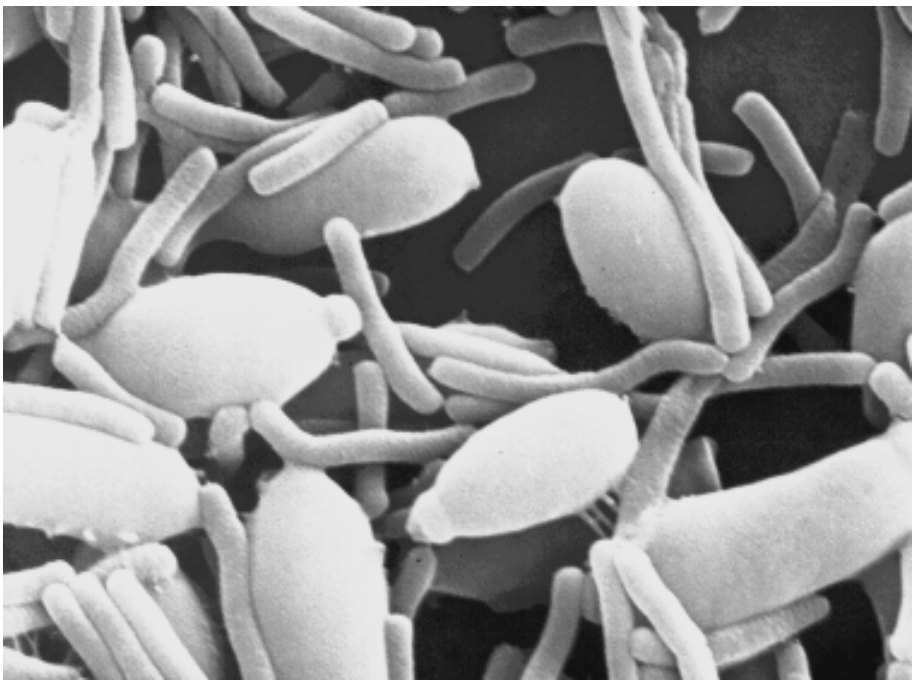
Erna Storgårds

Process hygiene control in beer production and dispensing



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PROCESS HYGIENE CONTROL IN BEER PRODUCTION AND DISPENSING

Erna Storgårds

VTT Biotechnology

Academic dissertation

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Abstract

Process hygiene plays a major role in the production of high quality beer. 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. The present study examined the growth of surface-attached beer spoilage organisms and the detection and elimination of microbial biofilms. Moreover, the detection and characterisation of *Lactobacillus lindneri*, a fastidious contaminant, was studied.

Beer spoilage microorganisms, such as lactic acid and acetic acid bacteria, enterobacteria and yeasts were shown to produce biofilm on process surface materials in conditions resembling those of the brewing process. However, attachment and biofilm formation were highly strain dependent. In addition, the substrates present in the growth environment had an important role in biofilm formation.

Different surface materials used in the brewing process differed in their susceptibility to biofilm formation. PTFE (polytetrafluoroethylene), NBR (nitrile butyl rubber) and Viton were less susceptible to biofilm formation than stainless steel or EPDM (ethylene propylene diene monomer rubber). However, the susceptibility varied depending on the bacteria and the conditions used in the *in vitro* studies. Physical deterioration resulting in reduced cleanability was observed on the gasket materials with increasing age. DEAE (diethylaminoethyl) cellulose, one of the carrier materials used in immobilized yeast reactors for secondary fermentation, promoted faster attachment and growth of contaminating *L. lindneri* than ceramic glass beads. Beer dispensing systems in pubs and restaurants were found to be prone to biofouling, resulting eventually in microbial contamination of draught beer and cleanability problems of the dispensing equipment.

Detection of surface-attached microorganisms is crucial in process hygiene control. *In situ* methods such as epifluorescence microscopy, impedimetry and direct ATP (adenosine triphosphate) analysis were the most reliable when studying surface-attached growth of beer spoilage microbes. However, further improvement of these techniques is needed before they can be applied for routine hygiene assessment. At present hygiene assessment is still dependent on detachment of microorganisms and soil prior to analysis. Surface-active agents and/or ultrasonication improved the detachment of microorganisms from surfaces in the sampling stage. The ATP bioluminescence technique showed good agreement with the plate count method in the control of working dispensing installations. Hygiene monitoring kits based on protein detection were less sensitive than the ATP method in the detection of wort or surface-attached microorganisms.

Effective process control should also be able to detect and trace fastidious spoilage organisms. In this study, the detection of *L. lindneri* was notably improved by choosing suitable cultivation conditions. *L. lindneri* isolates, which could not be correctly identified by API 50 CHL, were identified to the species level by automated ribotyping and by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) when compared with well-known reference strains. SDS-PAGE was also able to discriminate between different strains, which is a useful feature in the tracing of contamination sources.

Preface

This work was carried out at VTT Biotechnology during the years 1992–1998. The work was part of the research on brewing and process hygiene at this institute. I thank the former Laboratory Director, Prof. Matti Linko for encouraging me to take up my studies again and for ensuring a pleasant working atmosphere. I also thank the present Research Director, Prof. Juha Ahvenainen for providing excellent working facilities and possibilities to finalise this work.

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III Storgårds, E., Simola, H., Sjöberg, A.-M. & Wirtanen, G. 1999. Hygiene of gasket materials used in food processing equipment. Part 1: new materials. Trans IChemE, Part C, Food Bioproduction Processing, Vol. 77, pp. 137–145.

IV Storgårds, E., Simola, H., Sjöberg, A.-M. & Wirtanen, G. 1999. Hygiene of gasket materials used in food processing equipment. Part 2: aged materials. Trans IChemE, Part C, Food Bioproduction Processing, Vol. 77, pp. 146–155.

V Storgårds, E., Yli-Juuti, P., Salo, S., Wirtanen, G. and Haikara, A. 1999. Modern methods in process hygiene control – benefits and limitations. Proceedings of the 27th Congress of European Brewery Convention, Cannes, 29 May – 3 June 1999. Pp. 249–258.

VI Storgårds, E., Pot, B., Vanhonacker, K., Janssens, D., Broomfield, P. L. E., Banks, J. G. & Suihko, M.-L. 1998. Detection and identification of *Lactobacillus lindneri* from brewery environments. Journal of the Institute of Brewing, Vol. 104, pp. 47–54.

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Abbreviations

ATP	adenosine triphosphate
BOD	biological oxygen demand
BRI	Brewing Research International
CCFRA	Campden & Chorleywood Food Research Association
cfu	colony forming units
CIP	cleaning-in-place
COD	chemical oxygen demand
DEAE	diethylaminoethyl
DEM	direct epifluorescence microscopy
DNA	deoxyribonucleic acid
DOC	dissolved organic carbon
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellculturen GmbH, Braunschweig, Germany
EDTA	ethylene diamine tetra-acetic acid
EHEDG	European Hygienic Equipment Design Group
EPDM	ethylene propylene diene monomer rubber
EPS	extracellular polymeric substances
HACCP	Hazard Analysis Critical Control Point
HEPA	high efficiency particulate air filter
LMG	Laboratorium voor Microbiologie, BCCM/LMG Bacteria Collection, Universiteit Gent, Belgium
MRS	de Man – Rogosa – Sharpe medium
NBB-A	Nachweismedium für bierschädliche Bakterien, agar
NBB-C	Nachweismedium für bierschädliche Bakterien, concentrate
NBR	nitrile butyl rubber (Buna-N)
PAA	peracetic acid

PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PTFE	polytetrafluoroethylene (Teflon)
PU	pasteurisation units
PVC	polyvinyl chloride
QAC	quaternary ammonium compounds
RFLP	restriction fragment length polymorphism
RLU	relative light units
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SDA	Schwarz Differential Agar
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
TPC	total plate count agar
TQM	total quality management
UBA	Universal Beer Agar
UPGMA	unweighted-pair group method
UV	ultraviolet light
VTT	Valtion teknillinen tutkimuskeskus, Technical Research Centre of Finland

1. Introduction

Beer is generally regarded as safe in terms of food-borne illnesses, due to the belief that pathogens are not able to grow in beer (Ingledew 1979, Donhauser and Jacob 1988, Back 1994a). The biological stability of modern brewery products is also very good, with best before dates ranging from 6 to 12 months or more from production. Why then is hygiene still considered so important in the brewing industry?

