs that periodically purchases beer from retailers and has the sensory team evaluate how well the product is holding up. Consider a beer that was returned to the brewery and was tasted as part of a proactive buy-back. Once the sensory team has done its work, the differences can be easily contrasted with a sample that is considered True to Brand.

A quick look at the contrasting spider chart in Figure 18.4 shows an increase in diacetyl, oxidation, and sweetness levels and a decrease in bitterness and overall flavor in the aged sample. Depending on the brand and the sample's age, the changes noted in oxidation, sweetness, caramel notes, and bitterness may be expected transformations attributable to the aging process. The increase in diacetyl may be a cause for concern that warrants further investigation.

18.7.4 Proactive sensory evaluation

A buy-back program is one way in which a sensory program may extend beyond the brewery, but there is no reason that others who handle the brand cannot be involved in monitoring the product's shelf life. Distributors and sales representatives can be trained to evaluate beer. Business is better for everyone when beer is fresh and remains within its Brand Profile. If beer in trade is not handled correctly, microbiological issues may ensue (Spedding, 2013).

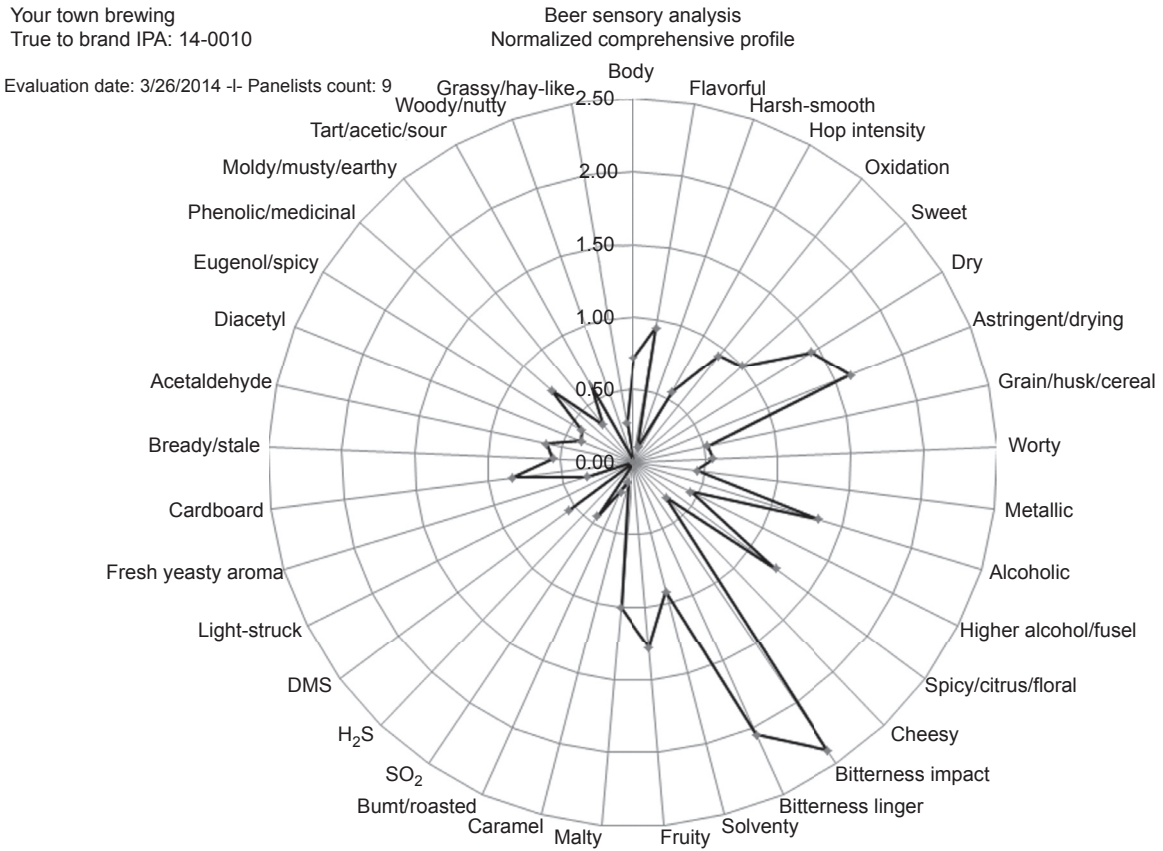

Figure 18.3 Graph of beer fitting the Brand Profile, provided courtesy of Data Collection Solutions.

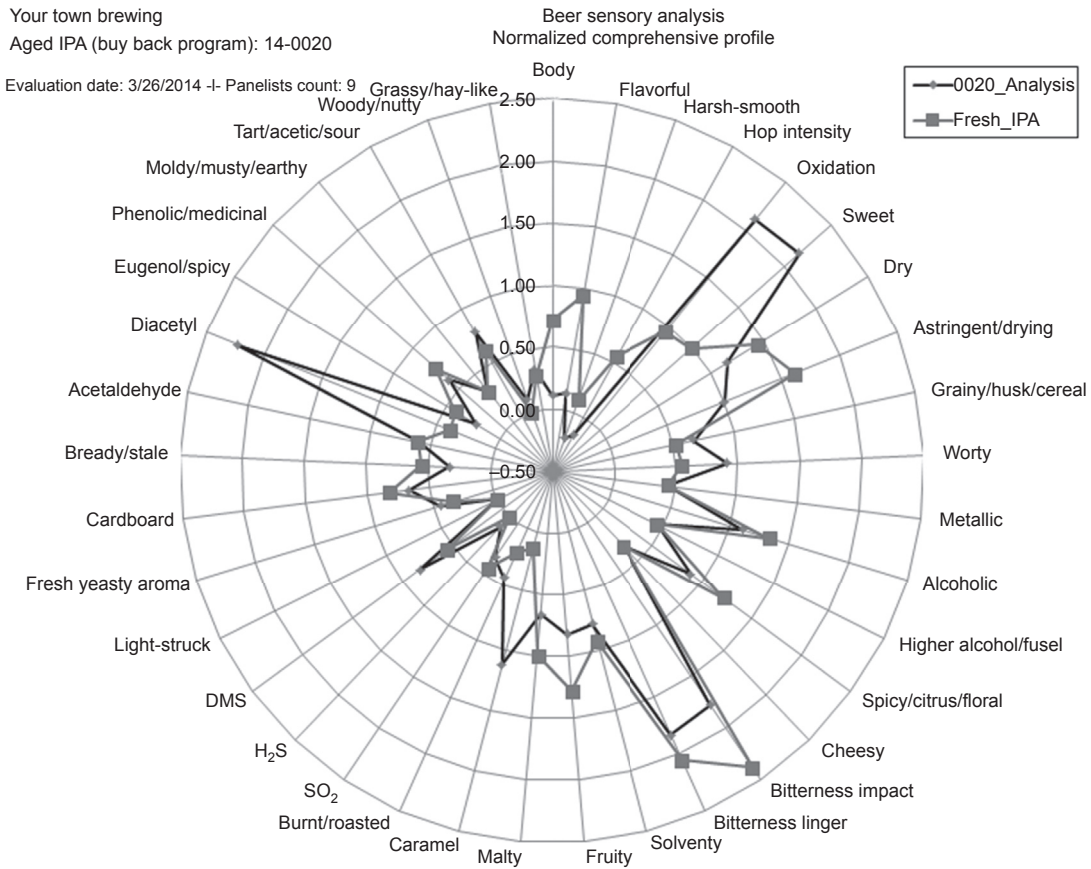

Figure 18.4 Graph of beer from the Buy Back program contrasted with sample fitting the Brand Profile, provided courtesy of Data Collection Solutions.

18.8 Sensory training

Of course, sensory panelists must be trained on the flavor and aroma characteristics of the key metabolites of microorganisms in order to make informed decisions about the desirability for release of tainted or contaminated beer or to recall product from the market. An effective means of introducing new flavor notes is the use of aroma/odor bottles. Training bottles are normally created by dosing a small amount of a standard reference compound into a brown glass bottle (~30 mL) containing a cotton ball. Optionally the cotton can be soaked in a light beer for background aromas (see the Flavor Sub-Committee Sensory Analysis Manual, 1995 for more on basic training with aroma bottles).

Training can then be extended to actual tasting of off-flavors in the brewery's own beers. For example, it may be unlikely that the beer will ever have DMS levels above threshold, but the panelist should be capable of identifying the defect if it ever does occur. This is where Sensory Training Kits can be very useful tools. These kits are for creating aroma bottles and spiking samples. Kits are available from several sources including the following:

- FlavorActiv (www.flavoractiv.com)
- AROXA™ (www.aroxa.com)
- The Siebel Institute of Technology (www.siebelinstitute.com)

Typically panel members are presented spiked beers (particular flavor characteristics added to a sample) alongside a standard beer (a control with no perceived defect) to understand the flavors versus a true-to-brand sample. Any material added to beer intended for human consumption must be food-grade quality and safe, to avoid any health hazard. The threshold values mentioned in Table 18.3 were based on light-style lagers, and as such, levels may have to be increased to accommodate their perception in more flavorful craft beers. Indeed threshold tests will become a part of routine sensory training practice, especially with more flavorful beers. Details on such testing are covered in many of the references that we cite in this section.

Sensory training must be an ongoing process. Validation of panelist performance is important to determine proficiency. It should be kept in mind, when testing panelists, that results should be used to better the panel and not to punish underperformers. A common way of assessing panelist accuracy is by introducing spiked samples alongside the panel's regular work. Validation should be designed to track not only team members who correctly identify off-flavors but to illuminate patterns in the incorrect responses. Failure to describe correctly the added character is indicative of a training requirement or a blind spot. For example, if multiple panelists are misidentifying acetaldehyde (green/bruised apple; see Table 18.3) as ethyl hexanoate (red apple/aniseed), there is a need for additional training. Panelist assessments may also point out that certain individuals are extremely sensitive to some attributes and below par on some others. This does not mean that the panelist is not of use to the team, but that it is important to note when faults are found by only some in the group. A good overview of motivating and evaluating panel members is provided by Meilgaard et al. (2007).

Difference testing can be useful tools for assessing panelist's skills. Methods such as the triangle and duo-trio tests are what are communally known as forced choice discrimination tests because panelists are presented with multiple samples and must decide which examples are the same and which are different. These tests can also be used in assessing the impact of changes in supply chain and production techniques. Succinct descriptions of these and other discrimination tests can be found in Bamforth (2014) and Simpson (2006). More in-depth discussions of these techniques are covered by Kilcast (2010), Meilgaard et al. (2007) and Stone et al. (2012).

18.9 Conclusion—part 2

Part 2 of this chapter has presented an overview of the sensory analysis of beer and beer wort, which also extends to brewing raw materials. Part 1 alluded to some areas in which sensory programs could provide answers to some interesting questions. Understanding beer when fresh or old (aged, oxidized) has been covered in sensory programs and training for more than 30 years with a tool known as the Beer Flavor Wheel (discussed above and by Bamforth, 2014 and Spedding, 2012b), being instrumental in providing clues and serving as a memory jogger for many important beer odor and flavor notes. Using the knowledge gained from the brief account of sensory programs here will lead readers into setting up their own programs for both fundamental research and for quality control of their beers.

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Part Five

Valorisation of microbiological brewing waste

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Anaerobic treatment of brewery wastes

19

J.C. Akunna
Abertay University, Dundee, United Kingdom

19.1 Introduction

The anaerobic digestion process is a biochemical process that occurs in the presence of readily biodegradable organic carbon and the absence of oxygen. This is similar to the process that occurs naturally in stomachs of ruminants and in marshes and sanitary landfill. The process results in the production of biogas, a complex mixture of carbon dioxide, methane and other gases, and a slurry or liquid by-product called digestate. The biogas is an important source of energy, which can be converted to electrical or mechanical energy for municipal or industrial use. The digestate is rich in nutrients that can enrich the soil. Anaerobic treatment technology is widely used throughout the world as a cost-effective treatment solution for biodegradable organic wastes and wastewater, from both municipal and industrial sources. The industrial wastes commonly treated using anaerobic digestion technologies include wastewater from food, including meat, and beverage production and processing, alcohol distilling, dairy and cheese processing, fish processing, fruit and vegetable processing, pulp and paper production, sugar processing, chemical manufacturing, and brewing wastes. This chapter discusses key principles important to the anaerobic digestion process and how these apply specifically to brewing waste.

The accepted biochemical pathway for the process is shown in Figure 19.1. It involves four main stages, namely, hydrolysis, acidogenesis, acetogenesis, and methanogenesis, with the last three stages catalysed by acidogenic, acetogenic, and methanogenic microbes, respectively, as shown in the figure. Acetogens and methanogens are strict anaerobes, whereas acidogens are mainly facultative fermentative microbes. The final products of each of these stages serve as substrates for other stages, as shown in the figure, with the final gaseous product comprising mainly methane gas and carbon dioxide, along with trace gases such as hydrogen sulphide and hydrogen.

Some of the common key microorganisms involved in the different degradation stages of the anaerobic digestion process can be seen in Table 19.1. In the absence of microbial inhibitory conditions, the distribution and balance of the various microorganisms in any anaerobic digestion system depend on the nature of the available substrates.

In comparison with aerobic treatment processes, in anaerobic digestion biodegradation occurs in the presence of oxygen; the advantages and disadvantages of anaerobic digestion are summarised in Table 19.2. As indicated in this table, anaerobic treatment can play an important role in cost-effective waste management and environmental protection.

