Microbiological stability of beer

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In ancient civilizations, the process of brewing developed as a means of producing a nutritious beverage that was safer to drink than water. Microbiological stability may therefore be regarded as inherent in its very creation. Beer has a spread of properties that impede microbial development: low pH, high alcohol concentration, antiseptic action of hop acids, low nutrient level, low oxygen concentration and carbonation. Haze has also been implicated in hindering bacterial contamination (Thelen et al., 2006).

To date no known human pathogens have been found to survive in beer. However, brewers cannot be complacent. A variety of yeasts and bacteria are able to flourish in beer causing product deterioration. There is also a range of new products containing lower levels of alcohol (or none at all), and/or fruit juices, that are more susceptible to spoilage than traditional ales and lagers. In addition, production of non-pasteurized beer is continuing to increase. Run losses, product retrieval, and the prohibitive cost effect on brand integrity excite the need for premium quality control mechanisms. Control of microbial contaminants is also important in ensuring consistently uniform and high quality beer.

In this chapter, we examine the microorganisms most commonly encountered at each stage of the brewing process and discuss their effect on fermentation and final product. We also assess the impact of new production methods and new products on the flora observed. Finally, we survey the methods available for microbiological detection and discuss techniques employed to reduce microbial contamination.

Overview of microbial spoilage

In general the bacterial contaminants brewers face today are the same as those encountered two centuries ago. This is despite modern brewing techniques and
new packaging methods. The names assigned to the various spoilage organisms have changed more frequently than the prevalence of different species!

There are literally millions of food spoilage microorganisms. However, in general those responsible for beer spoilage consist of a limited number of bacteria and a small number of “wild yeasts.” Moulds are not regarded as beer spoilage organisms due to their need for oxygen to grow, however they can indirectly cause spoilage through growth on raw materials. A number of fungi cause gushing of packaged beer and/or produce toxins.

Beer-spoiling bacteria are characterized as microorganisms capable of multiplying in beer, resulting in product deterioration. Bacteria may be divided into Gram positive or Gram negative depending on the structure of their cell wall. Gram positive bacteria appear purple under the light microscope following appropriate Gram staining. The Gram stain is of particular relevance in brewery microbiology as the cell wall structure determines which bacteria are able to grow in hopped wort. Growth of the vast majority of Gram positive organisms is inhibited by hop bitters, whereas growth of Gram negative bacteria is unaffected. A teichoic acid glycosylation protein, essential for Gram positive cell wall formation, has been identified as highly specific to Gram positive beer spoilage strains (Fujii and Hayashi, 2004). Of the Gram positive bacteria, the most dangerous members, in terms of beer stability, are lactic acid bacteria of the genera *Lactobacillus* and *Pediococcus*. Indeed, Lactobacilli are the most common beer-spoilage bacteria, regardless of beer type (Thelen et al., 2006). Some members of the genera *Micrococcus* and *Staphylococcus* can survive in beers and cause spoilage. The Gram positive Bacilli are generally not a serious threat, although have been problematic in unhopped worts. The occurrence of Gram negative bacteria in breweries is regarded as undesirable. The most significant are acetic acid bacteria, *Zymomonas* and certain members of *Enterobacteriaceae*, for example *Rahnella* and *Hafnia*, and *Acidaminococcaceae*, for example *Pectinatus*, *Megasphaera*, *Selenomonas* and *Zymophilus*.

Wild yeasts are generally defined as those yeasts “not deliberately used and not under full control” (Gilliland, 1971); these include contaminant yeast in the pitching yeast culture and those from the air or other raw materials. The major types of wild yeast encountered in the brewery are *Debaromyces*, *Dekkera*, *Pichia*, *Hanseniaspora*, *Kluyveromyces*, *Torulaspora*, *Williopsis* and non-brewing strains of *Saccharomyces*.

**Outline of the brewing process**

The microbiological stability of the final product can be compromised from a very early point in its production, with spoilage organisms able to access the brewing process at every stage, from raw materials to dispense. Figure 5.1 outlines a typical brewing process. Below we analyze the main parts of the process, detailing the most frequent spoilage microorganisms encountered and the consequences of their presence.
Raw materials

Most potential contaminants of beer originate from raw materials and/or unclean brewing equipment. Brewing raw materials, such as malt, hops and occasionally brewing water, may be infected by microorganisms and these have to be killed during the brewing process to prevent wort and beer spoilage.

Barley and Malt

The microorganisms that are normally associated with different barleys are remarkably similar (Flannigan, 2003). In the field, they are commonly infected with fungi from the *Alternaria*, *Cladosporium*, *Epicoccum* and *Fusarium* genera, whereas in storage the most commonly encountered fungi are members of *Aspergillus*, *Eurotium* and *Penicillium*. Moulds and bacteria are less commonly isolated from barley.

The effects on brewing and beer of contamination of growing, stored or malted barley are varied. The best known effect of the microbiota of both
barley and malt is that of reduced gas stability or gushing (spontaneous ejection of beer from its container). A variety of different fungi have been associated with gushing, the most notable of which are *Fusarium graminearum* and *F. moniliforme*. Small fungal proteins, hydrophobins, present in fungal cell walls have been isolated from strains of the genera *Fusarium*, *Nigrospora* and *Trichoderma* and shown to act as gushing factors in beer (Sarlin et al., 2005).