The brewing process itself is prone to growth of microorganisms because of the nutrient-rich environment of wort (Ingledew 1979) and the additional growth factors produced by the brewing yeast (Back 1994a). The comparatively long production run from wort boiling to beer packaging, with batch fermentations of up to several weeks, gives plenty of time for unwanted microorganisms to develop if they are given the opportunity. The microbiological sensitivity of continuous fermentation systems using immobilized yeast is also well documented (Kronlöf and Haikara 1991, Haikara and Kronlöf 1995, Haikara *et al.* 1997). However, work carried out for more than one hundred years in the field of brewery microbiology since the pioneering studies of Louis Pasteur (1876) and E.C. Hansen (1896) has resulted in the high hygienic standard of modern breweries. In small-scale pub or microbreweries with brews of 1.000 to 2.000 liters, it is still possible to discard the whole batch in case of microbiological spoilage. This is obviously impossible in large-scale breweries with fermentation tank volumes ranging from 200.000 to 500.000 liters, for both economical and environmental reasons. Thus at any price the breweries avoid the risk that the imago of a beer would suffer because of quality losses due to microbiological problems in the process.

The hygiene of vessels, machinery and other process surfaces crucially affects the quality of the final product. To ensure high quality, reliable detection of microorganisms that could have a detrimental effect on the product is essential as early as possible. Beer production and dispensing takes place mainly in closed systems, where cleaning-in-place procedures without the need for dismantling are applied. Long runs between cleaning are also typical for these systems. Such systems are susceptible to bacterial attachment and accumulation at surfaces, which is a time-dependent process (Notermans *et al.* 1991, Zottola 1994). Biofilms develop when attached microorganisms secrete extracellular polymers

such as polysaccharides and glycoproteins (Flemming *et al.* 1992). It is well established that microbes embedded in polymeric matrices are well protected against cleaning and sanitation (LeChevallier *et al.* 1988, Characklis 1990a, c, Holah *et al.* 1990, Wirtanen 1995, Gibson *et al.* 1995, McFeters *et al.* 1995). Areas in which biofilms mainly develop are those that are the most difficult to rinse, clean and disinfectant and also those most difficult to sample (Wong and Cerf 1995).

The method used for detection of adhering microorganims greatly influences the results obtained (Boulangé-Petermann 1996). Sometimes it is also necessary to detect product residues and soil in addition to living microbes. In these cases, high specificity of the method cannot be required. On other occasions, it is important to specifically identify the problem-causing microbe in question in order to be able to trace the source of contamination in the process. A demanding task in process hygiene assessment is the detection of low numbers of microorganisms after sanitation – especially because the surviving cells are often stressed and their metabolic activity is low (Carpentier and Cerf 1993, Duncan *et al.* 1994, Leriche and Carpentier 1995). The drawbacks of traditional methods based on cultivation are well known (Holah *et al.* 1988, Carpentier and Cerf 1993, McFeters *et al.* 1995, Wirtanen *et al.* 1995, Storgårds *et al.* 1998). Identification methods based on morphology and behaviour (e.g. carbohydrate utilisation tests) are of only little use when working with isolates from the brewing process (Campbell 1996, Gutteridge and Priest 1996, Priest 1996). To overcome the drawbacks of current methods, alternative methods are constantly being developed. However, the first applications of new methods are usually in the field of clinical microbiology or in the food industry facing the possibility of pathogens in their products. These applications can hardly be directly applied in the breweries where very low numbers of specific spoilage organisms are to be detected. Further work is still needed to solve the specific problems of process hygiene in the brewing industry. The present study is part of this work as it adapts theories and methodology from other fields of process microbiology to the specific needs of the brewing industry.

2. Literature review

2.1 Microorganisms associated with beer production and dispensing

The presence of inhibitors such as hop compounds, alcohol, carbon dioxide and sulphur dioxide as well as the shortage of nutrients and oxygen and the low pH all make beer resistant to microbial contamination. Moreover, processes such as filtration, storage at low temperatures and possible pasteurisation reduce contamination. The special environment in the brewing process restricts the range of microorganisms likely to be encountered to relatively few species (Ingledew 1979, Haikara 1984, Back 1994a, Dowhanick 1994). Although the contaminants found may cause quality defects, pathogens have not been reported to grow in standard beer products (Donhauser and Jacob 1988, Dowhanick 1994).

Back (1994a) divided the microorganisms encountered in the brewery into five categories depending on their spoilage characters:

- Absolute beer spoilage organisms (obligat bierschädlich)
- Potential beer spoilage organisms
- Indirect beer spoilage organisms
- Indicator organisms
- Latent organisms.

2.1.1 Absolute beer spoilage organisms

Absolute beer spoilage organisms tolerate the selective environment in beer. These organisms grow in beer without long adaptation and as a result cause off flavours and turbidity or precipitates. *Lactobacillus brevis*, *L. lindneri*, *L. brevisimilis*, *L. frigidus*, *L. coryniformis*, *L. casei*, *Pediococcus damnosus*, *Pectinatus cerevisiiphilus*, *P. frisingensis*, *Megasphaera cerevisiae*, *Selenomonas lacticifex* and *Saccharomyces cerevisiae* (ex. *diastaticus*) belong to this category (Seidel-Rüfer 1990, Back 1994a).

Previously unknown *Lactobacillus* sp. strains with beer-spoilage ability were described by Funahashi *et al.* (1998) and Nakakita *et al.* (1998). Nakakita *et al.* (1998) also described a Gram-negative, non-motile, strictly anaerobic bacterium with weak beer-spoilage ability which clearly differed from any of the previously known anaerobic beer-spoilage bacteria: *Pectinatus* spp., *M. cerevisiae* (Haikara 1992a), or pitching yeast contaminants: *S. lacticifex*, *Zymophilus raffinosivorans* and *Z. paucivorans* (Schleifer *et al.* 1990, Seidel-Rüfer 1990). The recent isolation of new beer-spoilage bacteria (Funahashi *et al.* 1998, Nakakita *et al.* 1998) suggests that previously non-characterised beer-spoilage bacteria still exist. The description of these 'newcomers' in the brewery environment could also be a consequence of the more exact identification methods constantly being developed.

The growth of lactic acid bacteria in beer depends on the pH of the beer and hop acids present (Simpson and Fernandez 1992, Simpson and Smith 1992, Simpson 1993). *Lactobacillus* strains with strong beer spoilage ability often belong to obligate heterofermentative species such as *L. brevis*, *L. lindneri* or the unidentified strain recently isolated by Japanese scientists (Ingledew 1979, Back 1981, Funahashi *et al.* 1998). Weak beer spoilage ability has been observed among facultative heterofermentive *Lactobacillus* strains (Back 1994a, Priest 1996, Funahashi *et al.* 1998, Nakakita *et al.* 1998).

2.1.2 Potential beer spoilage organisms

Potential beer spoilage organisms normally do not grow in beer. However, beers with high pH, low hop concentration, low degree of fermentation, low alcohol content or high oxygen content may be susceptible. The category of potential beer spoilers also includes organisms which can adapt to grow in beer after long exposure times. *L. plantarum*, *Lactococcus lactis*, *L. raffinolactis*, *Leuconostoc mesenteroides*, *Micrococcus kristinae*, *Pediococcus inopinatus*, *Zymomonas mobilis*, *Z. raffinosivorans* and *S. cerevisiae* (ex. *pastorianus*) are examples of organisms in this category (Seidel-Rüfer 1990, Back 1994a).

2.1.3 Indirect beer spoilage organisms

Indirect beer spoilage organisms do not grow in finished beer but they may start to grow at some stages of the process, causing off flavours in the final product. Typically they occur in the pitching yeast or in the beginning of fermentation, causing quality defects that must be avoided by blending. According to Back (1994a), enterobacteria and some *Saccharomyces* spp. wild yeasts as well as some aerobic yeasts belong to this category. *Obesumbacterium proteus* and *Rahnella aquatilis* are considered the most important enterobacterial spoilage organisms in the brewing process (Van Vuuren 1996). According to Van Vuuren (1996), brewery isolates of *Enterobacter agglomerans* probably belong to *R. aquatilis* but it is not clear whether *Pantoea agglomerans* (Gavini *et al*. 1989) should also be regarded as the same organism.