Brewing Microbiology. http://dx.doi.org/10.1016/B978-1-78242-331-7.00019-8

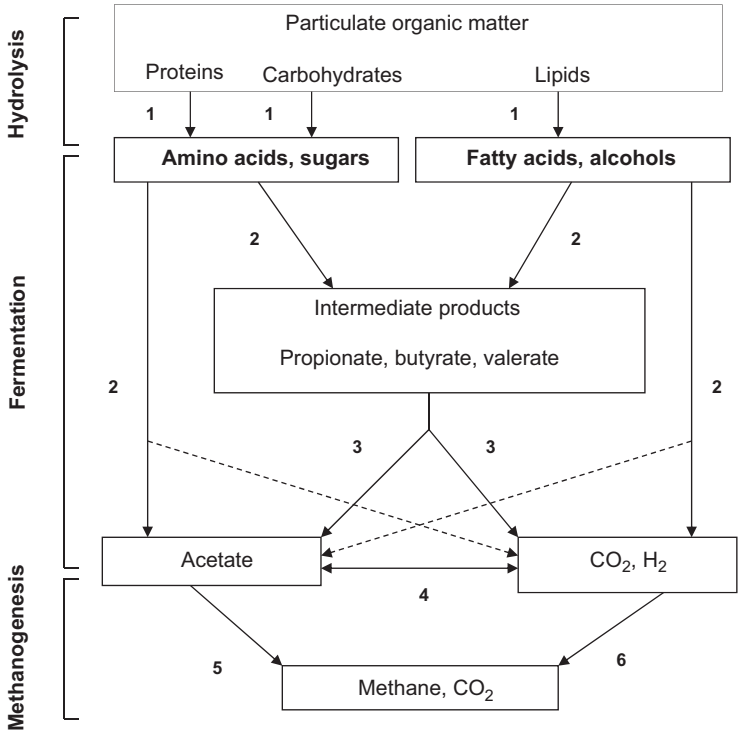

Figure 19.1 Simplified schematic diagram of different reactions involved in anaerobic digestion of complex organic matter. Key:

1. Hydrolysis of complex polymers by extracellular enzymes to simpler soluble products.
2. Fermentative or acidogenic bacteria convert simpler compounds to short chain fatty acids, alcohols, ammonia, hydrogen and carbon dioxide.
3. Break down of short-chain fatty acids to acetate, hydrogen and carbon dioxide, which act as substrates for methanogenic bacteria.
4. Reaction carried out by acetogenic bacteria.
5. About 70% of methane is produced by aceticlastic methanogens using acetate as substrate.
6. Methane production by hydrogenophilic methanogens using carbon dioxide and hydrogen.

Adapted from Kasper and Wuhrann (1978) and Gujer and Zehnder (1983).

19.2 Key factors affecting the anaerobic digestion process

19.2.1 Organic content

Anaerobic treatment is most suitable for wastewaters with chemical oxygen demand (COD) concentrations in the intermediate- to high-strength range (2000 to >20,000 mg COD/L) (Hall, 1992). Organic removal efficiencies tend to increase with increasing organic strength of the wastewater. However, in general, about 80%–90% COD

Table 19.1 Anaerobic digestion stages and typical associated microbial species

Process step	Typical microbial species and by-products
Hydrolysis	*Clostridium, Proteus vulgaris, Peptococcus, Bocteriodes, Bacillus* convert proteins to peptides and amino acids *Clostridium, Acetovibrio celluliticus, Staphylococcus, Bacteriodes* transform carbohydrates to soluble sugars *Clostridium, Micrococcus, Staphylococcus* convert lipids to higher fatty acids or alcohol as glycerol
Acidogenesis	*Lactobacillus, Escherichia, Staphylococcus, Bacillus, Pseudomonas, Desulfovibrio, Selenomonas, Sarcina, Veillonella, Streptococcus, Desulfobacter, Desulforomonas* transform amino acid to fatty acids, acetate and ammonia *Clostridium, Eubacterium limosum, Streptococcus* convert sugars to intermediary fermentation products
Acetogenesis	*Clostridium, Syntrophomonas wolfei* transform higher fatty acids or alcohols to hydrogen and acetate *Syntrophomonas wolfei, Syntrophomonas wolinii* convert volatile fatty acids and alcohols to acetate and hydrogen
Methanogenesis	*Methanosaeta, Methanosarcina* (aceticlastic methanogens) convert acetate to methane and carbon dioxide *Methanobacterium, Methanobreviacterium, Methanoplanus, Methanospirillum* (hydrogenophil methanogens) transform hydrogen and carbon dioxide to methane

Adapted from Stronach, Rudd, and Lester (1986).

Table 19.2 Advantages and disadvantages of anaerobic waste treatment processes compared to aerobic treatment

Advantages	Disadvantages
• Low sludge production • Low nutrient (nitrogen and phosphorus) requirement • Low capital cost and operating costs • Production of methane, a source of energy • Production of liquid and solid residues that may be used as soil conditioners • Inactivation of pathogens present in the waste • Survival of microbial biomass in anaerobic treatment reactors for long periods of little or no feeding	• Long start-up and retention times • Requires high temperatures for effective operation • Requires monitoring for smooth operation • Shock and variable load can upset microbial balance • Usually used as a pretreatment stage. Aerobic ‘polishing’ may be required before discharge to the aquatic environment

Adapted from Hall (1992) and Malina (1992).

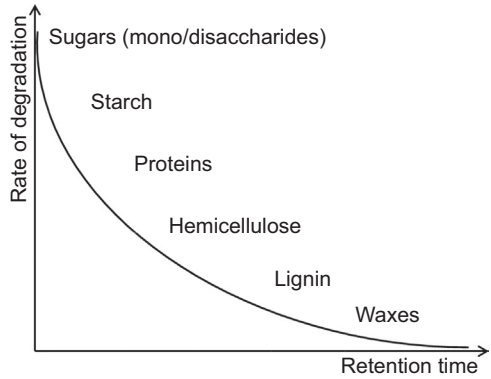

Figure 19.2 Relationship between the rate of degradation and retention time required according to the composition of the substrate.
Adapted from Eder and Schulz (2006).

removal is achievable in an efficiently operated anaerobic digestion system. To achieve higher COD removal, anaerobically pretreated effluent must be further treated with aerobic biological processes. If the wastewater is dilute (i.e. with COD <2000 mg/L), treatment using aerobic processes will be more cost-effective.

The types of compounds present in waste or wastewater are one of the primary indicators of the potential bioavailability of the organic matter for the anaerobic microbial population. Figure 19.2 shows the relative biodegradation rates and reaction times of various types of organic compounds. Biodegradability may be limited by the chemical structure of common compounds, such as lignin, cellulose and hemicellulose, which are not readily amenable to enzymatic hydrolysis. These compounds may require other types of treatment (herein referred to as pretreatment) before treatment by anaerobic processes. Pretreatment requirements for anaerobic digestion are discussed later in this chapter.

If the wastewater contains biodegradable particulate organic matter, usually expressed in total solids (TS) or volatile solids (VS), these must be hydrolysed in the first stage of anaerobic digestion process, as shown in Figure 19.1. TS is a measure of all solids in the wastewater, whereas VS measures only the organic fraction (i.e. both biodegradable and nonbiodegradable) of the TS. Hydrolysis of particulate biodegradable organic matter is a relatively slow biological reaction. Therefore, if the solid content of the waste is high, effective anaerobic treatment will require relatively long periods of contact (i.e. retention time) between the substrate and the anaerobic microbial consortium. Conversely, if the organic constituents of the waste are primarily soluble in nature, shorter retention times will be required.

19.2.2 Nutrients

The ideal feedstock composition ratio for carbon (C), nitrogen (N), phosphorus (P) and sulphur (S) for hydrolysis and acidogenesis phases (the C:N:P:S ratio) is

considered to be 500:15:5:3; for methanogenesis, the ultimate ratio is theoretically assumed to be 600:15:5:3 (Weiland, 2001). These elements are called macronutrients. The sulphur and phosphorous requirements are very low compared to the carbon and nitrogen requirements, and carbon is naturally abundant in organic waste streams. Therefore the limiting nutrient for the anaerobic digestion process is considered to be mainly nitrogen. The carbon/nitrogen (C/N) ratio is used to measure nitrogen suitability of the waste to be treated by the anaerobic digestion process, with appropriate values ranging from 20 to 30 (Deublein & Steinhauser, 2008; Polprasert, 1996). Higher C/N ratios can lead to decreased bacterial growth due to nitrogen deficiency, whereas low ratios may result in ammonia toxicity on the microbial population. For example, high protein content waste with low C/N ratio can produce high ammonia nitrogen during hydrolysis, which can lead to microbial toxicity at higher pH values. Where there is a nitrogen deficiency, nutrient supplement may be needed, and this is commonly achieved using urea, sewage sludge or animal manure. Where a phosphorus deficiency exists, phosphorous can be added as phosphate salt or phosphoric acid as necessary.

Another vital parameter in effective digestion is the availability of trace elements, or micronutrients, notably iron, cobalt, nickel and zinc, in the feedstock. These elements, when available in relatively small amounts, can stimulate methanogenic activities. The exact amount needed can vary for different types of wastewater, and prior trials are needed before they are added. More information on the importance of micronutrients on the anaerobic digestion process can be found in the literature (Banks, Zhang, Jiang, & Haven, 2012; Demirel & Scherer, 2011; Facchin et al., 2013).

19.2.3 pH and alkalinity

The stability of the anaerobic digestion process is highly dependent on the pH. Whereas the acidogenic bacteria are more tolerant to pH values below 6.0, the optimum pH values for methanogenic bacteria lie between 7 and 8 (Angelidaki & Sanders, 2004; Raposo, De la Rubia, Fernández-Cegrí, & Borja, 2012). Therefore the pH range of 6.5–7.8 is suitable for the main microbial groups involved in the process. Acidic pH can occur in anaerobic digestion systems where the methanogenesis rate is slower than the acidogenic rate, thereby bringing about accumulation of the volatile fatty acids. This situation commonly occurs where there is a sudden or excessive increase in the wastewater addition to the anaerobic system. On the other hand, alkaline pH can result in the treatment of wastes containing high amount of nitrogenous compounds, such as proteins. These compounds hydrolyse to produce ammonia, which can bring about alkaline pH. When pH value rises higher than 8.5, it begins to exert a toxic effect on the methanogenic bacteria (Hartmann & Ahring, 2006).

Wastewater alkalinity is also an important parameter in process control. It is a measure of the potential resistance of the digestion process to pH fluctuations. High alkalinity thus ensures process stability. Alkalinity concentrations in the range of 2000–5000 mg/L as for $CaCO_3$ are typically required to maintain the pH at or near neutral (Metcalf & Eddy, 2003; Polprasert, 1996).

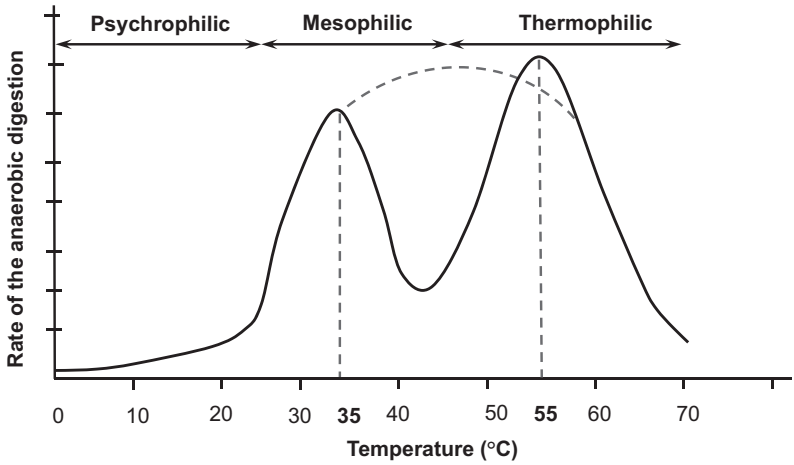

Figure 19.3 Temperature ranges for anaerobic digestion.
Adapted from Mata-Alvarez (2003).

19.2.4 Temperature

Like other biological processes, anaerobic biodegradation is also affected by temperature, as shown in Figure 19.3. The process can be operated at psychrophilic (<20 °C), mesophilic (25–40 °C) or thermophilic (45–60 °C) temperature ranges, with the optimum temperatures for the mesophilic and thermophilic process at about 37 °C and 55 °C, respectively (Abbasi, Tauseef, & Abbasi, 2012; Raposo et al., 2012). Psychrophilic temperatures are rarely used due to the resulting relatively low reaction rate. The choice of either mesophilic or themophilic digestion is dependent on the net economic gain that each can provide. In practice, most commercial anaerobic digestion plants operate at the mesophilic range.

19.2.5 Solid and hydraulic retention times

The solid retention time (SRT) refers to the average dwelling time of microorganisms within the reactor. This time depends on the growth rate of the microbes and also on the rate at which the microbial biomass is removed from the treatment system. The appropriate SRT varies from one microbial group to another, and is dependent on the degradation rate, which itself is dependent on the nature of organic compound in the waste, as discussed in Section 19.2.1 above. Methanogenic bacteria have significantly slower growth rates than other microbial groups in the anaerobic digestion process, as shown in Table 19.3. Consequently, the appropriate retention time of anaerobic digestion systems is controlled by the need to reduce the rate of removal of the methanogenic microorganisms from the anaerobic digestion system.

Operational temperature also plays a vital role in the microbial regeneration time and hence the retention time. The higher the operating temperature, the lower the retention time (Metcalf & Eddy, 2003). Thus, thermophilic systems tend to operate at shorter retention times than mesophilic systems.

Table 19.3 Average time of regeneration of some microbial groups

Microorganisms	Time of regeneration
Acidogenic bacteria	Less than 36 h
Acetogenic bacteria	80–90 h
Methanogenic bacteria	15–16 days

Adapted from Deublein and Steinhauser (2008).

The hydraulic retention time (HRT) is defined as the theoretical amount of time that the liquid is resident within the reactor. For completely mixed systems without biomass recycling, the HRT is the same as the SRT. For systems designed to encourage greater biomass retention, the SRT is generally longer that the HRT. Separating both parameters in this manner improves process stability and efficiency.

19.2.6 Organic loading rate

The organic loading rate (OLR) describes the relationships between the rate of treatment of the organic matter and the size of the treatment system or reactor. It is expressed as weight of organic matter in terms of COD or VS (or TS) per volume of reactor per day. The higher the OLR that a system can treat efficiently, the greater the cost-effectiveness of the application. Anaerobic treatment technologies or systems that can handle relative high OLR are usually referred to as high-rate systems.

19.2.7 Toxic compounds

The anaerobic digestion process can be inhibited by substances in the waste that are toxic to anaerobic microorganisms. The common inhibitors include ammonia, sulphide, long-chain fatty acids, salts, heavy metals, phenolic compounds and xenobiotics (Chen, Cheng, & Creamer, 2008; Mata-Alvarez, 2003).