A second consequence of fungal infection of barley and malt is the potential for release of mycotoxins, compounds toxic to man or animals. Aflatoxin B1, ochratoxin A, zearalene, deoxynivalenol (DON) and fumosins B1 and B2 are mycotoxins that may be transmitted from contaminated grains into beer (Scott, 1996; Schwarz et al., 1995). In addition to the potential harm to humans, mycotoxins may affect fermentation due to their influence on yeast activity. There is also an apparent relationship between the ability of strains to produce the mycotoxin zearalene and gushing.

Inoculation of malt or barley (during steeping or in the field) with *Fusarium* results in increased wort nitrogen and formol nitrogen in wort and beer. Bacterial growth on barley can contribute nitrosamines (which are carcinogens) and cause flavor and filtration problems. The effect of mould growth on the raw material is principally on beer flavor; they may cause a range of off-tastes and odors.

Accumulating evidence has indicated that the evolving microbial flora of the malting process may influence the final malt quality and its ultimate performance in the brewhouse.

Steeping does reduce the levels of naturally occurring mycotoxins although growth of moulds during germination increases mycotoxin production.

**Water**

Breweries and good water have long had a close association, and water quality is generally taken for granted. Large quantities of water are used in production (water making up over 90% of the final product) as well as for cleaning, washing and sterilizing of equipment. Current consumption in the UK averages approximately 5.0hl water used/hl beer produced, although there is large variation between breweries. Increasing costs of town supply have led to alternative, less expensive, sources of water being investigated, such as natural springs and boreholes. Water used for brewing must be fit for human consumption (potable). As such it must be free from contaminating organisms. However, what is fit to drink is not necessarily fit for brewing use.

Water for brewing is of course boiled during the process. From a microbiological point of view the main concern is the introduction of spoilage organisms from water introduced after fermentation, for example during dilution of beer following high gravity brewing or from vessels rinsed with contaminated water.

A variety of methods are available for water purification, and generally microorganisms may be removed very effectively. One of the most popular is
carbon purification for removal of organic, and some inorganic, compounds. However, this method does not remove microorganisms. Indeed, it can be a source of contaminants. Membrane filtration is generally used for complete removal of bacteria, viruses, proteins, salts and ions. Chlorine dioxide can be applied to water systems to reduce or eliminate brewery spoilage organisms. At levels of approximately 0.2 ppm chlorine dioxide significantly reduces microbial count while causing no off-flavors or odors in the final beer (Dirksen, 2003).

For public supplies, water quality is rigorously tested. Bacteriological analysis of water is designed to detect recent faecal pollution, for example by farm animals or by sewage effluent. Such analysis ensures absence of coliform bacteria, which may be spread by contaminated water supplies. The most dangerous, *Salmonella/Shigella* spp. and *Vibrio cholerae*, occur irregularly and in small numbers in contaminated water, and it is not normal practice to culture these pathogens directly. For brewers, the most significant of the coliform bacteria are *Aerobacter aerogenes* which may be the cause of biologically unstable wort.

Algae and fungi from water supplies are also able to create problems within a brewery causing undesirable odors and taints, clogging filters and providing nutrients for bacterial growth. The wild yeast *Pichia* may be found in some supplies; *Pichia* is quite tolerant to anaerobic conditions, is able to spoil wort, and grows readily in unpasteurized finished beer.

**Pitching yeast**

The most common source of bacterial contamination in the brewery is probably from pitching yeast, which can transfer contaminants from fermentation to fermentation. Any microbial contamination of pitching yeast compromises product quality and taste and can have a significant effect on the final beer. Wild yeast can cause a variety of off-flavours and aromas, damage by diastatic and proteolytic activity, altered flocculation characteristics of the pitching yeast and may posses killer activity. Wild yeast include members of *Dekkera* (*Brettanomyces*), *Candida*, *Debaryomyces*, *Hansenula*, *Kloeckera*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Saccharomycodes*, *Torulaspora* and *Zygosaccharomyces* (Back, 1987). *Brettanomyces* may be used for fermentation and is commonly used in secondary fermentation, for example for Belgian style beers and lambics.

Enteric, acetic and lactic acid bacteria may also contaminate pitching yeast causing serious problems in downstream processes. For example, *Pediococcus damnosus* contamination of pitching yeast can cause extension of the fermentation time and accumulation of high levels of diacetyl. The Gram negative bacteria *Hafnia proteus* (formerly *Obesumbacterium proteus*) and *Rahnella aqualitis* (formerly *Enterobacter agglomerans*) have also been detected in pitching yeasts. These bacteria retard fermentation and may cause excessive diacetyl and dimethyl sulfide in final beer.