Butyric acid-producing *Clostridium* spp. isolated from wort production or brewery adjuncts (Hawthorne *et al.* 1991, Stenius *et al.* 1991) could also be regarded as indirect beer spoilage organisms. *Z. paucivorans*, which was isolated from pitching yeast (Seidel-Rüfer 1990), probably also belongs to this group although the effects of yeast contamination were not reported.

The effects caused by different spoilage organisms during fermentation and in final beer are summarised in Table 1 (Schleifer *et al.* 1990, Stenius *et al.* 1991, Haikara 1992b, Prest *et al.* 1994, Van Vuuren 1996).

Table 1. Effects of contaminants during fermentation and on final beer.

Group or genera	Effects on fermentation	Turbidity	Ropiness	Off-flavours in final beer
Wild yeasts	Super-attenuation	+	–	Esters, fusel alcohols, diacetyl, phenolic compounds, H_2S
Lactobacillus, *Pediococcus*		+	+	Lactic and acetic acids, diacetyl, acetoin
Acetobacter, *Gluconobacter*		+ [1)]	+ [1)]	Acetic acid
Enterobacteria	Decreased fermentation rate, formation of ATNC	–	–	DMS, acetaldehyde, fusel alcohols, VDK, acetic acid, phenolic compounds
Zymomonas		+ [2)]	–	H_2S, acetaldehyde
Pectinatus		+	–	H_2S, methyl mercaptane, propionic, acetic, lactic and succinic acids, acetoin
Megasphaera		+	–	H_2S, butyric, valeric, caproic and acetic acids, acetoin
Selenomonas		+	–	Acetic, lactic and propionic acids
Zymophilus		+ [3)]	–	Acetic and propionic acids
Brevibacillus		–	+	–
Clostridium		–	–	Butyric, caproic, propionic, and valeric acids

ATNC; apparent total n-nitroso compounds, DMS; dimethyl sulphide, VDK; vicinal diketones, Fusel alcohols; n-propanol, iso-butanol, iso-pentanol, iso-amylalcohol
1) in the presence of oxygen, 2) in primed beer, 3) at elevated pH (5–6)

2.1.4 Indicator organisms

Indicator organisms do not cause spoilage but they appear as a consequence of insufficient cleaning or errors in the production. Their presence is often associated with the occurrence of beer spoilage organisms. *Acetobacter* spp., *Acinetobacter calcoaceticus*, *Gluconobacter oxydans*, *P. agglomerans* (Gavini *et al*. 1989), *Klebsiella* spp. and aerobic wild yeasts are representatives of this category (Back 1994a).

2.1.5 Latent organisms

Latent organisms are microbes which are sporadically encountered in the brewing process and which in some cases even can survive the different process stages and be isolated from finished beer. Usually members of this group are common organisms in soil and water and their presence in the brewery is often due to contaminated process water or to construction work inside the brewery. However, if they are found quite frequently they should be regarded as a sign of poor hygiene. Spore forming bacteria, enterobacteria, micrococci and film-forming yeast species are typical latent microorganisms in the brewery (Back 1994a).

2.1.6 Microorganisms associated with beer dispensing systems

A wider range of microorganims can cause problems in beer dispensing equipment than in the brewing process or in packaged beer. This is due to the higher oxygen levels and higher temperatures at certain points in the dispensing system. Aerobic conditions prevail at the dispensing tap and at the keg tapping head, and the pipe lines may also be comparatively oxygen permeable, e.g. low density polythene piping (Casson 1985). The dispensing lines are most often not totally cooled – at least close to the tap there may be a non-cooled area. These conditions favour contamination by microorganisms such as acetic acid bacteria, moderate levels of coliforms and aerobic wild yeast in addition to the oxygen-tolerant beer spoilage organisms found in the brewery environment (Harper 1981, Ilberg *et al.* 1995, Schwill-Miedaner *et al.* 1996, Taschan 1996, Storgårds 1997).

Bacteria and yeasts from the following genera have been isolated during surveys of beer dispensing systems: *Acetobacter*, *Gluconobacter*, *Obesumbacterium*, *Lactobacillus* (among them *L. brevis*), *Pediococcus*, *Zymomonas*, *Brettanomyces*/*Dekkera*, *Debaryomyces*, *Kloeckera*, *Pichia*, *Rhodotorula*, *Saccharomyces* (brewing and wild yeast strains), *Torulopsis* (Harper 1981, Casson 1985, Storgårds 1997, Thomas and Whitham 1997). Harper (1981) also reported that the acetic acid bacteria isolated from dispensing systems were able to grow in a microaerophilic environment, in contrast to corresponding laboratory strains.

The occurrence of coliforms in beer dispensing systems is a cause of concern due to the emerging enteric pathogen *Escherichia coli* serotype O157:H7. *E. coli* O157:H7 is unusually acid-resistant and has been associated with outbreaks of serious enteric infections after consumption of contaminated apple cider (Semanchek and Golden 1996, Park *et al.* 1999). This particular pathogen is infectious at a low dose, probably due to its acid tolerance, as it can overcome the acidic barrier of gastric juice and reach the intestinal tract with a low population number (Park *et al.* 1999). As it is common that pubs/inns/restaurants serve both beer and food, there may be an opportunity for cross-contamination from the food to the beer. Thus the possible survival in beer of acid-tolerant pathogens such as *E. coli* O157:H7 should not be overlooked.

2.2 Contamination sources

Contaminations in the brewery are usually divided into primary contaminations originating from the yeast, wort, fermentation, maturation or the pressure tanks, and secondary contaminations originating from bottling, canning or kegging (Fig. 1). About 50% of microbiological problems can be attributed to secondary contaminations in the bottling section (Back 1997), but the consequences of primary contaminations can be more comprehensive and disastrous. Absolute beer spoilage organisms may appear at any stage of the process, whereas indirect spoilage organisms are mainly primary contaminants. The spoilage character of a particular organism depends on where in the process it is found. After filtration, the brewing yeast should also be regarded as a contaminant (Haikara 1984, Eidtmann *et al.* 1998).

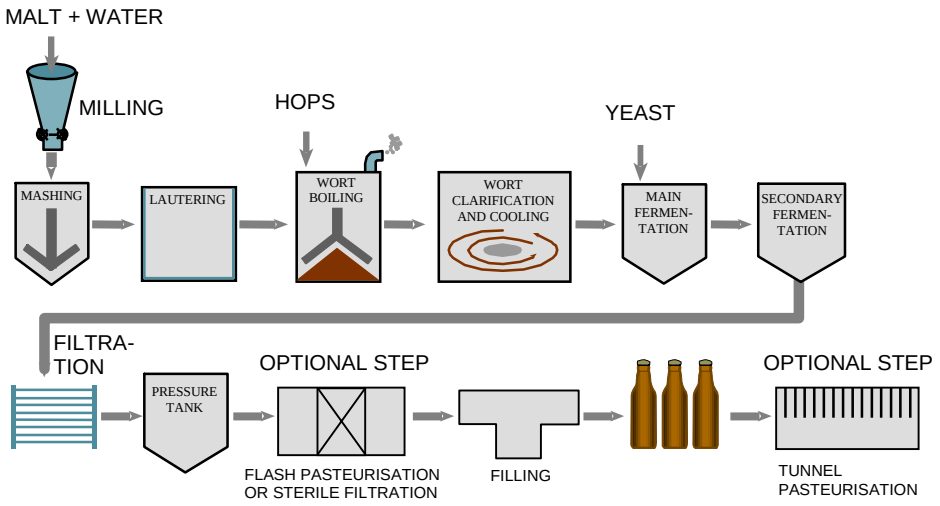


Figure 1. Simplified plan of the beer production process.

2.2.1 Primary contaminations

Little published material is available on the sources of contamination in breweries. Mäkinen *et al.* (1981) were able to show that recycled pitching yeast was the most frequent source of contamination in Finnish breweries 20 years ago. However, this situation has changed drastically along with the procedure to recycle only that yeast shown to be free of contaminating organisms in previous microbiological examination. Mäkinen *et al.* (1981) also found soiled equipment to be a significant source of contamination in brews pitched with pure culture yeast. The fact that the yeast is currently repitched 6 to10 times suggests marked improvement of the CIP procedures implemented in breweries.