Sulphide is produced during the hydrolysis of sulphates contained in the waste, and its microbial inhibitory effect is likely to occur where the COD/SO_4^{2-} ratio of the waste is less than 7.7. Where this is the case, the inhibition can be minimised by the following measures (Pohland, 1992):

- Dilution of the influent
- Addition of iron salts to precipitate sulphide from solution
- Stripping the reactor liquid or scrubbing and recirculation of the reactor biogas
- Biological sulphide oxidation and sulphide recovery

In general, the level of toxicity of a substance to microorganisms will depend on its nature, concentration and the degree to which the process has become acclimated to it. As with most microorganisms, anaerobic bacteria can develop a tolerance to a wide variety of inhibitors following an adequate acclimation period.

19.2.8 Treatment configuration: single- and multi-stage systems

In single-stage systems, all of the processes outlined in Figure 19.1 take place in a single reactor. The main advantage of a single reactor system is its relatively lower capital and maintenance costs. A major drawback is that the system cannot take sufficiently into account the differences in substrate and environmental requirements, and kinetic properties of the major microbial groups involved in the process, notably, the acidogenic and methanogen microorganisms. Providing and ensuring optimal conditions for both acidogenic and methanogenic microorganisms in a single reactor usually entails long retention times (SRT and HRT) and low treatment rates (i.e. low OLR).

Multi-stage systems involve separation of mainly the hydrolysis and acidogenesis stages from the methanogenesis stage, in different reactors connected in series or in a single compartmentalised reactor. The latter is also known as an anaerobic baffled reactor system (Akunna & Clark, 2000; Baloch & Akunna, 2003; Barber & Stuckey, 1999). Stage or phase separation enables each of the different processes to be maintained at their optimal conditions, to promote greater process stability and biogas yield (Sosnowski, Wieczorek, & Ledakiwicz, 2003). The main drawback associated with staged operation is the increased technical complexity and relatively higher capital and maintenance costs, which may not always lead to higher process rates and subsequent biogas yields (Weiland, 1992).

19.3 Factors affecting the application of anaerobic digestion in waste treatment

19.3.1 Pretreatment of wastes

As explained in Section 19.2.1, the hydrolysis stage is usually the limiting stage for the anaerobic digestion of organic solids. Increasing the rate of hydrolysis can lead to an increase in the rate of biogas production. A range of hydrolysis-enhancing pretreatment methods for organic solids have been developed. These include physical (e.g. thermal, ultrasound, mechanical, etc.), chemical (alkaline, acid, ozone, etc.), biological (enzymes, aerobic, etc.) and their various combinations. Determining the most appropriate pretreatment method(s) will depend on the characteristics of the waste. More information on pretreatment can be found in the literature (e.g. Al-Alm Rashed, Akunna, El-Halwany, & Abou Atiaa, 2010; Delgenes, Penaaud, & Moletta, 2003; Mallick, Akunna, & Walker, 2008, 2010).

19.3.2 Co-digestion

Co-digestion or co-treatment refers to the digestion of a mixture of two or more types of waste. The practice can provide the following benefits (Akunna, Abdullahi, & Stewart, 2007; Hartmann, Angelidaki, & Ahring, 2003):

- Waste dilution to reduce the inhibitory levels of certain constituents of the waste streams
- Increase in readily biodegradable organic matter and vital nutritional balance needed for increased biogas production and effective digestion
- Mitigation against seasonal and diurnal variations in quantity and quality of waste

In essence, co-digestion can lead to greater process stability and improved economic gains.

19.3.3 Technology selection

Various types of anaerobic technologies (or reactor systems) have been developed, and some are suitable only for certain types of wastes. Where the waste contains significant amounts of organic solids, a suitable technology will involve a longer SRT to allow sufficient time for effective hydrolysis of the solids. Long retention times result in large reactors, operating at relatively low OLR. Alternatively, the solids can be separated in a pretreatment stage, using physical or physico-chemical methods, and the liquid and semisolid fractions treated separately. This option can lead to a smaller overall reactor size. Solid–liquid separation pretreatment is a common practice in the anaerobic digestion of waste from the food- and beverage-processing wastewaters.

Technologies that can provide phase separation and/or ensure simultaneous high SRT and low HRT are usually referred to as high-rate systems. Other technological variations include operating at different temperatures (i.e. mesophilic, thermophilic or a combination of both in staged systems), use of inorganic or poorly biodegradable organic media support to enhance biomass retention (biofilm systems) and use of various methods to provide reactor mixing (use of biogas or treated effluent, fluidisation, etc.). More information on the range of available technologies can be found in the literature (e.g. Akunna & Clark, 2000; Baloch & Akunna, 2003; Barber & Stuckey, 1999; Hall, 1992; Letting & Hulshoff, 1992; van Lier, Mahmoud, & Zeeman, 2008; Metcalf & Eddy, 2003).

19.3.4 Biogas production and use

Irrespective of the technology adopted, the performance of an effectively operated anaerobic digestion system treating is similar to values shown in Table 19.4.

Table 19.5 shows a typical composition of biogas from a good functioning anaerobic treatment process.

Biogas can be used directly in an appropriate gas boiler for heating or can be burned in an engine to produce combined heat and power (CHP). In CHP units,

Table 19.4 Typical anaerobic treatment performance levels

Treatment parameter	Typical value
BOD removal (%)	80–90%
COD removal (mg/L)	1.5 × BOD removed
Biogas production	0.5 m^3/kg COD removed
Methane production	0.35 m^3/kg COD removed
Sludge production	0.05–0.10 kg VS/kg COD removed

BOD, biological oxygen demand; COD, chemical oxygen demand; VS, volatile solids; TKN, total Kjehldahl nitrogen.
Source: Pohland (1992).

Table 19.5 Typical biogas composition of a normal functioning anaerobic digestion process

Component	Typical range (% volume)
Methane (CH_4)	50–75
Carbon dioxide (CO_2)	25–50
Nitrogen (N_2)	0–10
Hydrogen (H_2)	0.01–5
Oxygen (O_2)	0.1–2
Water vapour	0–10
Ammonia (NH_3)	<1%
Hydrogen sulphide (H_2S)	0.01–3

Adapted from ADBA (2013) and DGS and Ecofys (2005).

about 70% of the energy contained in biogas is converted to heat and the rest to electricity. The heat can be used to maintain the digester to mesophilic or thermophilic operating temperature and also in supplementing industrial and residential heating requirements. The biogas can also be further cleaned to upgrade the methane content to up to 95%, for injection into the district gas supply network for domestic and industrial use. Upgrading of biogas to pure methane (termed biomethane) creates an option for the biogas to be used as transport fuel for vehicles. More information on options for biogas use can be obtained elsewhere (ADBA, 2013; DGS & Ecofys, 2005; Murphy et al., 2011).

19.3.5 Digestate handling and disposal

The anaerobic digestion process produces a semi-solid by-product, referred to as digestate, the properties of which will depend on the C/N ratio and the solids content of the raw waste. In the absence of significant amounts of toxic compounds such as heavy metals, the digestate is a good source of fertiliser (ADBA, 2013). Low-waste C/N ratios will produce relative high ammonia content digestate and vice versa. Where the conversion of the solids is relatively low, the moisture content of the digestate will be lower than where the solids are made up of readily biodegradable organic matter. Consideration must therefore be made as to the potential end uses of digestate and whether additional treatment is required, such as dewatering for volume reduction or aerobic digestion (or composting) (Abdullahi, Akunna, White, Hallett, & Wheatley, 2008).

19.4 Anaerobic treatment of brewery wastes

This section addresses some of the specific factors pertinent to the use of anaerobic processes as part of a treatment regimen for the management of brewery waste.

19.4.1 Waste production and collection

The brewing process is a significant water consumer and wastewater producer. It has been estimated that the volume of wastewater discharged from a brewery is within the range of 2.5–10 times the volume of beer being produced (Fakoya & van der Poll, 2013; Janhom, Wattanachira, & Pavasant, 2009; Reed, 2006; Simate et al., 2011).

Brewing involves unit processes, some of which are carried out daily in batch or discontinuous operations, with some breweries operating only a 5-day week and with weekends reserved for plant maintenance. Consequently, there are wide variations in the rates of wastewater production over a given period. If the wastewater is fed directly to an anaerobic treatment system, the variation in quality and quantity can upset the balance between the key microbial communities, notably the acidogenic and methanogenic microorganisms, thereby causing instability in the process. This problem can be prevented by collecting the wastewater in a balancing tank from which a consistent wastewater quality can be fed to the anaerobic digestion reactor.

19.4.2 Constituents of wastes

The basic beer production process involves mashing, boiling, and fermentation followed by cooling, clarification, pasteurisation, and packaging (Baloch, Akunna, & Collier, 2007; Mussatto, Dragone, & Roberto, 2006). Each of these stages produces wastewater with varying concentrations of organic compounds.

The particulate organic constituents of brewery wastes consist mainly of spent grain, spent hops and yeast. Spent grains or brewers' spent grains (BSG) is the most abundant brewing by-product, corresponding to about 85% of total by-products generated (Mussatto et al., 2006). BSG is typically more than 75% moisture, and the dry matter is composed of about 20% protein and about 70% fibre (Mussatto et al., 2006). It has a C/N ratio of less than 25, and hence is amenable to anaerobic digestion, either as a sole substrate or in co-digestion with other readily biodegradable organic compounds (Kuzmanova & Akunna, 2013; Sturm, Butcher, Wang, Huang, & Roskilly, 2012; Thomas & Rahman, 2006). Other sources of waste include waste liquor from the mashing process and 'out of specification' beer. The various waste streams in the brewing process can each therefore be characterised by one or more of the following properties:

- Highly variable (continuous or intermittent) flow and composition
- High or low organic strength, expressed in term of biological oxygen demand (BOD) or COD
- High or low organic solids content
- High or low nitrogen content
- High concentration of sulphates
- High pH or very low pH values

The characteristics of brewery waste will also be affected by the collection system and by housekeeping practice. Where the waste streams are separated according to their concentrations of organic contents, the wastewater from the fermentation and maturation vessels are likely to contain high levels of COD and suspended solids. Baloch et al. (2007) reported concentrations of wastewater from the tank bottoms,

which are the remains of the fermentation and maturation vessels after the beer has been held for a period of time to improve its flavour and allow any remaining yeast to settle, to be in the range of 115,000–125,000 mg/L for COD, 1400–16,000 mg/L for TS (composed of over 90% VS) and pH of about 4.2.

Where more mixing of various wastewater streams are carried out, the compositions of brewery waste can contain concentration ranges as reported in Tables 19.6 and 19.7. The yeast-enriched wastewater is usually the residues containing excess yeast used in the process.

As shown by the values reported in Tables 19.6 and 19.7, brewery waste is extremely variable in composition. In general, the waste can be classified (1) intermediate to high strength, (2) made up mainly of organic compounds and (3) does not contain compounds known to be toxic to the anaerobic treatment microorganisms. However, adequate attention should always be paid to ensure that the COD/SO_4^{2-} ratio is not within the range that can bring about sulphide toxicity. Mixing excess yeast residues with other waste streams can help dilute the sulphate concentration to appropriate levels (Akunna, 2010).

Table 19.6 Types and composition of brewery wastewaters

Parameter	Concentration(mg/L)[a]	
	Brewery effluent	Yeast-enriched wastewater
COD	3000–6000	50,000–110,000
Solids	50–1000	2000–3000
TKN	24–200	500–10,000
SO_4^{2-}	35	160
pH	5–11	8.3

COD, chemical oxygen demand; TKN, total Kjehldahl nitrogen.
[a]All parameters are in mg/L except pH.
Source: Akunna (2010).

Table 19.7 Characteristics of wastewater from a local brewery

Parameter	Range	Typical
COD (mg/L)	1800–50,000	10,000
BOD (mg/L)	2700–38,000	16,000
Solids (mg/L)	50–6000	500
TKN (mg/L)	20–600	50
$P_{(total)}$ (mg/L)	4–103	10
SO_4^{2-} (mg/L)	20–50	35
pH	5–11	9

BOD, biological oxygen demand; COD, chemical oxygen demand; P, phosphorus; TKN, total Kjehldahl nitrogen.
Source: Akunna (2003).

19.4.3 Treatment and pretreatment requirements

Depending on the composition of the waste and its discharge or reuse requirements, the following treatment processes can be applied, as stand-alone processes or as part of a treatment regimen:

- Solids separation (e.g. filter cake washing)
  - Sedimentation (with or without use of chemical to enhance process)
  - Filtration or flotation
- Neutralisation (e.g. for caustic wash)
- Biological treatment for medium/high strength streams (e.g. wort washings, tank bottoms, surplus yeast, kegging), consisting of aerobic or anaerobic processes, as stand-alone or combined.

Pretreatment to enhance the rate of the hydrolysis of the brewery solids is necessary in order to accelerate the digestion process and to increase biogas yield. In a recent study (Kuzmanova & Akunna, 2013), a VS reduction of about 24% was obtained after 40 days of batch digestion of the BSG. A common hydrolytic pretreatment operation for BSG is mechanical disintegration to reduce the size of the particles, which increases the available surface area available for biological process. Other methods include alkaline and acid, ultrasound, thermal and enzymatic treatments. Although some of these processes have been trialled in the laboratory with variable levels of success, full-scale application is hampered by their current high operational costs.

Nutrient correction and pH balancing may be part of pretreatment steps before anaerobic treatment. Nutrient correction may involve dosing with a solution of mainly nitrogen and phosphorus, micro-nutrients or compounds to provide alkalinity. Alkaline compounds (e.g. lime, caustic soda) could also be used for pH correction, dosed in the balancing tank and/or directly inside the reactor whenever the need arises.

19.4.4 Co-digestion

For some breweries, co-digestion with other easily available organic substrates may be the only way in which the operation will be economically viable. Co-digestion of brewery waste with animal slurry can bring about C/N ratio correction, which would have been more difficult to achieve with either of the substrates alone. Other appropriate substrates include source separated food wastes, agricultural and other food and beverage processing wastes, domestic wastewater treatment sludges, crop residues and grasses.

Effective co-digestion relies on proper determination of suitable blends that can provide the desired objectives, taking into consideration the biochemical pathways and kinetics of the individual components of the mixture. It is therefore important to be able to predict the outcome of digestion of a chosen waste mixture, and to manage the process carefully. Where one or more of the substrates have no history of successful co-digestion with brewery wastes, it is important to conduct laboratory- and pilot-scale trials to establish suitable design and operational data before embarking on a commercial-scale operation. When a full-scale plant is operational, it is always advisable to maintain laboratory scale models for quick and efficient assessment of

the various combinations of new substrates. Anaerobic digestion models combined with experimental results can be used to build and to validate co-digestion models to support effective feedstock management (Akunna et al., 2007; Hartmann et al., 2003; Hierholtzer & Akunna, 2012, 2014).