Acid washing has been shown to be effective in eliminating contaminating bacteria without adversely affecting the fermentation performance, and is common practice. However acid washing does not remove wild yeast.
Hops

The fourth brewing ingredient, hops, is known for its antiseptic properties. As described, the majority of Gram positive bacteria are inhibited by hops, although Gram negative bacteria are unaffected. The main cause of losses for hop growers are insect pests such as *Aphis humuli* (aphis blight) and *Tetranychus telarius* (red spider). However mould or mildew caused by the fungus *Podosphaera castagnei* is also problematic. Hop cultivars are generally bred to be resistant to the commonest pests and diseases, and any that do infect crops may be tempered by the use of pesticides or fungicides.

Whole hops are dried following harvesting. This process reduces the chances of subsequent microbial contamination, and for brewers who do use whole hops no beer spoilage effects attributable to infected hops have been reported. Similarly, no beer spoilage organisms have been reported to have been introduced by other herbs or plant-derived products used in brewing.

Sugars

Free flowing sugar, syrups or honey are commonly used adjuncts, generally added during wort boiling. Some are also added as non-fermentable sweeteners. Specially tailored syrups allow the production of better quality low alcohol beers. Sugars are in fact used to prevent microbial spoilage of preserves, jams, syrups and jellies, due to their effect on water activity (osmotic pressure), and problems from spoilage are rare. The main concern in brewing involves transfer of bacterial spores, principally from *Bacillus* sp., which can withstand heat treatment, including boiling, and may persist into the finished beer (although beer does not support the subsequent growth of these organisms).

Brewery surfaces

Microorganisms are all around us, and it is unrealistic to sterilize every surface within a brewery. The use of closed fermentation vessels and pipework does much to reduce the ingestion of potential spoilage organisms. Similarly, the use of automated cleaning systems that can apply high pressure, high temperature or high power cleaning agents is very effective in reducing the levels of contaminants within brewing equipment.

Despite rigorous cleaning regimes, most industrial plants are inhabited by biofilms. Biofilms usually have no impact on the final product (Timke et al., 2005). However, if they are colonized by product-spoiling microorganisms beyond wort boiling, the risk that the final product will be contaminated increases significantly. Different surface materials used in the brewing process differ in their susceptibility to biofilm formation. Polytetrafluoroethylene (PTFE), nitrile rubber (NBR) and viton (a fluoroelastomer), for example, are generally less susceptible to biofilm formation than stainless steel. The microorganisms present also affect biofilm formation.
Wort

The nutrient-rich environment of wort and the availability of yeast growth factors make it an attractive habitat for microbial growth. The most common contaminants are enteric bacteria, acetic and lactic acid bacteria and wild yeast. Enterobacteria may grow during the initial stages of the brewing process causing unwanted off-flavors in the final product. They are indirect beer spoilage microbes. The effects of contamination include nitrate reduction, off-flavors, such as hydrogen sulfide and acetaldehyde, vinegar flavors, diacetyl, acidic flavors, phenolic flavors, fruity solvent off-flavors and haze.

*Hafnia proteus* is the best-known enterobacterial contaminant which has only been found in breweries. It can grow together with pitching yeast. Furthermore, it has been isolated from wort and early fermentation stages where it causes a parsnip-like odor (Koivula, 2006). Two further wort-spoilage symptoms are celery flavor from *Aerobacter* and phenolic odors from *Escherichia*.

After boiling, the wort is largely free from microbiological contamination. However, as described above, some microorganisms are able to form spores which can withstand heat treatment, including boiling, and persist into the finished beer.

Fermentation

Traditional fermentation

The most troublesome bacterial contaminants during fermentation are members of the lactic acid bacteria, such as *Lactobacillus* and *Pediococcus*, which cause diacetyl formation, lactic acid formation (*Lactobacilli*) and ropiness (*Pedicocci*). However, *Hafnia proteus* and *Rahnella aqualitis* can also tolerate ethanol concentrations greater than 5% v/v. They can therefore contaminate harvested pitching yeast and spread throughout the production plant. Spoilage may also arise from the presence of acetic acid bacteria (*Acetomonas* and *Acetobacter*), *Zymomonas* spp., and strict anaerobes (*Megasphaera* sp., *Pectinatus cerevisiophilus* and *Bacteroides serpens*). Early infection (days 1–2 of fermentation) by Enterobacteria can retard or accelerate fermentation (Koivula, 2006).

High gravity fermentation

*Lactobacillus*, *Pediococcus*, *Acetomonas*, *Acetobacter* and *Zymomonas* contaminants have been shown to survive levels of ethanol of 12–13% (Magnus et al., 1986) and therefore remain a risk during high gravity fermentation. *Hafnia*, *Rahnella*, *Citrobacter* and *Klebsiella*, known to exist through most stages of the fermentation of traditional gravity wort, have been found to be completely eliminated by elevated ethanol levels. Very high gravity fermentations (>22°P) therefore narrow the range of bacteria capable of spoiling the beer, thereby reducing the risk of bacterial spoilage problems. Most brewers do not modify microbiological testing regimes for high gravity brewing despite the lower risk.
Low calorie fermentations

Low calorie beers may be produced by either the use of amylglucosidase to produce a wort which will ferment down to 8 or 9°P or lower, or by increasing wort fermentability via the mashing regime. Low or zero residual sugar and fairly high alcohol content leads to a fermentation product that is less prone to spoilage than traditional beers and as yet no specific microbiological issues in low calorie fermentations have been reported.