In Germany, data has systematically been assembled regarding contamination sources and most frequent contaminants. The pitching yeast, dirty return bottles and rest beer are the most important sources of contamination (Back 1994a). Weak points in the brewery which are reported as sources of contamination include measuring instruments such as thermometers and manometers, valves, dead ends, gas pipes (due to condensate) and worn floor surfaces (Paier and Ringhofer 1997). Contamination could possibly also occur when hot wort is cooled in plate heat exchangers, as a result of leaking plates, inadequate cleaning

of the plates or wort aeration (Back 1995). Contaminated filter powder or dirty filters or additives, such as finings, could probably also cause contamination.

Only very few species and strains can adapt to grow in beer. On the other hand, species adapted to the brewery environment have often not been isolated elsewhere (Haikara 1992a,b, Back 1994a). Beer spoilage organisms such as lactic acid bacteria, wild yeasts and even anaerobic bacteria are often present on the equipment, in the air or in raw materials. These organisms may survive for years in niches of the process, probably outside the direct product stream, without causing signs of contamination. Then suddenly, they may contaminate the entire process as a consequence of technological faults or insufficient cleaning (Back 1994a, Storgårds unpublished observations).

2.2.2 Secondary contaminations

Secondary contaminations are responsible for at least half of the incidents of microbiological spoilage in breweries not using tunnel pasteurisation (Back 1997, Haikara and Storgårds, unpublished observations). Thus, all points with direct or indirect contact with cleaned or with filled unsealed bottles are possible sources of contamination. Most common causes of secondary contamination are: the sealer (35%), the filler (25%), the bottle inspector (10%), the bottle washer due to dripping water (10%) and the environment close to the filler and sealer (10%) (Back 1994b).

According to Back (1994b), contaminations in the brewery filling area never occur suddenly but are always a consequence of sequential growth of microorganisms. First acetic acid bacteria and some enterobacteria start to grow in niches, corners etc. where residues of process intermediates, beer, or other products are collected. These bacteria are not considered harmful in the product but due to their slime formation they protect accompanying microorganisms from drying and disinfection. If product residues are present for a longer time, yeasts start to grow together with the acetic acid bacteria. Yeasts produce growth factors promoting the growth of lactic acid bacteria. The lactic acid produced by the latter organisms can then be metabolised to propionic acid by beer spoilage organisms such as *Pectinatus* spp.

Airborne contamination of beer can occur in the filling department during transport of open bottles from the bottle washer to the filler and until the bottle has been closed. This kind of contamination is significant in breweries which do not tunnel pasteurise their products. The distribution of microorganisms in the air is highly dependent on local air flow and in addition on humidity, temperature, air pressure and also on the settling properties of the microorganisms and their resistance to dehydration and UV from the sun (Henriksson and Haikara 1991, Oriet and Pfenninger 1998).

High numbers of beer-spoilage bacteria in the air have been associated with problems of microbiological spoilage of bottled beer (Dürr 1984, Henriksson and Haikara 1991). The highest numbers of potentially beer-spoiling bacteria were mainly encountered in the air close to the filler and crowner (Dürr 1984, Henriksson and Haikara 1991, Oriet and Pfenninger 1998). A relationship between air humidity and airborne microorganisms was observed confirming that high relative humidity leads to higher numbers of airborne microorganisms (Henriksson and Haikara 1991, Oriet and Pfenninger 1998).

2.2.3 Contamination of beer dispensing systems

The microbiological quality of draught beer has been shown to correspond to that of bottled or canned beer when leaving the brewery (Harper 1981, Taschan 1996, Storgårds 1997). However, kegs shown to be free from contaminants when delivered to retail outlets are often contaminated after being coupled to a dispensing system. Even the beer in the fresh keg itself may become contaminated (Harper 1981, Casson 1985, Ilberg *et al.* 1995, Storgårds 1997) and the ’one-way’ valves used apparently do not constitute a barrier. The dispensing system is exposed to microorganisms in the bar environment via the open tap and during changing of kegs. Draught beer from the tap has been found to contain different kinds of organisms than those common in the brewery (Harper 1981, Casson 1985, Ilberg *et al.* 1995), suggesting that the contamination originates rather from the bar than from the brewery.

Generally, microbial contamination is found throughout the dispensing system, particularly where ’dead’ areas are present such as in keg tapping heads, in dispensing taps, in manifolds etc. However, persistent contamination has always

been associated with organisms attached to surfaces. The largest available surface is the dispensing line itself, which therefore offers the greatest opportunity for adhesion and build-up of microorganisms (Casson 1985).

2.3 Significance of biofilms in the food and beverage industry

2.3.1 Microbial adhesion and biofilm formation

The formation of biofilm takes place when a solid surface comes into contact with a liquid medium in the presence of microorganisms. Organic substances and minerals are transported to the surface and create a conditioning film where nutrients are concentrated, allowing adhesion of the microorganisms (Characklis and Marshall 1990). The immobilized cells grow, reproduce and produce extracellular polymers. A biofilm is a functional consortium of microrganisms attached to a surface and embedded in the extracellular polymeric substances (EPS) produced by the microorganisms (Costerton *et al.* 1987, Christensen and Characklis 1990, Flemming *et al.* 1992). The attachment of bacteria to solid surfaces has been recognised to be a universal phenomen in all natural environments (Costerton *et al.* 1987, Notermans *et al.* 1991). In the case of the majority of microorganisms, adhering to a solid substrate is an essential prerequisite to their normal life and reproduction (Carpentier and Cerf 1993, Kumar and Anand 1998). Although bacteria may adhere to a surface within minutes, it is assumed that true biofilms take hours or days to develop (Hood and Zottola 1995).

Attachment of microorganisms may occur as a result of bacterial motility or passive transportation of planktonic (free floating) cells by gravity, diffusion or fluid dynamic forces. In irreversible adhesion, various short-range forces are involved including dipole-dipole interactions, hydrogen, ionic and covalent bonding and hydrophobic interactions (Characklis 1990a, Kumar and Anand 1998). Attachment of brewing yeast to glass was found to be significantly enhanced by starvation (Wood *et al.* 1992). The irreversibly attached bacterial cells grow and divide using the nutrients present, forming microcolonies. Attached cells also produce EPS, which stabilises the colony (Christensen and Characklis 1990).

Biofilms sometimes achieve uniform coverage of the surface but are sometimes quite 'patchy'. Biofilms may consist of less than a monolayer of cells, or may be as thick as 30–40 mm (Characklis and Marshall 1990). The microorganisms within the biofilm are not uniformly distributed. They grow in matrix-enclosed microcolonies interspersed within highly permeable water channels (Blenkinsopp and Costerton 1991, Carpentier and Cerf 1993, Costerton *et al.* 1994). A biofilm is largely composed of water. Reported biofilm water contents range from 87 to 99% (Christensen and Characklis 1990). Biofilms are generally very hydrophilic (Christensen and Characklis 1990). The EPS matrix could be regarded as a water-laden gel, which protects the microbial cells from desiccation (Blenkinsopp and Costerton 1991, Carpentier and Cerf 1993). Bacteria in biofilms in flowing systems are at an advantage because of increased delivery of nutrients and removal of inhibitory metabolites compared to biofilms in static conditions (Fletcher 1992a).

Many bacteria produce EPS whether grown in suspended cultures or in biofilms. Extracellular polymers are known as slime or capsule and are composed of fibrous polysaccharides or globular glycoproteins. The extent and composition of these polymers may vary with the physiological state of the organism (Christensen and Characklis 1990). Settled microbial cells undergo metabolic changes and begin to secrete large amounts of EPS. These extracellular polymers improve the adherence capacity to metal surfaces and promote further trapping of microorganisms in the substratum (Characklis and Marshall 1990). The biofilm EPS are critical for the persistence and survival of the microorganisms in hostile environments as they help in trapping and retaining the nutrients for the growth of biofilms and in protecting the cells from the effects of antimicrobial agents (Blenkinsopp and Costerton 1991, Kumar and Anand 1998).