19.4.5 Biogas production

Biogas yield reported in the literature for brewery wastes are very variable and depend on the wastewater characteristics. A range of 0.25–0.3 L CH_4/g VS (Agler, Aydinkaya, Cummings, Beers, & Angenent, 2010), 60–100 m^3/wet tonne of 20% TS (ADBA, 2013), 311 mL CH_4/g TS_{added} for BSG (Kuzmanova & Akunna, 2013) have been reported. The biogas production of various substrates that can be used in co-digestion with brewery wastes can be found in the literature (ADBA, 2013; DGS & Ecofys, 2005; Murphy et al., 2011). The various options for the use of biogas have been addressed in Section 19.3.3 above.

19.4.6 Digestate management

Digestates from the digestion of brewery wastewater have generally been found to be suitable for land application, preferably following posttreatment such as dewatering for volume reduction and aerobic digestion (composting) for further breakdown (or stabilisation). The latter is usually carried out alone or in combination with plant biomass (green wastes). Where co-digestion is practised, depending on the sources of the other constituting wastes, posttreatment for land application may include disinfection for pathogen reduction and reduction of potential toxic compounds (such as organic micropollutants and heavy metals). Other common disinfection methods include thermal treatment and lime addition. Further information on digestate handling and disposal can been found in the literature (ADBA, 2013).

19.5 Conclusion and perspectives

Anaerobic digestion is now widely regarded as a sustainable management approach for those high organic strength wastes that cannot be re-used for other purposes due to economic and public health reasons. The current worldwide quest for renewable sources of energy and the restrictions now in place in many countries regarding the disposal of organic wastes to landfill have also contributed to the uptake of anaerobic digestion for the management of municipal, agricultural and industrial organic residues. The brewing industry is one of the sectors that is embracing anaerobic digestion, both as a method of complying with environmental regulations and as a vital source of energy to offset its high dependence on fossil-fuel–based energy sources. Being environmentally friendly is now considered by many organisations as a tool to improve the corporate image, particularly for those sectors such as the food and beverage industries, which are heavy consumers of natural resources and energy as well as heavy producers of wastes.

In the brewing sector, one of the potential challenges to the uptake of anaerobic digestion for waste treatment is the economy of scale, particularly for breweries that do not produce sufficient organic waste to make the process viable. For these breweries, co-digestion can be the solution. There is therefore a need for more research on co-digestion of brewery organic waste with other amenable organic materials.

More research is also needed in finding cost-effective ways of enhancing the hydrolysis of the solid residues (BSG).

Finally, most of the current anaerobic digestion plants operate on mesophilic temperatures. More research is needed on process optimisation at lower temperatures and also at higher temperatures, to explore whether any of these or their combinations can result in greater net energy gains than existing practices.

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Water treatment and reuse in breweries

20

G.S. Simate
School of Chemical and Metallurgical Engineering, University of the Witwatersrand, Johannesburg, South Africa

20.1 Introduction

Brewing is a multibillion dollar industry that creates jobs, generates taxes, supports agriculture and attracts tourism (Richey, 2012). However, one of the major challenges faced by the brewing industry today is water consumption (Simate, 2012). It is estimated that up to seven litres of water are used for every litre of beer produced (Fakoya & van der Poll, 2013; Janhom, Wattanachira, & Pavasant, 2009; Simate et al., 2011). This water is used in various activities including beer production, heating and cooling, cleaning and sanitation (Braeken, Van der Bruggen, & Vandecasteele, 2004; Fakoya & van der Poll, 2013). However, most of this water is allowed to flow out to the drains after its intended use (Olajire, 2012). Furthermore, some of the water is lost with spent grains and some is lost through evaporation during wort boiling (Olajire, 2012). Besides large water consumption, the brewing industry is also a large producer of wastewater (Baloch, Akunna, & Collier, 2007). Though there are variations in the composition of brewery wastewater (Brito et al., 2007; Rao et al., 2007), typically, it has high organic components (Brewers of Europe, 2002; Goldammer, 2008). If the brewery wastewater is discharged into waterways, high levels of organic compounds can deplete dissolved oxygen needed for survival of aquatic species. Some organic compounds also cause serious physiological and neurological damage to the human body when ingested. Thus, it is imperative that brewery wastewater is subjected to some degree of pretreatment before discharge. Generally, the wastewater generated is pretreated within the brewery before being discharged into the waterway or municipal sewer system (Goldammer, 2008; Huige, 2006).

As a result of public perception and the possibility of the quality of beer deteriorating, beer brewing is characterised by the use of high-quality fresh water (Janhom et al., 2009). However, the use of fresh water is unsustainable because of an increase in water demand from various other sectors of society and a significant dwindling of fresh water sources. Therefore, it is important that appropriate processes are developed that should not only remove macro-, micro- and nano-pollutants from brewery wastewater, but also purify it to a suitable level so that it may be used in primary and/or secondary applications. This chapter focuses on the current and future processes for treating brewery wastewater including prospective applications for reuse. The chapter is divided into five themes: (1) production and composition of brewery wastewater, (2) pretreatment of brewery wastewater, (3) advanced treatment of brewery wastewater, (4) challenges and future prospects and (5) conclusions.

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20.2 Production and composition of brewery wastewater

Beer is one of the oldest alcoholic beverages humans have ever produced (Arnold, 1911; Hornsey, 2003; Wyatt, 1900). In fact, the antecedents of our modern day beer existed many years ago in several places, including Asia, Africa and Europe (Poelmans & Swinnen, 2011). Throughout the years, the brewing industry has developed systematically to include several new process developments and genetic inventions (Linko, Haikara, Ritala, & Penttilä, 1998). Batch-type operations are predominantly employed to process raw materials for the final beer product (van der Merwe & Friend, 2002). The five steps shown in Figure 20.1 dominate the brewing process (Harrison, 2009), though production methods will differ from brewery to brewery as well as according to the type of beer, brewery equipment and national legislation (Brewers of Europe, 2002). Mashing and fermentation are the two vital processes (Phiarais & Arendt, 2008). Mashing involves the breaking down of starch to sugar and fermentation is the conversion of the sugars to alcohol and carbon dioxide.

Due to a large number of steps in the brewing process and because of its batch-wise nature, an enormous amount of water is utilised in beer making itself, washing, cleaning and destruction of bacteria and other microorganisms from various units after completion of each and every batch (van der Merwe & Friend, 2002). Consequently, large volumes of brewery wastewater are produced.

The quantity and characteristics of brewery wastewater can differ significantly from time to time and location to location since it is dependent on several different processes that occur within the brewery (Driessen & Vereijken, 2003). Table 20.1 is a summary of some of the physicochemical characteristics of brewery wastewater (Driessen & Vereijken, 2003; Rao et al., 2007). As shown in Table 20.1, the composition of brewery wastewater is highly variable. However, the major component of brewery effluent is organic material (Brewers of Europe, 2002; Goldammer, 2008), as evidenced from high chemical oxygen demand (COD) and biological oxygen demand (BOD). Both of these parameters (i.e. COD and BOD) are important diagnostic parameters for determining the quality of water in natural waterways and waste streams (Mantech, 2011). BOD is a measure of the amount of oxygen required by microorganisms to degrade organic matter whilst COD is a measure of the total quantity of oxygen needed to oxidise organic as well as inorganic matter present in the wastewater into carbon dioxide and water (Metcalf & Eddy, 1991; ReVelle & ReVelle, 1988). Nevertheless, brewery wastewater is non-toxic, does not carry a considerable amount of heavy metals and is easily biodegradable (Brewers of Europe, 2002; Olajire, 2012). This implies that if properly treated, brewery wastewater may be reused as primary water and/or secondary water without harming public health and the environment. Primary water is the water that is utilised in producing beer itself whereas secondary water is water that does not have any physical contact with beer (Simate et al., 2011), for example water used for cooling utilities, water utilised in the packaging process and water used for general purpose cleaning.

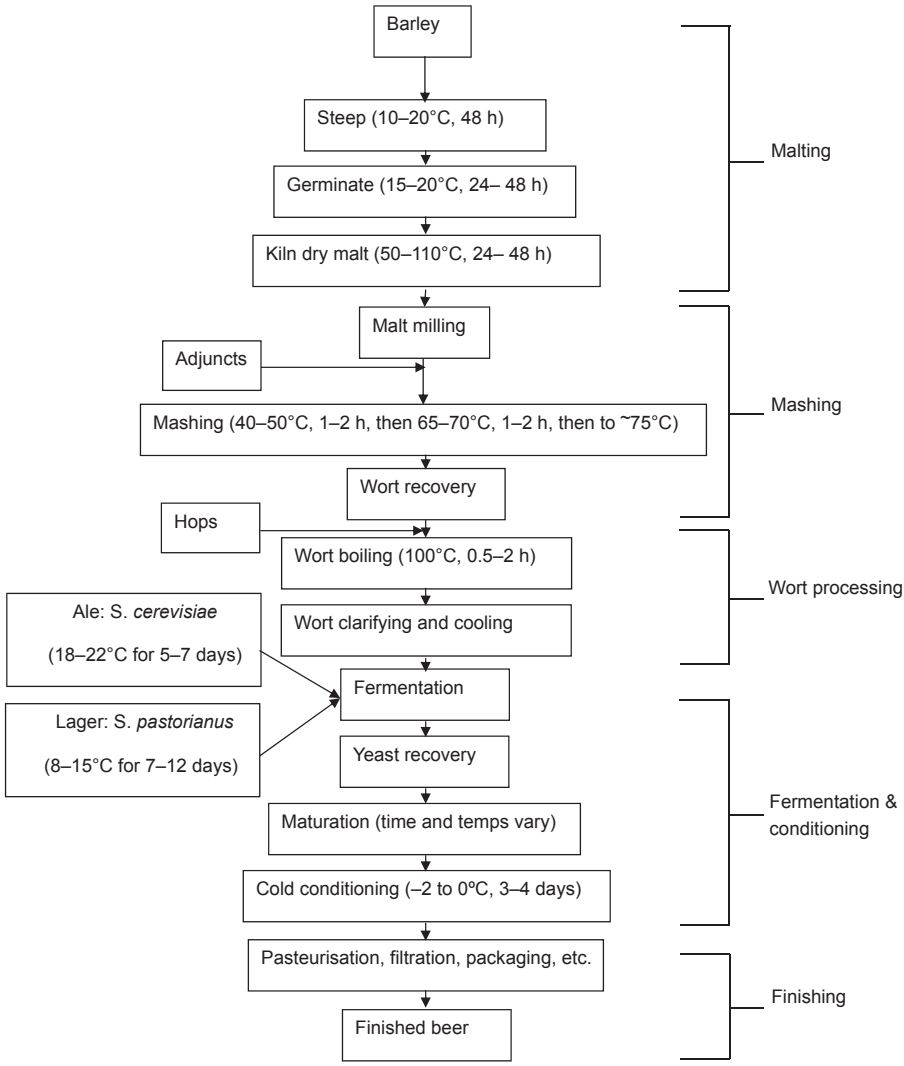

Figure 20.1 The brewing process.
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20.3 Pretreatment of brewery wastewater

Brewery wastewater is recognised as a significant environmental problem because of the considerable amount of impurities, particularly organic load, created by the brewing process. The disposal of wastewater with high organic load into water bodies can have severe consequences for the biota. This is because during the decomposition of organic pollutants, the dissolved oxygen in the receiving waterways may be used at a faster rate than it can be replenished, exhausting oxygen and thus depriving biota of oxygen needed for survival (Rashed, 2011). Furthermore, wastewater with high organic pollutants contains

Table 20.1 Physicochemical characteristics of brewery wastewater

Parameter	Value
pH	3–12
Temperature (°C)	18–40
COD (mg/L)	2000–6000
BOD (mg/L)	1200–3600
COD:BOD ratio	1.667
VFA (mg/L)	1000–2500
Nitrogen (mg/L)	25–80
Phosphates as PO_4 (mg/L)	10–50
TKN (mg/L)	25–80
TS (mg/L)	5100–8750
TSS (mg/L)	2901–3000
TDS (mg/L)	2020–5940

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a large amount of suspended solids that minimise the light accessible to photosynthetic organisms and, on settling out, significantly change the characteristics of the river bed, making it an inappropriate habitat for many invertebrates. More importantly, the disposal of untreated (or partially treated) brewery wastewater directly into waterways or municipal sewers costs more in municipal fees because there are environmental restrictions limiting the amount of contaminants in solution sent through to the municipal reticulation system. This section discusses the physicochemical and biological processes that are commonly used to remove as much particulate and colloidal contaminants from brewery wastewater as possible before it enters the waterways or municipal sewer systems. Table 20.2 lists the unit operations that are included within each category (Simate et al., 2011).

20.3.1 Physical pretreatment methods

Physical methods encompass all processes in which contaminants are removed by means of or through the application of physical forces. These are the first treatment methods that separate coarse solid matter, instead of dissolved pollutants (Simate et al., 2011). Large solids and grit are removed first so that they do not restrain treatment processes or cause excessive mechanical wear and increased maintenance on subsequent wastewater treatment equipment (EPA, 2003). In most cases, preliminary treatment consists of flow equalisation, screening, grit removal and gravity sedimentation (EPA, 2003). In general, physical pretreatment requires the least energy, but is also the least effective in removing contaminants.

20.3.2 Chemical pretreatment methods

Chemical methods are wastewater treatment processes in which contaminants are removed by means of or through chemical reactions (Metcalf & Eddy, 1991). Thus, in

Table 20.2 Wastewater treatment unit operations and processes

Physical unit operations	• Screening • Comminution • Flow equalisation • Sedimentation • Flotation • Granular-medium filtration
Chemical unit operations	• Chemical precipitation • Adsorption • Disinfection • Chlorination • Other chemical applications
Biological unit operations	• Activated sludge processes • Aerated lagoons • Trickling filters • Rotating biological contactors • Pond stabilisation • Anaerobic digestion • Biological nutrient removal

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this pretreatment method, different chemicals are mixed with the brewery wastewater to adjust the water chemistry (Huang, Schwab, & Jacangelo, 2009). Coagulation and flocculation and/or pH adjustment are some of the most commonly used chemical treatment methods for removing toxic materials and colloidal impurities at breweries (Olajire, 2012; Simate et al., 2011).