Non-alcoholic or low alcohol fermentations

Low alcohol beers may be produced by a variety of methods including distillation, dialysis, reverse-osmosis, ultrafiltration and pervaporation. However, the commonest method is by using wort that is not very fermentable and arresting fermentation at an early stage. A shorter fermentation time reduces the time available for any contaminants to “take hold,” however the final product will be more susceptible with fermentable sugar present and no alcohol.

Other non-traditional fermentation methods include immobilized yeast and continuous fermentations, accelerated fermentation and high pressure fermentation. No new microbiological risks have been reported for such fermentations and testing regimes should follow those for traditional methods, although increased attention should be paid to continuous fermentations due to the requirement for complete closure if any contaminants are found.

Storage and finishing

Despite the anti-microbial properties of finished beer, a variety of bacteria are able to cause off-odors or flavors given access to storage or finishing tanks, most commonly members of the acetic and lactic acid bacteria (which cause vinegary off-flavours and odors, and may cause excessive gassing and strong head retention). The approach taken to maintaining beer quality post-fermentation is generally that of avoiding contamination with air or oxygen. This may be achieved by holding beer under a carbon dioxide atmosphere, using de-aerated water for chasing and holding the pipelines, and filling pipelines with de-aerated water when not in use. In addition to assisting in maintaining shelf life, such methods also help to reduce microbial contamination of finished beer while in storage or transit.

Packaging and packaged beer

Beer bottling and canning plants are complex machines with a lot of areas in which biofilms can develop (Timke et al., 2005). Beer-spoiling organisms can be harbored and protected from desiccation, heat and disinfection within biofilms, that is biofilms are considered a reservoir of beer spoilage organisms.
Ribosomal RNA studies of bottling plant biofilms have revealed a remarkably diverse habitat, despite intensive cleaning (Timke et al., 2005). Such diversity could enable the biofilm community to react to changes in detergent use or cleaning procedures. Although biofilm studies do not show evidence of strictly anaerobic bacteria or indeed important beer spoilers, there is a need to ensure that biofilms are not allowed to mature.

Finished beer is largely an unfavorable environment for microorganisms and few bacteria can grow in this medium. However, it is believed that even an initial contamination of only a few bacterial cells in a bottle can lead to beer spoilage. Beer-spoiling lactobacilli cause acidity and turbidity. Due to the reduced oxygen content of packaged beer, the importance of the strictly anaerobic beer-spoilage bacteria, *Pectinatus frisingensis, P. cerevisiiphilus* and *Megasphaera cerevisiae*, has recently increased (Juvonen and Suihko, 2006). These bacteria are secondary contaminants of beer that is not pasteurized after packaging, and they spoil the product by producing foul-smelling metabolic end-products, such as methyl mercaptan, dimethyl sulfide, and hydrogen sulfide, and causing turbidity.

Changes that can be detected in packaged beer which has been contaminated by microorganisms include turbidity or haze formation, over attenuation, continued fermentation, acidification (lactic, acetic), diacetyl (buttery or butterscotch flavor), liquid manure odor, sulfurs, for example rotten egg, cooked vegetable, phenolic aromas, fusel alcohols, that is propanol, isobutanol and ropiness.

**Dispense**

Beer dispensing systems in pubs and restaurants are prone to biofouling, and microbial contamination of draught beer is frequently encountered, as are cleanability problems with dispense equipment. The most common bacterial contaminants are acetic and lactic acid bacteria, both of which cause haze and surface films. More worryingly, coliforms such as *E. coli* have been isolated from beer dispensing systems. The aerobic environment also allows growth of wild yeasts which may be found as surface growth on components of a beer system that is exposed to the air such as faucets, keg couplers and drains.

A summary of the main beer spoilage organisms at each stage and their effect(s) is given in Table 5.1.

**Detection**

Knowledge of microorganisms found in the brewery environment and the control of microbial fouling are both essential in the prevention of microbial spoilage of beer. Low sample volume in relation to huge batch volume (typically 250ml from >1000 hectoliter) and heterogeneity of the beer-spoiling bacteria makes detection of trace contaminants challenging. In addition, many of the “symptoms” of spoilage are identical to those of physical instability (see accompanying paper by K. Leiper).
Traditional methods of detection

Traditional methods of detection and identification are generally based on biochemical, morphological and physiological criteria (e.g. nutrient assimilation, microscopy and selective staining, respectively). Such methods tend to be time-consuming, often taking several days in order to first detect potential spoilage microorganisms. Further characterization (identification) can take several more days, once pure cultures have been isolated.

Generally, the first stage of detection involves sampling, either directly or through filtration, and direct microscopy. Samples are used to inoculate growth media and cultured for 2–3 days. Most commonly breweries use a combination of selective media, such as MRS (deMan, Rogosa and Sharpe), Raka-Ray and UBA (Universal Beer Agar). Cycloheximide (Actidione) may be used at a concentration of 0.004 g per liter, to suppress the growth of all brewery culture yeasts (it has no effect on bacterial growth). Anaerobic incubation is necessary to detect anaerobic bacteria such as *Megasphaera* and *Pectinatus*.