2.3.2 Microbial interactions in biofilms

Biofilms in most natural and many engineered environments consist of a complex community of microorganisms rather than a single species. Microbial communities often have capabilities greater than those of the individual members. Interspecies bacterial interactions have a profound influence on the formation, structure and physiology of biofilms (James *et al.* 1995). Interactions

between different species can influence the attachment of bacteria (Fletcher 1992b). As biofilm accumulation proceeds, stabilising interactions between species lead to increased biofilm thickness and stability. Physiological interactions between microbial populations increase the metabolic flexibility of the community and may influence biofilm architecture. Dual species biofilms of industrial isolates of *E. agglomerans* and *Klebsiella pneumoniae* were found to have greater strength of adhesion and higher resistance to disinfection than either single species biofilm (Skillman *et al.* 1997). As heterogeneity increases within the biofilm, chemical micro-gradients develop (Blenkinsopp and Costerton 1991). Oxygen gradients are often created in biofilms and pH gradients have been noted both vertically and horizontally within biofilms.

Biofilm stabilisation can be considered a commensal interaction, in which one species benefits from the ability of another to form a stable biofilm. Commensal interactions are probably common in biofilm systems (James *et al.* 1995). One type of commensalism involves the consumption of oxygen by aerobic and/or facultative microorganisms, allowing the growth of obligate anaerobes (Blekinsopp and Costerton 1991, Costerton *et al.* 1994). The microenvironment that results thus limits diffusion of oxygen through the layers of the biofilm. A great number of adhered anaerobic bacteria were found in a naturally established biofilm of an industrial cooling system (de França and Lutterbach 1996). The sequential growth of microorganisms on brewery surfaces, beginning with aerobic acetic acid bacteria and wild yeasts and culminating in the appearance of obligate anaerobic *Pectinatus* spp. is another example in which the consumption of oxygen by already established aerobic microorganisms and microaerophiles creates ideal conditions for the growth of anaerobic species (Back 1994b, Fig. 2).

Bacterial cells respond to changes in their immediate environments by a remarkable phenotypic plasticity involving changes in their physiology, their cell surface structure and their resistance to antimicrobial agents (Costerton *et al.* 1987). Bacteria that are attached to surfaces frequently appear to differ metabolically from their free-living counterparts. Thus bacteria in biofilms tend to be less susceptible to toxic substances, including disinfectants, than freely suspended cells (Fletcher 1992a). The difference between biofilm and planktonic bacterial cells in susceptibility to biocides may reflect the microenvironments of individual cells growing within biofilms and these may differ radically from

those of planktonic cells in the same ecosystem. Biofilm resistance to biocides is probably also due to the protective barrier provided by exopolysaccharide glycocalyx (Carpentier and Cerf 1993, Wirtanen 1995). Furthermore, antimicrobial agents are far more effective against actively growing cells (Holah *et al.* 1990).

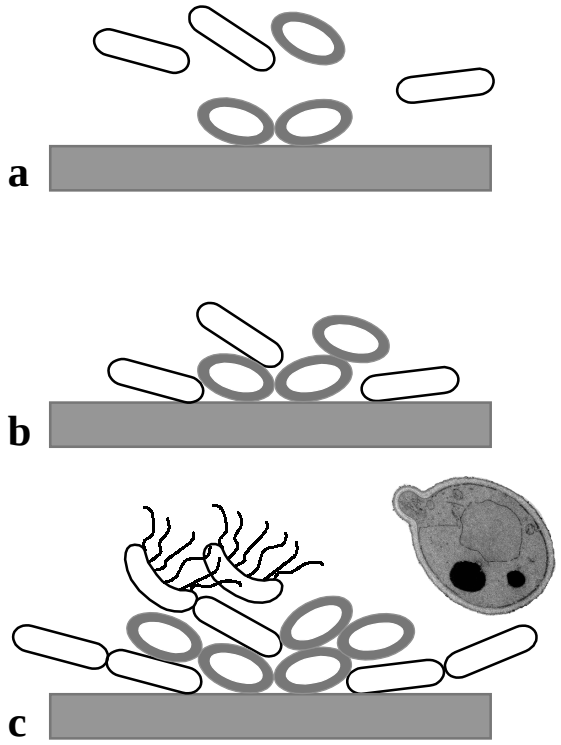


Figure 2. Sequential biofilm formation in the brewery environment according to the theory of Back (1994b). a) Attachment of capsule-forming acetic acid bacteria to a process surface, b) lactic acid bacteria attach to the surface carrying attached acetic acid bacteria, c) wild yeast and Pectinatus *cells attach to the biofilm consisting of acetic acid and lactic acid bacteria.*

Attached bacteria, in order to survive and colonise new niches, must be able to detach and disperse from the biofilm. Sloughing is a discrete process in which periodic detachment of relatively large particles of biomass from the biofilm occurs. This is influenced by fluid dynamics and shear effects, the presence of certain chemicals or altered surface properties of the bacteria or substratum (Characklis 1990a, Kumar and Anand 1998). Nutrients play a role in biofilm detachment, although contradictory results have been obtained concerning low or high nutrient conditions promoting detachment. Nutrient limitations were found to cause *Aeromonas hydrophila* to detach at greater rates in glass flow chambers (Sawyer and Hermanowicz 1998). The fact that biofilms may dislodge from a surface is a cause for concern in the food processing industry (Hood and Zottola 1995). The presence of 'floaters' in draught beer from the tap (Casson 1985) is probably a consequence of biofilm sloughing from the dispensing system. On the basis of microscopic examination such floaters frequently contain clumps of yeast and bacterial cells (unpublished observations).

2.3.3 The role of biofilms in different environments

Biofilms serve beneficial purposes in natural environments and in some engineered biological systems such as waste water plants, where they are responsible for removal of dissolved and particulate contaminants (Characklis and Marshall 1990). Another example of beneficial biofilms is the use of immobilized microorganisms in biotechnical processes (Bryers 1990), such as immobilized yeast in continuous beer fermentations (Kronlöf 1994).

Microorganisms remaining on equipment surfaces may survive for prolonged periods of time depending on temperature and humidity and on the amount and nature of residual soil. Gradually biofilm starts to build up in areas which are hard to access by cleaning and disinfection operations. Microbes growing as biofilms are far more resistant towards environmental stress than free cells, making such deposits ever more difficult to remove. Biofouling or microbial fouling refers to the undesirable formation of a layer of living microorganisms and their decomposition products as deposits on surfaces in contact with liquid media (Characklis 1990b, c, Kumar and Anand 1998). Biofilms cause fouling of industrial equipment such as heat exchangers and pipelines, which results in unsatisfactory equipment performance and reduces equipment lifetime, possibly

even causing corrosion (Characklis and Marshall 1990). Complex biofouling deposits, such as those found in industrial environments, often consist of biofilms in association with inorganic particles, crystalline precipitates or scale and/or corrosion products. These complex deposits often form more rapidly and are more tightly bound than biofilm alone (Characklis 1990b). 'Beer stone' is composed of deposits containing oxalate crystalline precipitates and must be removed regularly from brewing equipment using special treatments.

A food industry biofilm could be defined as a consortium of microorganisms developing within a defined period, dependent on the cycle of cleaning and disinfection programmes, or possibly as the core consortium surviving at low population densities after such cleaning cycles (Holah and Gibson 1999). Biofilms have been observed in bean processing factories, in dairies and breweries, in flour mills and malthouses, in sugar refineries and in poultry slaughter houses (Holah *et al.* 1989, Characklis 1990b, Mafu *et al.* 1990, Czechowski and Banner 1992, Mattila-Sandholm and Wirtanen 1992, Carpentier and Cerf 1993, Banner 1994, Kumar and Anand 1998). Biofilm accumulates on floors, waste water pipes, bends and dead ends in pipes, seals, conveyor belts, stainless steel surfaces and they can cause problems because:

- They are a source of contamination of food and beverages
- They degrade or corrode materials such as stainless steel or rubber
- The physical build up affects process efficiency – e.g. filtration units, heat exchangers.