Chemical pretreatment methods have an advantage of being easily applied as soon as it is required (Mohan, 2008). However, one of the inherent disadvantages of chemical treatment methods, as compared to physical methods, is that they are additive processes (Metcalf & Eddy, 1991). As a result, there is usually a positive increase in the dissolved constituents in the wastewater. This additive aspect is in contrast to physical and biological treatment methods that may be described as being subtractive because material is removed from the wastewater. Another drawback of chemical methods is that they are all intensive in operating costs (Metcalf & Eddy, 1991). The costs of some of the chemicals are tied to the cost of energy and thus can be expected to increase similarly.

20.3.3 Biological pretreatment methods

The objective of a biological pretreatment method is to eliminate or reduce the concentration of organic and inorganic compounds (Metcalf & Eddy, 1991). It is hinged on the activity of a variety of microorganisms, breaking down the biodegradable organic and inorganic pollutants in the wastewaters (Simate et al., 2011). The principal applications of these processes, also identified in Table 20.3, are (Metcalf & Eddy, 1991): (1) the removal of the carbonaceous organic matter, (2) nitrification, (3) denitrification and (4) stabilisation.

Table 20.3 Major biological treatment processes

Type	Common name	Use
Aerobic processes		
Suspended growth	Activated sludge process	Carbonaceous BOD removal; nitrification
	Suspended growth nitrification	Nitrification
	Aerated lagoons	Carbonaceous BOD removal; nitrification
	Aerobic digestion	Stabilisation; carbonaceous BOD removal
	High-rate aerobic algal ponds	Carbonaceous BOD removal
Attached growth	Trickling filters	Carbonaceous BOD removal; nitrification
	Roughing filters	Carbonaceous BOD removal
	Rotating biological contactors	Carbonaceous BOD removal; nitrification
	Packed bed reactors	Nitrification
Combined processes	Trickling filter, activated sludge	Carbonaceous BOD removal; nitrification
	Activated sludge, trickling filter	Carbonaceous BOD removal; nitrification
Anoxic processes		
Suspended growth	Suspended growth denitrification	Denitrification
Attached growth	Fixed film denitrification	Denitrification
Anaerobic processes		
Suspended growth	Anaerobic digestion	Stabilisation; carbonaceous BOD removal
	Anaerobic contact process	Carbonaceous BOD removal
Attached growth	Anaerobic filter	Carbonaceous BOD removal; stabilisation
	Anaerobic lagoon (ponds)	Denitrification
Aerobic/anoxic/anaerobic processes		
Suspended growth	Single stage	Carbonaceous BOD removal; nitrification; denitrification
Attached growth	Nitrification–denitrification	Nitrification–denitrification
Combined processes	Facultative lagoons (ponds)	Carbonaceous BOD removal
	Maturation or tertiary ponds	Carbonaceous BOD removal; nitrification
	Anaerobic facultative-lagoons	Carbonaceous BOD removal
	Anaerobic facultative-aerobic lagoons	Carbonaceous BOD removal

Source: Metcalf and Eddy (1991).

Table 20.4 Anaerobic treatment as compared to aerobic treatment

	Aerobic systems	Anaerobic systems
Energy consumption	High	Low
Energy production	No	Yes
Biosolids production	High	Low
COD removal (%)	90–98	70–85
Nutrients (N/P) removal	High	Low
Space requirement	High	Low
Discontinuous operation	Difficult	Easy

Source: Driessen and Vereijken (2003).

Biological methods of treating wastewater can be either anaerobic (without oxygen) or aerobic (with air/oxygen supply) (Goldammer, 2008). Another process which is not very different from anaerobic is anoxic process (used for the removal of nitrogen from wastewater). The individual processes are subdivided further, depending on whether treatment is performed in suspended-growth systems, attached-growth systems or combinations thereof (Metcalf & Eddy, 1991). Table 20.4 compares aerobic and anaerobic biological treatment systems such as activated sludge (Driessen & Vereijken, 2003; Simate et al., 2011). Compared with physicochemical or chemical methods, biological treatment methods possess three main advantages (Dai, Yang, Dong, Ke, & Wang, 2010): (1) the treatment technology is fully developed, (2) high COD and BOD removal efficiency (80–90%), and (3) low cost of investment. While biological treatment processes are highly effective in reducing conventional pollutants, they also require a high energy input (Feng, Wang, Logan, & Lee, 2008).

20.4 Advanced treatment of brewery wastewater

Water of drinking quality is one of the most important resources in breweries (Blomenhofer, Groß, Procelewska, Delgado, & Becher, 2013). This water is required for brewing, rinsing or cooling purposes (Braeken et al., 2004; Fakoya & van der Poll, 2013). Brewing water is utilised during the brewing process itself; rinsing water is needed for the cleaning of bottles, vessels and installations, and cooling water is applied at different stages of the brewing process (Braeken et al., 2004). However, with a growing human population, water resources are under stress both quantitatively and qualitatively (Manios, Gaki, Banou, Ntigakis, & Andreadakis, 2006), making operations in the brewery industry very difficult. Therefore, the perpetual necessity for high-quality, but ever insufficient water in the brewery industry has continued to drive the need to find other sources of water (Simate, 2012). One option that needs serious consideration is wastewater reclamation and reuse (Simate, 2012). In fact, the future reuse of water appears to be inescapable, as the concern of water shortage has become a grave global and environmental problem (Janhom et al., 2009).

It must be noted, however, that due to expected high standards, reuse of treated water in breweries is considered unacceptable and would thus require that drinking water standards are complied with (Braeken et al., 2004). In many countries, standards for wastewater reuse have been influenced by the World Health Organisation (WHO) health guidelines (WHO, 1989) and the United States Environmental Protection Agency (US–EPA/USAID) guidelines for water reuse (EPA, 1992). The WHO health guidelines focus mainly on the presence of pathogens, while the EPA guidelines also include physiochemical parameters such as organic load (BOD or COD), total suspended solids (TSS) and residual chlorine concentration (Manios et al., 2006). Table 20.5 shows some of the vital quality requirements for rinsing, cooling and drinking water. Amongst the parameters in Table 20.5, the most important parameter for recycling water is the COD; this is also the most important parameter for measuring (Braeken et al., 2004; Ince, Ince, Sallis, & Anderson, 2000).

Because of the tighter water quality regulations coupled with unsatisfactory results from conventional or pretreatment processes, the use of intensive treatment processes is necessary if brewery wastewater is to be reused. Therefore, after the brewery wastewater has been subjected to physical, chemical and biological treatments, the wastewater can then go through advanced treatment. This section will discuss some of the current and future advanced treatment processes needed to improve the overall water quality.

20.4.1 Membrane filtration technologies

In the last two decades, various membrane filtration technologies have been used in water and wastewater treatment because of proven solid–liquid separation efficiency and more importantly because of drastic cost reduction of manufacturing membrane materials (Xie, Zhou, Chong, & Holbein, 2008). In addition, this technology has a lot of other advantages including stable and quality effluent, a small area (Hua et al., 2007), low-energy requirements, a small volume of retentate to be handled and selective removal of pollutants (Wu, Li, Wang, Xue, & Li, 2012). Moreover, no chemical addition is required. According to Mallevialle, Odendall, and Wiesner (1996),

Table 20.5 Quality standards for rinsing and cooling water and aimed value for drinking water

	Quality standard rinsing water	Quality standard cooling water	Quality standard drinking water
COD (mg O_2/L)	0–2	0–2	0–2
Na^+ (mg/L)	0–200	/	20
Cl^- (mg/L)	50–250	/	25
pH	6.5–9.5	6.5–9.5	6.5–9.5
Conductivity (μS/cm)	/	/	400

/: Not specified.
Reprinted with permission from Braeken et al. (2004).

membrane filtration is a process that uses a semipermeable membrane to separate the feed stream into two portions: a permeate that contains the species passing through the membrane and a retentate consisting of materials left behind. In other words, a membrane is a semipermeable barrier existing between two homogeneous phases and has the ability to transport one component more readily than another because of differences in physical and/or chemical properties between the membrane and the permeating components (Mulder, 1997).

There are four groups of membrane filtration that depend on the effective pore size of the membrane (Gregory, 2006). In the order of decreasing pore size, the four groups are as follows: microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and hyperfiltration (HF) or reverse osmosis (RO). Apart from the size range of permeating species, membrane filtration can be classified further in terms of the mechanisms of rejection of permeating species, the driving forces employed, the chemical structure and composition of membranes and the geometry of construction (Zhou & Smith, 2002). Table 20.6 summarises the essential features of the common membrane filtration processes.

Practically, membranes should have a high permeate flux, high contaminant rejection, great durability, good chemical resistance and low cost (Zhou & Smith, 2002). The other property that is also important in the selection and/or classification of a membrane process is pore size or molecular weight cutoff (MWCO) (Zhou & Smith, 2002). The MWCO expresses the retention characteristics of the membrane in terms of molecules of known sizes (Brock, 1983) and defines the maximum molecular weight of a solute to be rejected (Zhou & Smith, 2002).

The performance of membrane processes also relies on the use of correct module configurations (Zhou & Smith, 2002). Typical commercial membrane geometries are flat sheet and tubular. There are five module types: plate-and-flame and spiral-wound modules, based on flat membranes, and tubular, capillary and hollow-fibre modules, based on tubular membrane geometries (Basile, 2013). A qualitative comparison amongst some of the different model configurations is presented in Table 20.7.

Typically, NF and RO are of spiral-wound configuration so as to promote turbulence, thereby reducing concentration polarisation fouling and particle cake deposition (Zhou & Smith, 2002). However, this type of membrane configuration is vulnerable to biofouling. The weakness of seals and glue lines also prevents the use of vigorous backwashing and may lead to loss of module integrity. In contrast, MF and UF usually use hollow-fibre geometry to facilitate backwash and yield a high surface area-to-volume ratio. A major drawback is the high energy consumption necessary to maintain high crossflow velocity (CFV).

As stated at the beginning of this section, various membrane technologies have been used successfully for water and wastewater treatment applications. Fakhru'l-Razi (1994) used UF membranes of 10,000 nominal molecular weight limit in conjunction with an anaerobic reactor to treat wastewater from a brewery. The percentages of COD removal achieved were above 96%. The results indicated that the UF membranes are capable of efficient biomass–effluent separation, thus preventing any biomass loss from the reactor, and have potential for treating industrial wastewaters. In an attempt to treat brewery wastewater for recycling, Braeken et al. (2004) used

Table 20.6 Typical characteristics of common membrane filtration processes

Process	Operating pressure (bar)	Pore size (nm)	Molecular weight cut-off range	Size cut-off range (nm)	Main mechanisms	Permeate flux
Microfiltration (MF)	<4	100–3000	>500,000	50–3000	Sieving	High
Ultrafiltration (UF)	2–10	10–200	1000–1,000,000	15–200	Sieving	High
Nanofiltration (NF)	5–40	1–10	100–20,000	1–100	Diffusion + exclusion	Medium
Reverse osmosis (RO)	15–150	<2	<200	<1	Diffusion + exclusion	Low

Source: Gregory (2006), Zhou and Smith (2002).

Table 20.7 Comparison of different membrane configurations

Criteria	Spiral wound	Hollow fibre	Tubular	Plate and frame	Rotating disc
Packing density (m^2/m^3)	++	+++	–	+	–
Wall shear rate	++	+	+++	+	+++
Permeate flux ($L/(m^2\,h)$)	++	++	+++	+	+++
Holdup volume	+	+	–	+	–
Cost per area	+++	+++	–	–	–
Replacement cost	++	++	–	+++	–
Energy consumption	+	++	–	+	++
Fouling tendency	+	++	+++	++	+++
Ease of cleaning	–	+	++	+	+
Pretreatment requirement	–	+	+++	+	+++

Note: The configurations are ranked from clear disadvantage (–) to clear advantage (+++).
Source: Zhou and Smith (2002).

NF. Four different water streams (wastewater after biological treatment, bottle rinsing water, rinsing water of the brewing room, and rinsing water of the bright beer reservoir) were filtered with four different NF membranes. The results for the biologically treated wastewater were the most promising with removal of COD, Na^+ and Cl^- averaging 100%, 55% and 70%, respectively. The other three wastewater streams were not suitable for recycling using NF. These results clearly show the significance of pretreatment processes.

RO membranes have been used to remove both organics and inorganics in various wastewaters for wastewater reclamation. Compared to other processes, RO offers several advantages (Williams, 2003): (1) high removal rates for many contaminants and pollutants, and can remove both inorganic and organic pollutants simultaneously, (2) simple to design and operate with low maintenance costs, and (3) often consume less energy. As a result of these advantages and many others, RO has been employed for treating wastewaters in chemical, textile, petrochemical, electrochemical, pulp and paper, mining and food industries as well as municipal wastewater (Ghabris, Abdel-Jawad, & Aly, 1989; Williams, 2003). A review of RO applications has shown that COD of the effluent may decrease by 90% or may be completely removed (Madaeni & Mansourpanah, 2006; Williams, 2003). Madaeni and Mansourpanah (2006) evaluated various polymeric RO and NF membranes for COD (900–1200 mg/L) removal from biologically treated wastewater from an alcohol manufacturing plant. A complete COD removal (100%) and high flux ($33\,kg/m^2\,h$) were obtained from the hydrophilic polyethylene terphetalate PVD RO membrane. These results illustrate that RO is the best method for separating organics from water.

RO systems can also replace or be integrated with other treatment processes such as oxidation, adsorption, stripping or biological treatment to produce a high-quality product water that can be reused or safely discharged (Simate et al., 2011; Williams, 2003). For example, a combination of UF and RO resulted in very high removals of COD (98–99%), colour and conductivity from the pulp and paper industry effluents (Koyuncu, Yalcin, & Ozturk, 1999; Yalcin, Koyuncu, Oztürk, & Topacik, 1999). Shao, Wei, Yo, and Levy (2009) applied UF and RO for mine wastewater reuse. A study was carried out in two plants treating copper and coal mine wastewater that were characterised by high levels of total dissolved solids (TDS), COD, hardness and TSS as well as high concentrations of sulphates, silica, iron and other metals. The results showed that by integrating UF with RO, suspended solids, bacteria and colloids could be removed effectively. UF membranes could provide feed water for RO with low silt density index (SDI) and turbidity, even in difficult applications where raw water quality fluctuates. The study demonstrated that careful design of a multistage treatment process, and especially the combination of UF and RO membranes, can allow efficient and cost-effective reuse of wastewater that otherwise would be discharged to the environment. Actually, there are many advantages of using UF membrane technology as a pretreatment for RO (Shao et al., 2009; Yeung, Chu, Rosenberg, & Tong, 2008): (1) stable quality of UF permeate independent of raw water quality, (2) low SDI and turbidity of the UF permeate, and (3) reliable removal of bacteria and viruses by UF, thus reducing biofouling of the RO membranes.