Traditional methods can yield inconclusive or incomplete results depending on the degree of characterization required and there is a growing need for more
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sophisticated methods. Further reasons to improve detection techniques in the brewing industry include:

- Increased consumer awareness on product quality
- Tightened government regulations
- Increased competition among brewers
- Increasing trend to avoid pasteurization of small-packed beer
- Technological advancements.

Rapid methods of detection

The basic premise of rapid methods is to significantly reduce the time required to establish the presence and/or nature of microorganisms found in particular samples of interest. Rapid microbiological detection systems should be faster than “traditional” methods, accurate, affordable, capable of being utilized for either low or high volume testing, easy to learn and user friendly. Fortunately for the brewing industry, research into such methods has been intense and a wide variety of new techniques, suitable for a range of budgets, are available. Here we examine rapid methods under three categories: physical, biochemical and molecular.

Physical methods

1. Impedance/Conductance measurement: Growth of microorganisms in culture media is usually measured by the increase in cell number. However, an alternative is to monitor changes in the chemical and ionic composition of the medium. If an alternating electric current is applied across a growth medium, the medium displays resistance to the flow of the current through it (impedence). Impedence is affected by the conductance (the ability of the medium to allow electricity to pass through it) and the capacitance (the ability to store electrical energy) of the medium. If the impedence or conductance of an actively growing culture is measured and the results plotted against time, a curve almost identical to the growth curve is produced. As such, impedimetric technology may be used as an alternative method to plating to measure microbial spoilage in breweries.

2. Microcalorimetry: As microorganisms grow they generate heat as a result of their metabolic reactions. The small fluctuations in temperature may be measured and recorded over time using a microcalorimeter. Microcalorimeters are instruments designed specially for microbial measurements that generally function by coupling heat to the production of an electric current. Heat production by microbial activity begins during exponential growth. As the culture reaches stationary phase the metabolic activity decreases accompanied by a decrease in heat generation. Microcalorimetry is often used in clinical applications to detect microorganisms, however it is rarely used in brewing.

3. Turbidometry: Spectrophotometry, or turbidometry, is commonly used in food and dairy industries and also in brewing. A number of automated methods are
currently available for detection of microbial contaminants such as *Lactobacillus casei*, *Rahnella aqualitis*, *Pediococcus damnosus* and *Saccharomyces diastaticus*, in pitching yeast. Such methods work within 2–4 days.

4. **Flow cytometry**: Flow cytometry is a modification of spectrophotometry that combines both microscopic and biochemical analyses. Cells are introduced into the center of a rapidly moving stream and then forced to flow at a uniform speed and in single file through an orifice of 50–100 μm in diameter. As they move through the orifice they are illuminated by a laser. The illuminating light is scattered by the cells and this scattering is characteristic of cell shape, size, viability, density and surface morphology. More advanced cytometry methods involve the use of fluorescent dyes to selectively stain different cells. In this way specific cells may be counted in a mixed population, for example ChemScan™. Fluorochromes may also be used to selectively label specific cellular components. When excited by the illuminating laser beam the fluorescence emitted can yield information on the expression of the component. Newer instruments are capable of counting well over 1000 cell per second and if “gating” a numerical boundary is applied, subpopulations can be differentiated from the cell population (dead/live). Also, a threshold applied to the gain will remove debris and noise, which is of particular importance to brewers as wort and beer contain a lot of debris (Paul Chlup, Personal Communication). There are a number of disadvantages to using cytometry including slow process time and interference by small particulate debris. However its major advantage is sensitivity.

5. **Microcolony method**: This method uses microscopy to detect growing cells that have not yet reached visibly discernible colony forming units. Samples are filtered through a membrane and microcolonies are selectively stained and then examined under the microscope following approximately 24 hours of culture. The first stains used for the microcolony method included janus green, methylene blue and safranin for detection of yeasts. A method whereby both the membrane and the cells were stained was also developed for identification of bacteria. However, the early methods had the disadvantage that cells were killed using the stains and could therefore not be cultured for further examination. Nowadays a range of fluorescent dyes may be used. Such dyes are taken up by both yeast and bacterial cells and incorporated into the cell components, and may be visualized using fluorescence microscopy. Modern fluorescent dyes, or optical brighteners, may be used not only to identify yeasts (both brewing and wild) and bacteria (including member of the genera *Lactobacillus*, *Pediococcus*, *Bacillus*, *Hafnia* and *Escherichia*), but also differentiate between living and dead yeast cells. This technique is very useful for the rapid detection of very low numbers of microbes, but it does not give any indication of the organism’s ability to spoil beer.