2.3.4 Biofilms in beer production and dispensing

There are very few published studies concerning biofilms in brewing environment. However, biofilms are of significance in beer production especially if the products are not pasteurised in their packages. Biofilms at different stages of the brewing process can also result in severe off-flavours due to the long process time, often 2 to 3 weeks. Biofilms are readily found in brewery pasteurisers and on conveyor systems, and brewery isolates of *L. brevis*, *E. agglomerans* and *Acetobacter* sp. were found to attach to surface materials used in breweries, such as Buna-N, Teflon and stainless steel (Czechowski and

Banner 1992). The most heavily contaminated areas in the brewery filling area were the points on the track systems near the fillers and can and bottle warmers (Banner 1994). Biofilms were also found on side rails, wearstrips, interior and exterior surfaces of conveyor carriages, drip pans, struts linking the chains and on the bottom of and between chain links. The microorganisms present in biofilms associated with conveyor tracks and bottle and can warmers were generally bacteria of the genera *Pseudomonas, Enterobacter, Klebsiella, Alcaligenes, Flavobacterium, Lactobacillus, Bacillus* and *Arthrobacter*. Yeast and moulds representing the genera *Saccharomyces, Candida, Rhodotorula, Trichosporon, Cladosporium, Penicillium, Geotrichum, Trichoderma, Mucor, Hormonconis, Aureobasidium* and *Paecilomyces* were also observed (Banner 1994).

Biofilms have been observed on dispensing system lines made of polyvinyl chloride (PVC), polythene and nylon (Harper 1981). Casson (1985) studied the colonisation of dispensing systems and found that an organic conditioning film adsorbed onto the PVC pipe after 24 h exposure to beer. He concluded that the adsorbed organic material consisting of polysaccharides or glycoproteins may arise from the original wort or yeast cell wall material. Contaminants introduced into the dispensing system are attracted to the pipe surface by electrostatic interactions but cannot actually adhere on the conditioning film due to close range charge repulsion. The yeasts overcome this charge barrier by extending surface fimbriae, which anchor them to the conditioning film. Subsequently more fimbriae are produced and finally the cells produce EPS to consolidate their position and protect the cells. According to Casson (1985), this polymeric matrix may then harden and become rigid, making the removal of these deposits very difficult. Even if the cells in the film are killed during cleaning, the remaining deposit provides perfect sites for recolonisation when new viable cells are introduced into the dispensing system.

Thomas and Whitham (1997) found that PVC tubing inserted into trade dispensing lines carrying cask ale contained adhering microorganisms after two weeks at levels comparable to control samples of dispensing lines used for more than 18 months. Average levels of adhesion in these samples after washing ranged from 10 to $3.5 \cdot 10^4$ cells per cm^2. Approximately comparable numbers of bacteria and yeast were found to be adhered. *Pediococcus* spp. and acetic acid

bacteria were common contaminants in many lines, along with brewing and wild yeast (Thomas and Whitham 1997).

2.4 Control strategies

According to Hammond *et al.* (1998), control of microbial spoilage of beer is best achieved by eliminating the sources of contamination. However, the brewing process is not aseptic and contaminants will often be encountered. Contaminations can be minimised by reducing the susceptibility of beer to spoilage and by using rapid techniques to determine low numbers of contaminating organisms (Hammond *et al.* 1998).

Traditional control strategies in the food and beverage industry include:

- Increasing the resistance of the product to microbial attack by pH adjustment, addition of antimicrobial compounds, reducing water activity, increasing osmotic pressure etc
- Processes aimed at reducing the microbial load, such as filtration, the use of elevated temperatures (cooking, pasteurisation etc) and storage at reduced temperatures
- Hygienic design of equipment used for production, including the choice of suitable materials and elimination or minimisation of dead spaces and rough surfaces
- Physical separation of high care areas in which critical operations are undertaken and in which barrier technologies are practised to prevent the entry of microorganisms from e.g. raw materials, people, air or utensils.
- Effective, regular cleaning and disinfection of equipment and facilities.

2.4.1 Resistance of beer to microbial spoilage

The beer type determines its the ability to resist microbial spoilage. The most resistant beers are strong beers and beers with a pH below 4.3 (Back 1994a). These beers can be spoiled only by certain strains of absolute beer spoiling

lactobacilli, pediococci, *Pectinatus* spp. or some *Saccharomyces* wild yeasts. Also quite resistant are all malt beers with pH 4.4–4.6 and beers with a high hop content (>30 EBC bitter units). Most prone to spoilage are beers with low acidity, low alcohol beers, beers with added sugar or a high fermentable rest extract and beers with a low carbon dioxide concentration. According to Back (1994a), these beers can also be spoiled by potential and indirect beer spoilage organisms. The biological stability of beer is also negatively affected by high levels of malic acid (>30 mg/l), manganese, pantothenic acid, folic acid and some sugars (mannose, ribose, arabinose) (Back 1997). The growth of fastidious lactobacilli and pediococci is stimulated by growth factors produced by yeast during the fermentation (Haikara 1984, Back 1997).

Carbon dioxide, which is considered a growth promoter for *Lactobacillus* spp. at low concentrations, has been shown to be inhibitory at the concentrations typically found in beer (Hammond *et al.* 1998). Thus beers with lower levels of dissolved carbon dioxide will be more prone to spoilage than conventional products. Such beers include e.g. cask-conditioned beers with low carbon dioxide content and beers dispensed with nitrogen gas, especially if they are unpasteurised. Phytic acid and phenolic compounds (ferulic acid, 4-vinyl guaiacol) were shown to have significant antimicrobial activity in beer (Hammond *et al.* 1998). Unfortunately 4-vinyl guaiacol is of little relevance for most beers, because of its strong aroma and flavour attributes.

The sensitivity of different beers to spoilage by lactic acid bacteria varies. Parameters found to correlate with the spoilage potential include pH, beer colour, content of free amino nitrogen, total soluble nitrogen, a range of amino acids, maltotriose, undissociated forms of sulphur dioxide and hop bitter acids (Fernandez and Simpson 1995). Fernandez and Simpson (1995) were able to predict the spoilage potential of 17 lager beers using a predictive model based on undissociated sulphur dioxide content, undissociated hop bitter acids content, polyphenol content, free amino nitrogen content and colour intensity. They concluded that earlier attempts to explain sensitivity of beers to spoilage (Dolezil and Kirsop 1980, Pfenninger *et al*. 1979) had failed because the bacteria had not been adapted to grow in beer prior to inoculation.

2.4.2 Processes for reduction of microorganisms

Processes used for removal of the pitching yeast and/or reduction of contaminating microorganisms in beer production are listed in Table 2.

Table 2. Processes used for reduction of microorganisms in beer production.

Process	Purpose
Acid washing of pitching yeast	Reduction of contaminating microorganisms in pitching yeast
Cooling	Retardation of the growth of contaminating microorganisms during fermentation and maturation
Filtration	Removal of pitching yeast, reduction of contaminating microorganisms
Pasteurisation	Elimination of vegetative cells in final beer
Aseptic or hygienic packaging	Prevention of contamination during packaging

Pitching yeast is one of the most important contamination routes in the brewery (Haikara 1984, Back 1994a) and it is therefore essential to keep the yeast free of contaminating organisms. Washing the pitching yeast is a controversial practice because of the negative effect of acid washing on the yeast viability (Back 1997, Johnson and Kunz 1998). Therefore many breweries, among them the Finnish breweries, do not use yeast washing but instead rely on careful yeast handling and efficient sanitation of equipment. However, in the UK acid washing is applied (Cunningham and Stewart 1998, Anon. 1999).

Acid washing of yeast is usually performed by lowering the pH of the yeast slurry to pH 2–3 with phosphoric acid and incubating for 2 hours to overnight (Campbell 1996, Cunningham and Stewart 1998, Johnson and Kunz 1998). An alternative way to wash the yeast is by using chlorine dioxide at a concentration of 20–50 ppm activated sodium chlorite. This method is less harmful to the yeast

than acid washing and it also destroys lactic acid bacteria more effectively. However, neither acid washing nor chlorine dioxide treatment was effective against wild yeast contaminants in the pitching yeast (Johnson and Kunz 1998).