Many studies of membrane separation have also been reported for oily wastewater treatment from various industries such as oil fields, petrochemical, metallurgical, pharmaceutical and others. Oil concentrations in wastewater generated in such industries may go up to 1000 mg/L or above (Chakrabarty, Ghoshal, & Purkait, 2008); however, the acceptable discharge limit is only 10–15 mg/L (Maphutha, Moothi, Meyyappan, & Iyuke, 2013). Using ceramic MF membrane, Hua et al. (2007) studied the effects of transmembrane pressure (TMP), CFV, oil concentration in feed, pH and salt concentration on the permeate flux, and total organic carbon (TOC) removal efficiency during the separation of oily wastewater. The high permeate flux was achieved under high TMP, high CFV, and low oil concentration. The TOC removal efficiencies were higher than 92.4% for all experimental conditions. The results also indicated that the permeate flux decreased either under high salt concentration or under low pH value in the feed solution. Maphutha et al. (2013) used a carbon nanotube integrated polymer composite membrane with a polyvinyl alcohol barrier layer to treat oil-containing wastewater. The permeate through the membrane contained oil concentrations below the acceptable 10 mg/L limit with an excellent throughput and oil rejection of over 95%.

Since brewery wastewater contains high levels of organic impurities, the results discussed in this section show that membrane technologies may be considered as preferred treatment methods for the brewing industry because of their environmentally friendly results, simplicity regarding design, user-friendly aspects in terms of operations and the small amount of space they require. Furthermore, no regenerating chemicals are required, which means no additional salts have to be added for wastewater neutralisation.

20.4.2 Membrane bioreactor technologies

Membrane bioreactor (MBR) technology combines biological-activated sludge processes and membrane filtration technologies, as shown in Figure 20.2. This technology has become more popular, abundant and accepted in recent years for the treatment of many types of wastewaters where the conventional-activated sludge (CAS) process cannot cope with either composition of wastewater or fluctuations of wastewater flow rate (Radjenovic, Petrovic, & Barceló, 2007). Depending on how the membrane is integrated with the bioreactor, the process may be carried out either by pressure-driven filtration in side-stream MBRs or with vacuum-driven membranes immersed directly into the bioreactor in submerged MBRs (Radjenović et al., 2007; Simate et al., 2011). Figure 20.3 shows the two MBR process configurations (Simate et al., 2011), and Table 20.8 gives a comparison of the two process configurations (Côté & Thompson, 2000). As can be seen in Table 20.8, the side-stream MBRs are more energy intensive compared to submerged MBRs due to higher operational TMPs, and the elevated volumetric flow required to achieve the desired CFV (Jeison, 2007). However, submerged MBRs use more membrane area and operate at lower flux levels (Seneviratne, 2007).

Several studies have investigated the efficiencies of MBR and CAS processes operating under comparable conditions, and results have shown significantly improved performance for an MBR in terms of COD, NH_3–N and suspended solids

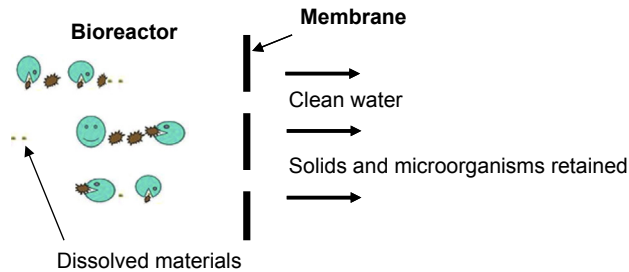

Figure 20.2 Simplified schematic description of the membrane bioreactor process. Reprinted with permission from Simate et al. (2011).

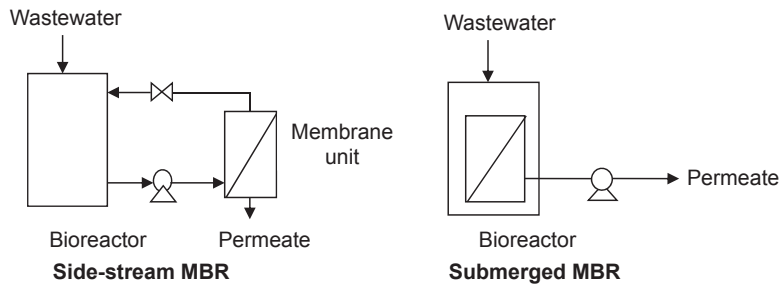

Figure 20.3 Membrane bioreactor configurations. Reprinted with permission from Simate et al. (2011).

Table 20.8 Comparison of filtration conditions for side-stream and submerged membrane bioreactors

	Side-stream tubular membrane	Submerged membrane
Manufacturer	Zenon	Zenon
Model	Permaflow Z-8	ZeeWeed ZW-500
Surface area (m^2)	2	46
Permeate flux ($L/(m^2 h)$)	50–100	20–50
Pressure (bar)	4	0.2–0.5
Air flow rate (m^3/h)	–	40
Energy for filtration (kWh/m)	4–12	0.3–0.6

Source: Côté and Thompson (2000).

(SS) removal (Bailey, Hansford, & Dold, 1994; Muller, Stouthamer, van Verseveld, & Eikelboom, 1995; Ng & Hermanowicz, 2005; Yamamoto, Hiasa, Mahmood, & Matsuo, 1989). In fact, MBR has been studied not only for wastewater, but also for drinking water treatment (Fan & Zhou, 2007; Li & Chu, 2003), and is applied to municipal wastewater treatment at full scale (Lyko et al., 2007).

Li and Chu (2003) used MBR to treat raw water supply that was contaminated by domestic sewage discharge. The results showed that nearly 60% of influent TOC was removed by MBR, accompanied by more than 75% reduction in trihalomethanes formation potential (THMFP). The MBR was also highly effective in removing turbidity, microorganisms and UV_{254} absorbance. The MBR technology was also applied to the brewery wastewater for the purpose of reuse (Dai et al., 2010). Dai et al. (2010) investigated various operating parameters during the process of brewery wastewater treatment in an MBR. The COD reduction in MBR influent (500–1000 mg O_2/L) of up to an average of 96% was achieved. Ammonium and phosphorus impurities were also reduced by 92% and 98%, respectively. Treatment of brewery wastewater in MBR was also conducted by various other researchers (Fakhru'l-Razi, 1994; Kimura, 1991; Nagano, Arikawa, & Kobayashi, 1992). In most of these studies, significant amounts of COD removals (~90%) were reported. Improved COD removal in MBR applications is attributed to the prevention of biomass washout problems commonly encountered in activated sludge processes as well as to complete particulate retention by the membrane (Côté, Buisson, Pound, & Arakaki, 1997, 1998). In another study, brewery bioeffluent was obtained using an internal aerobic MBR (internal MEMBIOR) which was superior to a conventional wastewater treatment plant (Cornelissen, Janse, & Koning, 2002). In this study, the COD of brewery wastewater varied from 1500 to 3500 mg/L, but after the internal MEMBIOR treatment the COD was reduced to around 30 mg/L regardless of the COD fluctuations of the influent. The suspended solids were also completely retained by the flat plate membrane. This made the effluent perfectly suited for reuse via RO as process water, omitting the

need for expensive pretreatment methods. This demonstrates that proper process design can provide a visible and feasible solution to the treatment of brewery wastewater.

With these promising results, it can be seen that the MBR process is an attractive option for the treatment and reuse of brewery wastewaters.

20.4.3 Electrochemical technologies

Electrochemical methods of treating wastewaters have gained increasing interest due to their outstanding technical characteristics for eliminating a wide variety of pollutants such as refractory organic matter, nitrogen species and microorganisms (Anglada, Urtiaga, & Ortiz, 2009). Furthermore, electrochemical methods of treatment are favoured because they are neither subject to failure due to variation in wastewater strength nor due to the presence of toxic substances, and require less hydraulic retention time (Simate et al., 2011). This method of treating wastewater came into existence when it was first used to treat sewage generated onboard by ships (Bockris, 1977), but extensive investigation of this technology commenced in the 1970s, when Nilsson and others investigated the anodic oxidation of phenolic compounds (Nilsson, Ronlan, & Parker, 1973). Figure 20.4 shows a conceptual diagram of an electrochemical reactor for wastewater electro-oxidation (Anglada et al., 2009).

Electrochemical oxidation of pollutants can take place directly or indirectly. In direct oxidation (or anodic oxidation), the pollutants are destroyed (or oxidised) at the anode surface. The anodic oxidation can take place through two different pathways (Anglada et al., 2009; Drogui, Blais, & Mercier, 2007): electrochemical conversion where organic compounds are only partially oxidised; therefore, a subsequent treatment may be required, or electrochemical combustion where organic compounds are transformed into water, carbon dioxide and other inorganic or biodegradable components. In indirect oxidation, the mediator species (e.g. HClO, $H_2S_2O_8$ and others) are electrochemically generated to carry out the oxidation (Anglada et al., 2009). As far as

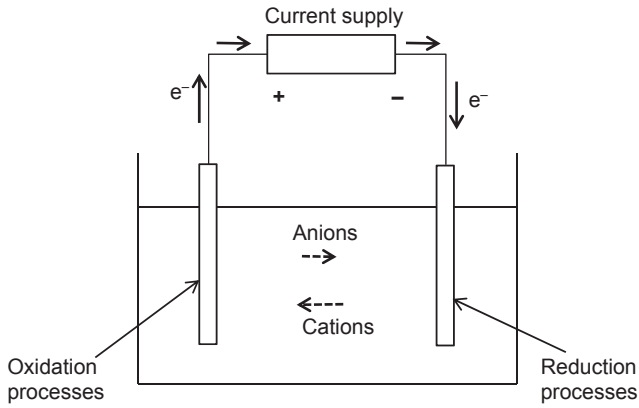

Figure 20.4 Conceptual diagram of an electrochemical reactor.
Reprinted with permission from Anglada et al. (2009).

the indirect oxidation is concerned, the most used electrochemical oxidant is probably chlorine (HClO, in neutral or alkaline media), as a result of the ubiquitous character of Cl^- species in wastewaters and due to their effective action (Martínez-Huitle & Ferro, 2006).

The efficiency and flexibility of electrochemical methods have been studied in a wide spectrum of effluents (e.g. in chemical industry, textile industry, tannery industry, food industry, agro-industry, landfill leachate and urban wastewater) (Anglada et al., 2009). Overall, the aim of these studies was mainly to eliminate nonbiodegradable and/or toxic organic pollutants and ammonia nitrogen contained in the effluent. In the food industry, Vijayaraghavan, Ramanujam, and Balasubramanian (1999) studied electrochemical oxidation of high-strength organic waste of distillery spentwash in the presence of sodium chloride. The COD removal from the spentwash was found to be 99% for an initial COD concentration of 15,000 mg/L within 4 h. Because the graphite anode and stainless steel cathode were kept in an undivided electrolytic reactor, chlorine produced from sodium chloride during electrolysis underwent a disproportionation reaction, forming hypochlorous acid. The hypochlorous acid formed thus oxidised the organic matter present in the wastewater. Later, Vijayaraghavan, Ahmad, and Lesa (2006) developed a novel brewery wastewater treatment method also based on in situ hypochlorous acid generation. The generated hypochlorous acid served as an oxidising agent that destroyed organic compounds present in the brewery wastewater. An influent COD value of 2470 mg/L was reduced to only 64 mg/L (i.e. over 97% COD reduction). In the same period, Piya-areetham, Shenchunthichai, and Hunsom (2006) investigated the removal of colour and COD from distillery wastewater by using electro-oxidation processes. The commercial Ti/RuO_2 grid was used as the cathode, and two voluminous surface area materials including graphite particles and the commercial titanium sponge were used as the anode. Effects of several parameters including the initial pH of wastewater (1–5), dilution factor, current intensity (1–10 A), type of additive (H_2O_2 or NaCl) and additive concentration were investigated. The results showed that the optimum condition for treating effluent from distillery wastewater with 10 times dilution was found at the current intensity of 9 A at an initial pH of 1 with a titanium sponge anode in the presence of 1.0 M NaCl. At this condition, approximately 92.24% and 89.62% of colour and COD were removed, respectively.

Another electrochemical method that has the potential to be an effective alternative to the various traditional techniques employed for the distillery and/or brewery effluent treatment is electrocoagulation. Electrocoagulation is based on the in situ formation of the coagulant as the sacrificial anode dissolves due to the applied current, while the simultaneous evolution of gases at the electrodes allows for organic pollutant removal by flotation (Khandegar & Saroha, 2012).

Electrocoagulation cells consist of pairs of parallel metal plate electrodes separated by a few millimetres with a low voltage applied at high current densities, as shown in Figure 20.5 (Global Advantech, 2011; Xu & Zhu, 2004). The current flowing between the electrodes destabilises electrical charges, which maintain suspensions of particulates, e.g. clays, and emulsions/microemulsions of hydrocarbons and insoluble organic compounds. The particulates coagulate together into flocs. The hydrocarbons and insoluble organic compounds coalesce into larger droplets and rise in the cells.