**Biochemical methods**

1. **Direct Epifluorescence Filter Technique (DEFT)**: DEFT is an improved version of the microcolony method. Initially developed as a quality control method for counting bacteria in raw milk, DEFT combines filtration, using a polycarbonate
membrane, with epifluorescent microscopy to detect fluorochrome-stained cells within approximately 30 minutes. This method does not involve pregrowth, as required in the microcolony method, and may be used to detect single cells. The polycarbonate membrane used in DEFT has a very smooth and flat surface that is better suited for microscopy than the cellulose acetate filters used above. The most commonly employed dye for DEFT is acridine orange, a fluorescent dye that binds to nucleic acids. When the dye binds to single-stranded DNA or RNA, which are found in high numbers in growing cells, it stains orange. Double stranded DNA naturally fluoresces green, therefore actively growing cells may be distinguished from dead cells by the difference in color. Automated methods for DEFT analysis are currently available with throughput rates of over 150 samples per hour.

2. **ATP bioluminescence:** A number of commercially produced kits for detecting yeast and bacteria in wort or beer based on assaying ATP production by microbial metabolism, for example Microstar™, Bev-Trace™, Aqua-Trace™. The assay involves a two-step reaction employing the luciferin-luciferase enzyme reaction that is the basis of bioluminescence in fireflies. The light generated is measured using a luminometer. An enhanced kit is currently available that can detect a single yeast cell or 50 lactic acid bacteria (LAB) cells per sample (Nakakita et al., 2002). LAB produce a substantial amount of ATP in spoiled beer and detection could be used as a method of screening (Suzuki et al., 2005). ATP bioluminescence is now routinely used in surface swabbing, water analysis and beer analysis, and is considerably quicker and also comparable in cost to conventional plate-count techniques.

3. **Protein fingerprinting:** Expression of the microbial genome produces more than 2000 protein molecules. However, not all of the proteins are expressed at the same time. The cellular proteins may be divided into two different groups: the constitutively synthesized structural proteins, and a group of polypeptides, for example enzymes, that are either induced or repressed as a result of the environmental conditions. As such, under defined conditions, the complement of proteins in a cell is characteristic and may be used as a method of identification. In protein fingerprinting, polyacrylamide gel electrophoresis (PAGE) is used to separate cellular proteins based on size or on differences in ionic charge. Separation may be carried out in one dimension, based on size, or in two dimensions, based on both size and ionic charge (Figure 5.2).

The pattern of proteins on the gel following separation is unique (and therefore referred to as the “protein fingerprint”) and may be analyzed for relative similarities or differences to other strains. PAGE is still largely refined to research laboratories and is not generally used as a microbial identification method in breweries. This technique involves growth of cells followed by extraction of proteins. Subsequent electrophoretic separation and staining bring the total time to 1–2 days. In order to make comparisons between strains, the culture conditions must be absolutely exact.

4. **Immunooanalysis (ELISA):** The use of antibodies to detect contaminating microorganisms in breweries is growing in popularity due to its potential to identify microbes in a quantitative way. A range of assays has been designed employing polyclonal or monoclonal antibodies to differentiate microorganisms.
A number of kits are commercially available for rapid colorimetric differentiation or identification of relatively abundant numbers of intact cells ($>10^4$). These kits involve the use of secondary antibodies to which enzymes such as alkaline phosphatase or horseradish peroxidase have been attached. This enzyme linked immunoassay (ELISA) combined with chemiluminescence detection can be used to detect microbial contaminants with high sensitivity and selectivity both during the brewing process and in the finished beer. A number of monoclonal antibodies are commercially available in kits for the detection of lactic acid bacteria such as *Lactobacilli* and *Pediococci*. Monoclonal antibodies for *Pectinatus* have been developed, but there are problems with cross-reactivity.

5. **Gas chromatography**: Metabolic end products, including volatile and non-volatile organic acids, may be analyzed qualitatively using gas chromatography. The organic acid profile generated is unique. The time taken is only a few hours, although further selection in recovery media may be needed to improve sensitivity. The advantage is that all important beer spoilage organisms may be detected, such as *Lactobacillus*, *Pediococcus*, *Pectinatus* and *Saccharomyces diastaticus* (Schisler et al., 1979).

**Molecular methods**

1. **Molecular probes (DNA/RNA)**: Specific sequences of DNA can be detected by hybridization. Hybridization is the formation of a double-stranded nucleic acid (either DNA to DNA or DNA to RNA) between single-stranded nucleic acids from different sources. Short sequences of DNA generated either from a microbial cell or by chemical synthesis, may be labeled and then used to “probe” for cells that contains the complimentary sequence of DNA. Hybridizations may be carried out at different stringencies by altering conditions such as temperature or salt concentration. Under high stringency the probe will only hybridize to sequences that match it perfectly. Under low stringency the sequence of
nucleotides in the hybridized DNA may differ slightly from the order in the probe. DNA probes are sensitive and can detect $10^4$–$10^7$ organisms per test sample. The advantage of DNA hybridization over protein analysis is that the detection of the target organism is not dependent on the products of gene expression, which can vary depending on the environmental conditions. DNA hybridizations may be performed using single colonies, cells on membrane filters or on nucleic acid purified from cells, digested with restriction endonucleases, and separated by agarose gel electrophoresis. A number of different DNA probes have been designed specifically for brewing spoilage organisms such as *Lactobacillus brevis*, *Pediococcus damnosus* and wild strains of *Saccharomyces cerevisiae* and are available in kit form, for example Vermicon identification technology (VIT). Recently kits have become available that are able to identify the most common beer-spoiling bacteria with no-cross-reactivity with naked DNA, dead bacteria or non-beer–spoiling bacteria, and with no influence from possible inhibitory components of the beer. Such tests take a maximum of 2 days, although some require pre-enrichment.