Filtration is used to remove the yeast and possible contaminants after fermentation. Very tight filtration is not possible due to macromolecules in beer (glucans, dextrins and proteins) which would block a tight filter and have negative effects on the taste, colour, foam and bitterness (Duchek 1993, Gaub 1993). The filtration process is generally carried out stepwise. First yeast, haze particles and the majority of bacteria are removed in the clarification step in which kieselguhr (diatomaceous earth) filtration is applied. The logarithmic reduction value in kieselguhr filtration is >8 for yeast and >3 for bacteria (Kiefer and Schröder 1992). In a second filtration step, filter sheets, filter cartridges or pulp filters can be used. In the production of unpasteurised beer, a sterile filter can eventually be applied with the purpose of removing any possible residual microorganisms from the beer (Ikeda and Komatsu 1992, Ryder *et al.* 1994). However, this step can be avoided by maintaining strict process hygiene (Gaub 1993).

According to Back (1995, 1997), modern filter lines combining kieselguhr, sheet and final filters achieve almost the same degree of safety as flash pasteurisation. Filters are adequate if 10^3 cells per ml are separated quantitatively during running dosage and at least 10^7 are removed during daily contaminations of about 10^{11} cells (Back 1997). A satisfactory separation of beer spoilage bacteria in the final filtration was attained with a 0.45 µm membrane, but 0.65 µm membranes did not ensure a sufficient degree of safety (Back *et al.* 1992).

Pasteurisation is used to eliminate the beer spoilage organisms in final beer. The treatment is dependent on the time and temperature used as expressed as pasteurisation units (PU). A PU refers to the thermal treatment equivalent to 1 minute at 60°C, although higher temperatures and shorter times are usually applied to save the product from adverse chemical reactions (Enari and Mäkinen 1993). All beer spoilage organisms including yeasts are killed at 30 pasteurisation units (PU) (Back *et al.* 1992). Most beer spoilage lactobacilli and pediococci are already killed below 15 PU. *Lactobacillus lindneri* can tolerate up to 17 PU and *L. frigidus*, because of mucus encapsulation, even up to 27 PU. Heat resistant beer spoilage organisms practically do not occur. The only

exception is *Clostridium acetobutylicum*, which may multiply in beers with low alcohol content and pH >4.2 (Back *et al.* 1992). Minimum temperatures of 66°C and minimum effective times of 15 seconds should be maintained when setting pasteurisation units. Pasteurisation also improves the physical chemical stability of beer by deactivation of yeast proteinases, resulting in long-term foam stability (Back *et al.* 1992).

Bottle pasteurisation guarantees complete microbiological safety of the product, provided that the pasteurisation units are set correctly to 27–30 PU (Back 1995). However, this involves high costs and thermal stresses and is mostly used for very sensitive beer types such as low alcohol beers. Flash pasteurisation can be used to eliminate primary contaminants, leaving the possibility for secondary contaminations. Moreover, fine crevices or pitting in the plate heat exchangers may cause cross contaminations (Back 1995). According to Back (1995, 1997), the microbiological safety of packaged beer is reduced from 100% to 50% when flash pasteurisation is used instead of bottle pasteurisation and a further reduction to 35–40% is to be expected when relying entirely on filtration processes.

'Aseptic packaging' or strict ensuring of hygiene during filling is applied in breweries that do not tunnel-pasteurise their products. Saturated steam, hot water flooding, disinfectant spraying and/or clean room technology are used to reduce secondary contaminations at bottling, canning and kegging (Haikara and Henriksson 1992, Ikeda and Komatsu 1992, Takemura *et al.* 1992, Watson 1992, Takagi 1993, Back 1994b, Rammert *et al.* 1994, Roesicke *et al.* 1994, Ryder *et al.* 1994). In hot water flooding the temperature must be between 80 and 95°C and the frequency should be every 2 hours in summer and every 4 hours in winter (Back 1994b). The frequency of disinfectant spraying at the filler and crowner was also shown to be important: disinfecting at the beginning and the end of production was not sufficient to reduce the number of beer spoilage organisms in the air (Haikara and Henriksson 1992).

The filling operation can also be carried out in aseptic rooms (Ikeda and Komatsu 1992, Takagi 1993) or in an aseptic envelope (Ryder *et al.* 1994). In these applications the incoming air is HEPA-filtered (HEPA; high efficiency particulate filters capable of removing >99.97% of all particles >0.2µm) and the air pressure in the room is higher than outside. Special clothing is used in the

filling area and all packaging material is sanitised by UV, hot water or a disinfectant. The ventilation ensures at least 20 changes of air per hour and the room temperature is maintained below 20°C. Machinery constructions are modified to make them more easily cleanable (Ikeda and Komatsu 1992, Takagi 1993, Ryder *et al.* 1994).

2.4.3 Hygienic design

Hygienic design practices are important aspects essential in controlling biofilm formation and/or minimising the biotransfer potential in food processing equipment such as tanks, pipelines, joints and accessories. These mainly include suitable choice of equipment, materials and accessories, correct construction, process layout and process automation (Holah 1992, Mattila-Sandholm and Wirtanen 1992, Kumar and Anand 1998). The requirements for hygienic design are well documented and they state in detail how equipment should be constructed so that all surfaces in contact with the food or beverage are easy to clean (Timperley *et al.* 1992, EHEDG 1993a, b, c, 1994, Chisti and Moo-Yong 1994, Felstead 1994). Generally, all product-contact surfaces should be smooth (preferably $R_a \leq 0.8$ µm), pits, crevices, sharp edges and dead ends should be avoided and all equipment and pipelines should be self-draining (EHEDG 1993a, b, c, 1994).

Valves cause a significant risk of contamination in the production process and the risk increases with each valve installed in the process plant (EHEDG 1994, Chisti and Moo-Young 1994). For bioreactors, either valves with metal bellows sealed stem or diaphragm and pinch valves are recommended (Chisti and Moo-Young 1994). Plug valves and traditional ball valves are not suitable for CIP (EHEDG 1994). Accumulation of debris at gaskets and valve spindles has been documented for ball valves, butterfly valves and gate and globe valves which are also difficult to clean using CIP methods (Chisti and Moo-Young 1994). There should be as few seals in a valve as possible and the maximum compressibility of the sealing material should not be exceeded during processing, cleaning or thermal treatments (EHEDG 1994).

In the filling hall, constructions should be open to facilitate cleaning and should not allow any liquid to remain on surfaces. Drop plates should be avoided when

possible since they collect dirt. Cable installations should be avoided in the wet area whenever possible or they should be in closed pipes with access from below (Paier and Ringhofer 1997). Drains must be correctly sized and placed in order to avoid any water and organic residues and floor coverings must be chosen so that they can be effectively cleaned and maintained (Ryder *et al.* 1994).

Because the air is one possible contamination route in beer production it is recommended to ensure good air quality especially in the filling department. The location of machinery has an impact on the microbiological quality of the air. The bottle washer should preferably be located at some distance from the filler because of the generation of heat and humidity, and the same applies for the labelling machine because of the organic load caused by the glue (Henriksson and Haikara 1991, Haikara and Henriksson 1992). Improvement of air quality can be achieved e.g. by separation of clean rooms from other areas, sanitation of ceilings, floors and drains, regular removing of wastes (labels, splinters) or installation of laminar flow in the filling area (Oriet and Pfenninger 1998).

In the construction of beer dispensing systems, hygienic design is equally important as in the construction of production equipment. However, many weak points have been identified in these systems, including the dispensing tap and tap armature, fittings and joints (Schwill-Miedaner *et al.* 1996, Schwill-Miedaner and Vogel 1997). The dispensing systems should be constructed so that pipes, pumps and refrigeration equipment are self draining and no gas pockets or dead ends are left in the system (Hauser 1995).

2.4.4 Cleaning and disinfection

The role of cleaning and disinfection for both small and large breweries has grown immensely due to the production of non-pasteurised products (Kretsch 1994) and due to new products low in alcohol and bitterness. In larger breweries, all functions for cleaning and disinfection are computer-controlled, with chemical additions, cycle times and cleaning/rinsing cycles automatically programmed, monitored and recorded. The chemicals, equipment and procedures are designed and controlled so that the results are reproducible. The cleaning solutions are recovered and reused as much as possible and discharges to the sewage system are minimised and neutralised (Kretsch 1994).

In general, chemical cleaners have been found to be more effective in eliminating attached bacteria from surfaces than disinfectants. In experimental conditions, complete biofilm removal and inactivation was obtained when the surface was first cleaned prior to exposure to disinfectant (Krysinski *et al.* 1992). Furthermore, disinfectants are generally most effective in the absence of organic material (Donhauser *et al.* 1991, Czechowski and Banner 1992, Krysinski *et al.* 1992). Thus the control and inactivation of adherent microbes or biofilms requires detergent cleaning of the surface followed by treatment with a disinfectant (Zottola and Sasahara 1994).