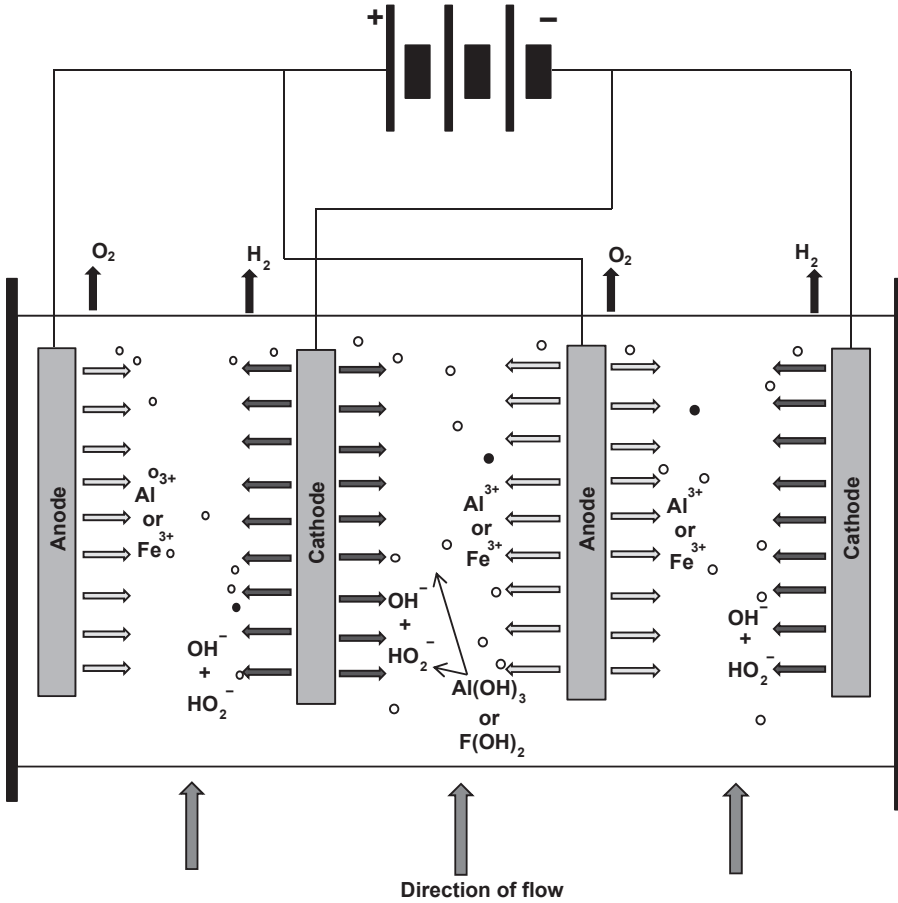

Figure 20.5 Conceptual diagram of an electrocoagulation reactor. Adapted with permission from Xu and Zhu (2004).

Electrochemical reactions at the electrodes produce very fine H_2 and O_2 gas bubbles and highly chemically reactive hydroxyl (OH^-) and superoxide (HO_2^-) radicals. The gas bubbles promote the flotation of coagulated solids and coalesced hydrocarbons, etc. The hydroxyl and superoxide radicals cause precipitation of hydroxides of heavy metals and the breakdown of many soluble organic molecules.

Only a few studies have been reported in the literature on the use of electrocoagulation for the treatment of distillery and/or brewery wastewater. Manisankar, Rani, and Viswanathan (2004) used electrocoagulation to remove COD, BOD and colour from distillery effluent using graphite electrodes and studied the effect of pH, current density and the halides (sodium fluoride, sodium chloride and sodium bromide) as supporting electrolytes on the treatment of distillery effluent. An influent COD value of 12,000 mg/L was reduced by 85.2% in the presence of sodium chloride electrolyte. Colour and BOD were also reduced by

98% and 93.5%, respectively. Thakur, Srivastava, and Mall (2009) investigated the removal of COD (9310 mg/L) and colour from a two-stage aerobic treatment effluent using stainless-steel electrodes and studied the effect of pH, current density, interelectrode distance and electrolysis time. At the optimum conditions (pH = 6.75; current density = 146.75 A/m^2; interelectrode distance = 1 cm and electrolysis time = 130 min), 61.6% and 98.4% COD and colour were removed, respectively. The results also showed that for pH < 6, the primary mechanism for COD and colour removal was charge neutralisation by monomeric cationic iron species, while sweep coagulation with amorphous iron hydroxide was the dominant mechanism for higher pH.

Khandegar and Saroha (2012) employed different combinations of electrodes in the treatment of alcohol distillery spentwash having very high COD (120,000 mg/L). The tests were performed to study the effect of current density, pH of the spentwash, agitation speed, electrolysis time and the distance between the electrodes on the COD removal efficiency. It was observed that aluminum electrodes were more suitable for treatment of distillery spentwash as compared to iron electrodes. The maximum COD removal efficiency of 81.3% was obtained with aluminium anode and cathode electrodes at the current density of 0.187 A/cm and pH 3 for an electrolysis time of 2 h. In this study, the COD reduction of the distillery spentwash happened due to two mechanisms (Khandegar & Saroha, 2012). Firstly, the coagulants [$Al(OH)_3$ and $Fe(OH)_2$] were generated in situ in the electrocoagulation process (see Figure 20.5), which helped in coagulation of the organic content. The coagulants were generated through the electrochemical reactions occurring at the aluminum and iron electrodes as follows (Khandegar & Saroha, 2012).

For aluminium electrodes:

Anode: $$Al \rightarrow Al^{3+} + 3e^- \quad (20.1)$$

Cathode: $$3H_2O + 3e^- \rightarrow 1½H_2 + 3OH^- \quad (20.2)$$

Overall: $$Al \rightarrow Al^{3+} \rightarrow Al(OH)_n^{(3-n)} \rightarrow Al(OH)_2^{4+} \rightarrow$$

$$Al(OH)_4^{5+} \rightarrow Al_{13}\ (\text{complex}) \rightarrow Al(OH)_3 \quad (20.3)$$

For iron electrodes:

Anode: $$Fe \rightarrow Fe^{2+} + 2e^- \quad (20.4)$$

$$Fe^{2+} + 2OH^- \rightarrow Fe(OH)_2 \quad (20.5)$$

Cathode: $$2H_2O + 2e^- \rightarrow H_2 + 2OH^- \quad (20.6)$$

Overall: $$Fe + 2H_2O \rightarrow Fe(OH)_2 + H_2 \quad (20.7)$$

Secondly, the presence of chlorides in the distillery spentwash and the application of electric current led to the generation of chlorine and hypochlorite ions, which reacted with the organic molecules and oxidised them. The hypochlorous acid and hypochlorite ions can decompose organic matter due to their high oxidative potentials (Krishna, Murthy, Manoj, & Lokesh, 2010; Vijayaraghavan et al., 1999, 2006). The reactions at anode and cathode were as follows:

$$\text{Anode:} \quad 2Cl^- \rightarrow Cl_2^+ 2e^- \tag{20.8}$$

$$\text{Cathode:} \quad 2H_2O + 2e^- \rightarrow H_2 + 2OH^- \tag{20.9}$$

$$\text{Bulk solution:} \quad Cl_2 + H_2O \rightarrow HOCl + HCl \tag{20.10}$$

$$HOCl \rightarrow OCl^- + Hl^+ \tag{20.11}$$

This section of the chapter has shown that electrochemical methods are efficient and versatile processes that are able to handle a wide variety of wastewaters. The coupling of electron-driven reactions (direct oxidation) with in situ generation of oxidants (indirect oxidation) makes this technique a valuable treatment alternative (Anglada et al., 2009). It must be noted that if any chlorinated organics are formed during electrolytic treatment of wastewater, they can be removed by passing the treated effluent through activated carbon before the discharge (Vijayaraghavan et al., 1999). Moreover, any excess concentration of chlorine can be reduced by the addition of bisulphite (Vijayaraghavan et al., 2006).

20.4.4 Microbial fuel cell technologies

Microbial fuel cells (MFCs) have gained a lot of attention as a means for converting organic waste, including low-strength wastewaters and lignocellulosic biomass, into electricity (Pant, van Bogaert, Diels, & Vanbroekhoven, 2010). Actually, MFCs are considered to be the major type of bioelectrochemical systems that convert biomass spontaneously into electricity through the metabolic activity of the micro-organisms (Pant et al., 2010). In other words, MFCs allow a direct conversion of chemical energy from the biodegradable organic matter into electricity via microbial catalysis (Liu, Liu, Zhang, & Su, 2009). Though the idea of using micro-organisms as catalysts in an MFC has been explored since the 1970s (Roller et al., 1984; Suzuki, 1976), the MFCs used to treat domestic wastewater were only introduced relatively recently by Habermann and Pommer (1991).

An MFC typically consists of a porous anode chamber, a porous cathode chamber and a membrane (or an electrolyte) sandwiched between the two. In a two-chamber setup, the anode and cathode compartments are separated by a proton exchange membrane (PEM) that allows proton transfer from anode to cathode, but prevents oxygen diffusion to the anode chamber (Pant et al., 2010). In the single-chamber MFC, the cathode is exposed directly to the air (Pant et al., 2010).

The basic operation of an MFC is as follows: micro-organisms oxidise organic matters in the anode chamber under anaerobic conditions and produce electrons and protons (Köroğlu, Özkaya, & Çetinkaya, 2014; Liu et al., 2010; Pant et al., 2010). Electrons transfer via the external circuit to the cathode chamber (thus generating electric current) where electrons, protons and electron acceptors (mainly oxygen) combine to form water (Köroğlu et al., 2014; Liu et al., 2009; Logan, 2008; Pant et al., 2010). Essentially there are three configurations amongst MFCs with a PEM (Figure 20.6): (a) bioreactor separated from the MFC: the micro-organisms generate hydrogen that is then used as fuel in a fuel cell, (b) bioreactor integrated into the MFC: the micro-organisms generate hydrogen that is converted into electricity in a single cell, and (c) MFC with direct electron transfer: microbiological electricity generation and direct transfer to the anode (Alzate-Gaviria, 2011; Rabaey, Lissens, & Verstraete, 2005).

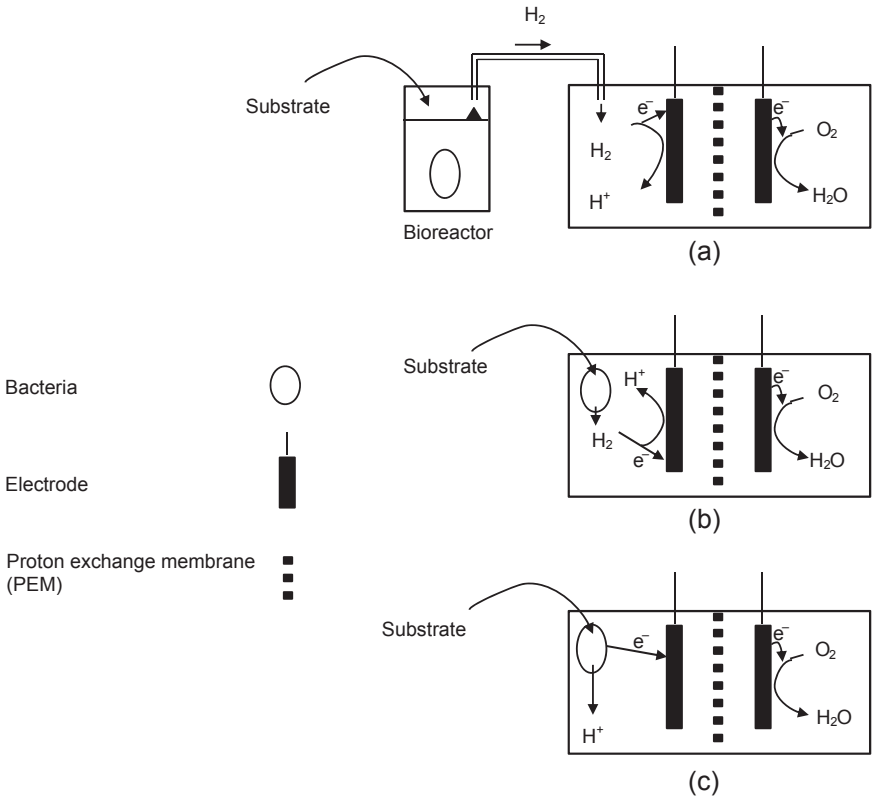

Figure 20.6 Three typical microbial fuel cell configurations: (a) bioreactor separate from the MFC: the micro-organisms generate hydrogen that is then used as fuel in a fuel cell, (b) bioreactor integrated into the MFC: the micro-organisms generate hydrogen that is converted into electricity in a single cell and (c) MFC with direct electron transfer: microbiological electricity generation and direct transfer to the anode.

Reprinted with permission from Rabaey et al. (2005).

MFCs can be monitored via electrochemical parameters, such as power density, generated electrical current and voltage (Alzate-Gavira, 2011). Equally, a very important parameter is the organic load of the substrate to be used (Rabaey, Lissens, Siliciano, & Verstraete, 2003). Actually, in MFCs, a substrate is regarded as one of the most important biological factors affecting electricity generation (Liu et al., 2009; Pant et al., 2010). As already stated, brewery wastewater is characterised by the presence of organic material; therefore, it is a suitable substrate for MFCs. In fact, wastewater from breweries has been a favourite substrate for MFCs among researchers, primarily because of its low strength (Pant et al., 2010) compared to other organic substrates. Besides, it is suitable for electricity generation in MFCs due to the food-derived nature of the organic matter and the lack of high concentrations of inhibitory substances (Feng et al., 2008; Pant et al., 2010). In other words, it is an ideal substrate for MFCs due to its nature of high carbohydrate content and low ammonium nitrogen concentration (Pant et al., 2010).

Feng et al. (2008) investigated the treatment of beer brewery wastewater using air cathode MFC. In this study, the efficiency of wastewater treatment was examined in terms of maximum power densities, Coulombic efficiencies (CEs) and COD removal as a function of temperature. It was found that with an influent COD of beer brewery wastewater of 2250 ± 418 mg/L, the COD removal efficiency was 85% and 87% at 20 °C and 30 °C, respectively. Decreasing the temperature from 30 °C to 20 °C reduced the maximum power density from 205 to 170 mW/m^2, while CEs decreased only slightly with temperature. The performance of electricity production from beer brewery wastewater in a single-chamber membrane-free MFC was investigated by Wang, Feng, and Lee (2008). Experimental results showed that the MFCs could generate electricity with the maximum power density of 483 mW/m^2 at 30 °C and 435 mW/m^2 at 20 °C, from wastewater with influent COD of 2239 mg/L. Wen, Wu, Zhao, Sun, and Kong (2010) used an MFC model based on a polarisation curve to investigate the performance of brewery wastewater treatment in conjunction with electricity generation. With influent COD of 1250 ± 100 mg/L, this sequential anode–cathode MFC achieved COD removal efficiency of more than 90%. This study also showed that the most important factors which influenced the performance of the MFC with brewery wastewater were reaction kinetic loss and mass transport loss. However, these can be avoided by increasing the concentration of brewery wastewater and by increasing the reaction temperature, and using a rough electrode to provide more reaction sites (Pant et al., 2010). Mathuriya and Sharma (2010) also used an MFC to simultaneously treat brewery wastewater and produce electricity. This study reported 93.8% COD removal efficiency and up to 10.89 mA electric current generation. The study also showed that the addition of readily utilisable substrates like glucose and sucrose to the wastewater can enhance the electricity production and COD removal.