2. **Ribotyping/Riboprinting:** Ribotyping is a hybridization method identical to that described above. It is used for the comparison of similarities in ribosomal RNA (rRNA) gene sequence. Automated methods of ribotyping have been developed that allow identification of fastidious strains, such as *Lactobacillus lindneri*, and obligate anaerobes, such as *Pectinatus*, within 8 hours of isolating a pure culture (Barney et al., 2001; Amann et al., 2001). Recent innovations, known as FISH (fluorescent *in situ* hybridization), use fluorescent-labeled oligonucleotide probes that hybridize directly to the target region of nucleotides inside the bacteria, without extraction of the nucleotides. The results of FISH analysis reflect real-time physiological characterization and quantification of target bacteria in beer and are well suited to determine whether the spoilage bacteria would cause beer spoilage or not. Definition is greater than previous immunological, LPS and cell-surface protein analyses. For example, the detection limit for *Pectinatus* has been found to be approximately 1000 cells per 100 ml of beer (Yasuhrara et al., 2001). FISH technology could potentially eliminate the need for culture-based detection systems traditionally adopted in microbiological quality control in breweries.

3. **Karyotyping:** Electrokaryotyping is a more recent method of characterizing and identifying yeast strains. It is an electrophoretic method that separates chromosomal DNA. It may be used to distinguish different strains of *Saccharomyces*, and therefore to detect non-brewing strains (Fologea et al., 2002).

4. **End-point PCR:** The development of the polymerase chain reaction in 1986 revolutionized molecular biology. This technique is used to amplify small amounts of nucleic acid by several orders of magnitude over the space of only a few hours. As the DNA sequence is unique to each cell, primers may be designed to complement DNA specifically for different strains of microorganisms. PCR primers have already been designed based on sequences found in *Lactobacillus, Megasphaera, Pectinatus, Leuconostoc* and *Saccharomyces diastaticus*. More recently, primers have been designed based on hop-resistance genes, such as *horc*, from *Lactobacilli*, which gives an indication of the spoilage capability of the strain (Iijima et al., 2006). In PCR technology, the design of the primers is important
because microorganisms that should be detected are not always monospecies (Tsuchiya et al., 1994). The total time taken from DNA extraction to PCR result may be less than 5 hours. In this way a microbial contaminant may be detected before the end of fermentation, in time to decide whether or not to collect the yeast for re-pitching. There may, however, be losses of target molecules during DNA extraction from brewing samples, therefore relatively high numbers of microbes are needed for detection. Inhibitive compounds in beer also decrease sensitivity levels.

5. **Real-time PCR (RT-PCR):** In RT-PCR, the products of each PCR cycle are quantified as they are produced, as opposed to quantifying reaction products at the end of 30 or more PCR cycles in End-Point PCR. The total time for RT-PCR detection is typically below 5 hours with limits of detection currently around $10^2$ to $10^3$ cfu $100\text{ml}$ for *Hafnia proteus* (*Obesumbacterium proteus*; Koivula et al., 2006). Cultivation methods are still more sensitive, being able to detect 1–5 cells in a beer sample. Pre-enrichment has been shown to be a suitable method to improve the sensitivity of PCR-based detection of beer spoilage bacteria, but this additional step increases the total detection time by 1–4 days.

6. **RAPD-PCR:** An alternative to using specific primers for PCR is to use a set of randomly designed primers in random amplified polymorphic DNA PCR (RAPD PCR). The product of RAPD PCR is a set of different sized DNA fragments that may be separated electrophoretically to give a characteristic pattern of DNA segments. This pattern is termed a “DNA fingerprint” because it may be genus or even species specific. A number of brewery microorganisms, such as *Pediococcus, Lactobacillus, Hafnia*, and a number of ale and lager yeasts have already been characterized by RAPD PCR and primers are commercially available. A mixture containing 600 primers has been used in reactions resulting in the identification of a highly specific region of DNA from beer-spoilage strains (Fujii et al., 2004; Fujii and Hayashi, 2004). The procedure requires only a small amount of crudely extracted DNA from a single colony isolate, however, the method is not useful for the characterization of mixtures of unknowns (Tompkins et al., 1996).

### Improving microbiological stability

We have found that a wide variety of methods are available for detecting microorganisms. However, such methods do not predict the ability of a given contaminant to grow in and spoil beer. The simplest method of determining this is the forcing test, where a pure culture is re-inoculated into beer or beer enriched with concentrated nutrient medium. This test is unfortunately too time consuming for quality control purposes, and more recently attention has focused on developing databases of common physiological properties responsible for beer spoilage ability; detection of the genes responsible for determining the physiological properties using PCR or immunoassays is much faster than culture methods.

Unfortunately, both rapid detection and prediction methods are expensive. Realistically a pro-active response to microbiological instability would seem
preferable to reactive testing, and research into methods of reducing microbial contamination remains strong. Generally, strict cleaning routines are employed to maintain a microbe-free plant. This does involve large amounts of cleaning agent and water, in addition to staffing.

A second major route to reducing microbial access is through use of closed vessels and pipework, limiting exposure to air. Filtered nitrogen is preferable to filtered air for any mixing of purging so that oxygen levels are kept to a minimum. Over the past decade, considerable progress has been made in packaging technology in breweries, which has led to a substantial decrease in the dissolved oxygen content of beer (Yasuhara et al., 2001). This trend has resulted in significant reduction of aerobic spoilage organisms, such as acetic acid bacteria. However, the frequency of detection of strictly anaerobic beer-spoiling bacteria, such as Pectinatus and Megasphaera, has increased dramatically.

A further method of minimizing bacterial growth is to ensure that the yeast starter culture is healthy and contains an adequate quantity of yeast cells. Bacterial growth is slowed under acidic, anaerobic conditions, and the quicker yeast can achieve this during fermentation the better the chance of limiting growth of contaminants.

For finished beer, there are two fundamental strategies to avoid microbial contamination: pasteurization or the application of preserving agents. Thermal pasteurization is the most common method used in beer treatment despite the disadvantage of high capital outlay and energy costs. Another aspect is the loss of quality, particularly taste, because oxidation processes run faster under increased temperatures. Problems also exist at filing and with heat-resistant microorganisms (Beveridge et al., 2004).

Over the past few years a number of non-thermal pasteurization methods have been investigated. These include the use of electric fields and hydrostatic pressure. The application of pulsed electric fields (PEF) as a non-thermal pasteurization method in food processing is of growing interest because of the inactivation of microorganisms and the maintenance of heat sensitive compounds and sensory properties (Evrendilek et al., 2004). One of the main advantages of this alternative processing technology is that the organoleptic and nutritional quality of foodstuffs sustains little or no degradation as a result of treatment. The application of PEF induces a relatively large transmembrane potential that can lead to electroporation of microbial cell walls.

The application of hydrostatic pressure ranging from 100 to 1000 MPa also allows the preservation of foods without altering food quality to the same extent as thermal treatments with a comparable preservation effect. Mild pressure treatments with little effect on cell viability have been shown to increase the permeability of the cytoplasmic membrane, and inactivate specific hop resistance mechanisms in Lactobacillus plantarum (Ganzle et al., 2001). Pressure treatment of both ale and lager beers at 600 Mpa for 5 minutes have been found to have no effect on pH, ethanol, bitterness or phenolics, and the beers displayed permanent haze similar to untreated beer. The microbiological stability of pressure treated beers has also been found to be comparable to heat-treated beer (Castellari et al., 2000). However the feasibility and cost of such treatments have not been defined.
An alternative to the above pasteurization methods is filtration. Advances in the understanding of the factors influencing cold beer filterability as well as the quality assurance procedures necessary for success have now made sterile filtration a viable alternative to flash pasteurization for the production of draft beer. The two most important considerations are the cost of filtration and the guarantee of the microbiological stability of the beer. Studies have shown that all beer-spoilage microbes are retained on either 1.2\(\mu\)m or 0.22\(\mu\)m filters. Membrane filtration may be applied at final packaging, bottle washing, blanketing/top pressure blending, line cleaning, carbonation, sparging, aseptic fill, bottle purging and tank charging.

The second strategy to reduce microbial contamination is the use of preserving agents, although this is not possible under German purity laws. A few manufacturers of low-alcohol and non-hopped beers have used preservatives such as sorbate or benzoate. Chitosan has been found to enhance the lag period of two strains of *Brettanomyces*. Growth rate of *Saccharomyces cerevisiae* is inversely proportional to chitosan concentration and in mixed fermentations *Brettanomyces* strains fail to grow whereas *Saccharomyces* is unaffected. Other naturally occurring “preservatives” have also been investigated, including zymocins, toxins produced by certain yeasts, which are lethal to sensitive yeast strains. Bacteriocins, produced by lactic acid bacteria, also have the potential to be used as antimicrobials (Vaughan et al., 2001).

**Quality control**

To summarize, there is a constant battle with microbes, and brewers have a range of tools available to detect and to limit access of undesirable microorganisms. Quality control recommendations vary in the strength of cleaning regimes and in types and number of testing procedures. However, the basic ingredients for good microbiological control may be regarded as good plant design, efficient plant maintenance/renewal, use of cleaning-in-place, effective detergents and sterilants, and strict microbiological monitoring.

The following locations are regarded as areas where any microorganism should either be completely absent or present in very low numbers.

1. Raw materials
2. Bright beer
3. Finished product
4. Strategic surfaces of process machinery, for example filler heads.

There are also three locations where brewing yeasts are present and where selective conditions that exclude the brewing yeast strain are required to detect contaminants: yeast slurry, fermentation and ageing.

For many companies, practical realities are far removed from desired sampling plans and detection methods, and the cost of imposing quality control systems is prohibitively high. However, awareness of threats, simple hygiene and “good practice” are often the most effective methods of managing microbiological risk.
References