Since high COD removal efficiencies were achieved in these studies, it can be ascertained that MFCs can provide a new approach for brewery wastewater treatment while offering a valuable alternative to energy generation. Thus, the use of brewery wastewater as a substrate for MFCs has great development potential not only in terms of wastewater treatment, but also in terms of energy self-sufficiency as well as reducing competition with food production as is the apprehension with conventional biofuels.

20.4.5 Electric discharge plasma technologies

One of the vital developments of advanced oxidation processes is concerned with using electrical discharges to generate very powerful and nonselective oxidising agents. The class of advanced oxidation processes involving electric discharge that will be discussed in this section is referred to as gliding arc discharge plasma. Plasma is a highly ionised gas that occurs at high temperatures (Moreau, Orange, & Feuilloley, 2008; Simate et al., 2011). The gas consists of positive and negative ions and electrons as well as neutral species (Kaunas, 2012). Plasmas are energetically the strongest, and are characterised by their acidic, oxidising and complexing properties (Abba, Gongwala, Laminsi, & Brisset, 2014). The plasma gas can be directly cooled and projected onto the target or quenched by a reaction with water. In both cases, highly reactive oxidative species are formed locally and can react with the macromolecules of contaminants (Moreau et al., 2008). Like gas, plasma does not have a definite shape or a definite volume unless enclosed in a container; unlike gas, in the influence of a magnetic field it may form structures such as filaments, beams and double layers (Simate et al., 2011).

There are two categories of plasma: those in thermal equilibrium and those not in thermal equilibrium (Kaunas, 2012), defined according to the conditions in which they are created (Moreau et al., 2008). Thermal equilibrium implies that the temperatures of active species (electrons, ions and neutrals) are the same. In the case of nonthermal equilibrium plasmas, the temperatures of active species are not the same. To be more precise, electrons are characterised by much higher temperatures compared to heavy ions (Kaunas, 2012). Thermal plasmas are obtained at high pressure (≥105 Pa) and need substantial power (up to 50 MW) to be observed. This type of plasma is found, for example, in plasma torches and in electric arcs. Nonthermal plasmas are obtained at lower pressures and use less power. Such plasma can be generated by electric discharges in lower pressure gases.

A third category of plasmas is an intermediate between thermal and nonthermal discharges (Moreau et al., 2008). Usually, these plasmas are included in the category of the nonthermal plasmas because they are formed near atmospheric pressure and ambient temperature. These low temperature and medium pressure plasmas are of particular interest technically and industrially because they do not require extreme conditions. Typical examples of these plasmas are the corona discharge and the gliding arc discharge. As already mentioned, of particular interest in this chapter is the gliding arc discharge plasma.

A gliding arc is an electrical discharge formed between two or more thin diverging electrodes with a high-velocity (>1 m/s) gas flowing between the electrodes (Burlica, Kirkpatrick, & Locke, 2006). An arc forms at the narrowest gap between the electrodes (Djepang, Laminsi, Njoyim-Tamungang, Ngnintedem, & Brisset, 2014). A gas flow directed along the axis of the electrodes gently pushes the arc feet along the conductors, so that the arc length increases until breaking in a plasma plume. Its temperature decreases, as does its energy, when the arc is short-circuited by a new one (Brisset et al., 2008; Djepang et al., 2014). In other words, the gliding arc plasma generator consists of two divergent electrodes, where the arc starts at the shortest distance between the

electrodes and then moves with the gas flow. The length of the arc column increases together with the voltage (Kaunas, 2012). The arc discharge disappears when arc maintenance voltage exceeds input voltage. This process of generating an arc, movement (gliding), and disappearance is repeated continuously (Burlica & Locke, 2008). The gliding arc generates regions of both thermal and nonthermal plasma at the conditions of atmospheric pressure and ambient temperature (Fridman, Nester, Kennedy, Saveliev, & Mutaf-Yardimci, 1995).

Gliding arc discharges have been investigated as a potential treatment technology for gas-phase pollution treatment (Krawczyk & Motek, 2001) and for liquid-phase pollution treatment (Moussa & Brisset, 2003). Ghezzar, Abdelmalek, Belhadj, Benderdouche, and Addou (2007) used a nonthermal gliding arc at atmospheric pressure to remove anthraquinonic acid green 25 (AG 25) from an aqueous solution. The removal of the dye was carried out in the absence and presence of TiO_2 as photocatalyst. The gaseous species formed in the discharge (particularly OH* radicals) induced strong oxidising effects in the target solution. At the optimum concentration, the dye (80 μM) was totally decolourised within 15 min of plasma treatment time, and 93% of the initial COD was removed after 180 min of plasma treatment time. In the absence of catalyst, colour removal was 46% after 15 min, while COD abatement reached 84% after 180 min. The results have shown that the combined plasma–TiO_2 method is a rapid and cost-effective means that might prove well adapted to the removal of other organic pollutants such as those in brewery wastewater.

The plasma chemical treatment of wastewater has also been applied to brewery wastewater. Doubla et al. (2007) reported the use of humid air plasma created by an electric gliding arc discharge in humid air to lower organic pollutants in brewery wastewater. The gliding arc discharge in humid air generates NO and OH radicals, which have strong oxidising characteristics. The OH radical is a very powerful oxidising agent [$E^0(\cdot OH/H_2O) = 2.85$ V/SHE] and is responsible for oxidation reactions with organic targets, both due to its own properties and to its derivative and/or parent molecule H_2O_2, as shown in Eqn (20.12) (Doubla et al., 2007):

$$H_2O_2 \leftrightarrow 2OH \tag{20.12}$$

The nitrate ions also participated in the oxidising characteristics of the humid air plasma. It must be noted that, initially, NO in humid air led to the formation of nitrite in neutral mediums, but was further oxidised to stable nitrate ion species. As can be seen, the high standard oxidation–reduction potentials of the HNO_2/NO (1.00 V) and NO_3^-/HNO_2 (1.04 V) systems reflect the oxidising power of the nitrate ions (Doubla et al., 2007). In the study by Doubla et al. (2007), the BOD removal efficiency of the gliding arc discharge process with brewery wastewaters of BOD values of 385 and 1018 mg/L were 74% and 98%, respectively. The alkaline wastewaters were also rapidly neutralised due to the pH-lowering effect of the plasma treatment emanating from the production of nitrate ions (Benstaali, Moussa, Addou, & Brisset, 1998). This process can be coupled with other methods, such as biological processes, to further lower the organic pollutant concentration more easily and rapidly to an acceptable level for reuse (Doubla et al., 2007).

This section has shown that nonthermal plasma technology (or the gliding arc discharge specifically) is one of the most attractive of the advanced oxidation techniques for treating wastewaters because of low equipment and energy costs and greater efficiency (Benstaali et al., 1998). In summary, the rapid interest in the application of gliding arc discharges results from the unusual chemical properties and enhanced reactivity of the activated species (atoms, radicals and excited molecules) produced in the plasma. These activated species formed are responsible for acid and oxidising effects in the target solution (Abba et al., 2014).

20.5 Challenges and future prospects

This section explores the existing and emerging challenges in relation to water treatment and reuse in breweries. The section will also discuss future prospects. Water reuse has been dubbed the 'greatest challenge of this century' as water supplies continue to dwindle and water demands increase because of the increase in population (Fatta et al., 2005). In the brewery industry, this statement is exacerbated by public perception and possible product quality deterioration problems (Janhom et al., 2009). In fact, most studies investigating public acceptance of recycled water come to the same conclusion – that people are very open to using recycled water for uses with low personal contact, such as watering trees and shrubs in their garden, but are reluctant to adopt recycled water for uses with high personal contact, such as drinking or bathing (Dolnicar, Hurlimann, & Grün, 2011). Moreover, concern for human health and the environment are the most important constraints in the reuse of wastewater (Fatta et al., 2005). Nevertheless, the main problem preventing the safe reuse of treated wastewater in breweries is the nonexistence of the reuse criteria related to hygiene, public health and product quality control.

Notwithstanding the obstacles, several promising results have shown that new wastewater treatment processes such as gliding arc discharge plasma, electrochemical methods, and MBRs have great capacity to be used for the treatment of brewery wastewater for reuse. In other words, there have been several technological advances and innovations that can achieve significant improvements in the treatment of brewery wastewater to guarantee its reuse. Furthermore, integrating these processes together as two or more stage processes would be more suitable thus giving the brewery wastewater treatment processes good economics and a high degree of energy efficiency (Simate et al., 2011).

Previous research has shown that integration of several processes may be able to partially or completely eliminate a wide range of contaminants (Dobias, 1993; Harrelkas, Azizi, Yaacoubi, Benhammou, & Pons, 2009). For example, integrated anaerobic and aerobic processes in brewery wastewater treatment (Driessen & Vereijken, 2003) have resulted in up to 98% COD and nutrient removal (Biothane, 2014). Besides wastewater treatment, the other benefit from the anaerobic–aerobic system is the production of biogas. When biogas is burned in brewery boilers or in a combined heat and power unit, the whole treatment can create a positive energy balance (Biothane, 2014). Thus, the combination of wastewater treatment together with power production may help in reducing the cost of wastewater treatment.

In view of environmental problems accompanied by the use of nonrenewable fossil fuels and an urgent need for renewable energy, it is suggested that MFCs are used as the first pretreatment stage of every integrated process, particularly with membrane filtration techniques. Besides generating electricity, MFCs would substantially reduce the organic load, thus minimising membrane fouling. It must, however, be noted that despite the potential to treat brewery wastewater as well as produce electricity, MFCs have a lot of limitations. A major drawback associated with MFCs is the start-up time that may vary from just days to months depending on the inoculum, electrode materials, reactor design and operating conditions (Pant et al., 2010). Furthermore, scaleup is still a big challenge; the high cost of cation exchange membranes, the potential for biofouling and associated high internal resistance restrain the power generation and limit the practical applications of MFCs (Hu, 2008; Pant et al., 2010). Therefore, before the potential of MFCs is fully realised additional research and development is needed.

Electrochemical methods can be well suited to be coupled in the latter stages of the integrated process (Simate et al., 2011). Sanitising agents (often called disinfectants), which are present in brewery wastewater, contain chlorine compounds. These compounds produce chlorine during electrolysis and, thereafter, chlorine generates hypochlorous acid, which may oxidise organic compounds. Furthermore, chlorine produced may also deactivate pathogenic micro-organisms. Therefore, electrochemical methods coupled in the latter stages can serve as an organic oxidation and disinfecting stage. Nevertheless, to achieve an efficient and cost competitive electrochemical treatment process, the wastewater should have relatively high conductivity (Anglada et al., 2009). The main obstacles that require attention before the full scale implementation of electrochemical oxidation are the high operating cost and lack of efficient and stable electrode materials (Anglada et al., 2009). Therefore, a major area for future research is the improvement of the electrocatalytic activity and electrochemical stability of the electrode materials, which will result in lower operational and capital costs (Anglada et al., 2009).

Though Doubla et al. (2007) recommend integrating gliding arc discharge plasma techniques with other treatment processes in order to lower the organic pollutant concentrations more easily and rapidly to an acceptable level for reuse, the processes can be very expensive (Simate et al., 2011). This is because of the high ionisation energy requirements and the cost of energy sources such as lasers (Simate et al., 2011). However, the gliding arc discharge can easily be powered by a DC or AC power supply source. The DC gliding arc plasma generator is characterised by stability of discharge and a simple design. The main advantages of the AC gliding arc discharge plasma generator are simplicity of the power supply system and its low cost (Lie, Bin, Chi, & Chengkang, 2006).

Membrane filtration processes, particularly RO, have been demonstrated to be very effective in removing organic and inorganic materials. The COD removal efficiencies up to 99%, TSS removal efficiencies up to 100% and complete removal of pathogens have been reported. Nevertheless, in order to improve the operations of membrane processes, the following measures are required (Zhou & Smith, 2002): (1) better understanding of membrane fouling mechanisms, (2) effective fouling control strategies, (3) better membrane materials and module designs, and (4) membrane integrity management. The high cost of membranes is still a significant issue impeding a faster commercialisation (Skouteris, Hermosilla, López, Negro, & Blanc, 2012). So, even though the membrane costs have been dramatically reduced over time, they are still a critical issue.

To date, much progress has been achieved in research and applications of both anaerobic and aerobic MBRs. Just like conventional membranes, fouling is one of the main disadvantages of MBRs, because it hinders the operation of the systems in a constant, reliable way (Skouteris et al., 2012). The deposition of solids on anaerobic MBR membrane surfaces is lower than on aerobic MBR membrane surfaces. However, since anaerobic MBRs are usually operated at lower membrane permeate fluxes, they are characterised by lower sludge filterabilities, which favour membrane fouling (Skouteris et al., 2012). Therefore, it is imperative that further research is carried out to mitigate this problem.

The energy produced from biogas in anaerobic MBRs could be used to cover the energy required for membrane filtration and the excess energy could be used elsewhere. However, more research is required to investigate in detail to what extent the biogas produced in anaerobic MBR can lead to sustainable energy operations. For example, little information is available regarding the energy that is consumed by anaerobic MBRs as a whole or by each of their components (Skouteris et al., 2012).

20.6 Conclusions

Though the conventional or pretreatment processes for getting rid of many pollutants from brewery wastewater have long been established, their effectiveness is limited. This chapter discussed various processes that can be used individually or coupled with others to treat brewery wastewater for reuse. The chapter has shown that most of the processes studied could be successfully implemented for high-level treatment of brewery wastewater for reuse. In summary, these processes may have the much needed solution for the future because, if properly utilised, they can give the most efficacious and cost-effective approach to treating brewery wastewater for reuse. A number of hybrid treatment methods have also been proposed that are formed by integrating these processes with other traditional treatment processes or amongst themselves. However, there is a need to carry out extensive research so as to understand both synergistic and antagonistic effects of the suggested hybrid processes.

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