Cleaning-in-place (CIP) procedures are employed in closed processing lines of the brewing process (Table 3). However, the limitation of CIP procedures is the accumulation of microorganisms on the equipment surfaces (Mattila *et al.* 1990, Czechowski and Banner 1992). Fermenters operated with yeast cells represent cleaning problems of intermediate difficulty (Chisti and Moo-Yong 1994). The mechanical input in cleaning has been shown to be critical in removing biofilms (Exner *et al.* 1987, Characklis 1990c, Blenkinsopp and Costerton 1991, Carpentier and Cerf 1993, Wirtanen *et al.* 1996). Mechanical force can be achieved by turbulence flow in the pipelines and spray nozzles in the cylindrical tanks, but in practice there are places in the process where the mechanical action is low. Bacteria attached in pits and crevices are difficult to remove by cleaning agents because of poor chemical penetration and possibly also because of surface tension (Holah and Thorpe 1990). Furthermore, high temperatures can only partly be employed in cleaning of brewery vessels. Low cleaning temperatures have been found to be ineffective in the removal of biofilms (Holah and Gibson 1999).

In breweries, acid-based detergents may be preferred for tank cleaning because of the following practical advantages (Gingell and Bruce 1998):

- Acids are not affected by carbon dioxide and hence do not loose their cleaning efficiency when used on a recovery system
- They prevent carbon dioxide losses by allowing cleaning and sanitising to take place without the need to vent down tanks and they facilitate carbon dioxide top pressure cleaning

- There is less risk of tank implosion compared to the case of caustic soda reacting with carbon dioxide and due to the use of ambient temperatures
- They are efficient in removing and preventing beer stone and hard water deposits
- They are more cost effective than alkaline detergents because the high detergent losses due to carbonation of alkalis do not occur
- They are more efficient in terms of water consumption since they are more quickly rinsed away
- They are energy efficient because hot cleaning is not necessary.

Table 3. Typical CIP programmes used in the brewery. The programmes are adapted to the part of the process to be cleaned, and some of the steps: alkalic, acidic, or disinfection, can be left out.

Action	Temperature	Duration
Prerinsing	cold or hot	5–10 min
Alkali cleaning; sodium hydroxide (1.5–4%)	cold or hot (60–85°C)	10–60 min
Intermediate rinsing	cold or hot	10–30 min
Acidic cleaning; phosphoric, nitric or sulphuric acid (1–2%)	cold	10–30 min
Intermediate rinsing	cold	10–30 min
Disinfection		
– by disinfectant solution	cold	10–30 min
– by hot water	85–90°C	45–60 min
Final rinsing if necessary – may contain a disinfectant at low concentration	cold	5–10 min

However, increase in pH and to a lesser extent, increase in temperature has been shown to enhance biofilm removal (Notermans *et al.* 1991, Czechowski and Banner 1992, Carpentier and Cerf 1993). Chlorinated alkaline detergents were found to be the most effective in removing biofilms of brewery-related species in CIP (Czechowski and Banner 1992).

The cleaning of open surfaces in the brewery, such as e.g. bottle inspectors, fillers and conveyor belts in the bottling hall, is usually performed using low-pressure foam systems or thin film cleaning (Table 4). The use of hot solutions or strong chemicals is limited for safety reasons, but disinfectants also effective in cold conditions can be used to ensure the hygiene. Back (1994b, 1997) recommended foam cleaning and subsequent spraying with a disinfectant after every production day and regular basic cleaning including dismantling of components that are difficult to inspect visually. However, care must be taken to avoid transmission of spoilage organisms resulting from aerosols produced during pressure-cleaning (Holah 1992).

Table 4. Foam cleaning and disinfection programme (Kluschanzoff et al. *1997).*

Action	Agent
Prerinsing	Water
Foaming	Foam cleaner
Soak time	Foam cleaner
Intermediate rinsing	Water
Spraying	Disinfectant solution
Final rinsing	Water

Mechanical or chemical breakage of the polysaccharide matrix is essential for successful biofilm control, as the matrix protects the microorganisms from the effects of detergents and disinfectants (Blenkinsopp and Costerton 1991, Czechowski and Banner 1992, Wirtanen 1995). When the deposits also consist

of inorganic scale, mechanical treatment alone may be inadequate (Characklis 1990c). Detergents containing chelating agents such as EDTA (ethylene diaminetetra-acetic acid) have been used to break biofilms (Carpentier and Cerf 1993, Wirtanen *et al.* 1996, Kumar and Anand 1998) and EDTA has excellent beer stone removal properties (Kretsch 1994). Enzymes have been demonstrated to cause effective breakage of the EPS matrix, thus helping in the removal of biofilms, and oxidoreductases have bactericidal activity against biofilm bacteria (Carpentier and Cerf 1993, Johansen *et al.* 1997, Kumar and Anand 1998). Multicomponent enzymes could provide a supplement to the present cleaning and disinfection agents. Physical methods could also be used for the control of biofilms, including ultrasound treatment, super-high magnetic fields and high and low pulsed electrical fields, and they could be applied both on their own and as enhancers of biocides (Zips *et al.* 1990, Stickler 1997, Kumar and Anand 1998, Mott *et al.* 1998, Pagan *et al.* 1999).

The aim of disinfection is to reduce the surface population of viable microorganisms after cleaning and to prevent microbial growth on surfaces during the interproduction time. Microorganisms that are exposed to the disinfection on food processing surfaces are those that remain after the cleaning stage and are thus likely to be surface attached (Holah 1992). However, adherent cells have been shown to be more resistant to disinfectants and heat than planktonic cells (Frank and Koffi 1990). Disinfectants effective against bacteria in suspension are not necessarily the most successful against biofilm bacteria (Carpentier and Cerf 1993, Wirtanen 1995). The concentrations of some disinfectants must be increased ten to one hundred fold in order to obtain the same degree of inactivation of biofilm bacteria as for cells in suspension (Holah *et al.* 1990). Biofilms grown under static conditions were found to be more resistant to disinfectants than biofilms produced under flow conditions, probably due to stagnation and starvation effects causing increased EPS production (Blanchard *et al.* 1998). In the brewery, environments where biofilm may form in static and in flow conditions are both present and it is equally important to keep both free from microorganisms.

The borderline between cleaning and disinfection is somewhat diffuse because microorganisms are to a great extent eliminated already during the cleaning stage. Some detergents are bactericidal and some disinfectants depolymerize EPS, causing detachment of biofilms from surfaces, e.g. oxidants such as

chlorine and hydrogen peroxide (Carpentier and Cerf 1993). Sodium hydroxide, the most common cleaning agent in CIP, was shown to have microbicidic activity against organisms encountered in the brewery. In a suspension of 0.5% sodium hydroxide at 20°C, a 5 log reduction of brewer's yeast was achieved in 2 min, of *L. brevis* in 3 min and of *P. damnosus* in 5 min (Donhauser *et al.* 1991).

In choosing disinfectants for use in the brewery (Table 5), the following characteristics are of importance (Donhauser *et al.* 1991):

- Effective against Gram-positive and Gram-negative bacteria and against yeasts (preferably also against moulds)
- Effective in the presence of proteins
- Effective at low temperatures (often contradictory with efficiency against proteins)
- Wetting ability (contradictory with rinsability)
- CIP-suitability (low foam formation, compatibility with carbon dioxide, concentration measurable by conductibility, reusable/not easily de-composable)
- Environmental aspects (easily rinsable, readily biodegradable*)
- Economy (effective at low concentrations, reusable, easily rinsable)
- Health aspects – safe to use
- Product compatibility – no adverse effects on the product.

Formulations based on peracetic acid and hydrogen peroxide are frequently used for post-cleaning disinfection. Peracetic acid (PAA) penetrates the cell and oxidises enzymes and other proteins irreversibly (Donhauser *et al.* 1991). PAA has been shown to be effective against biofilms (Exner *et al.* 1987, Holah *et al.* 1990). Because of its acidic and non-foaming properties, PAA is suitable for CIP disinfection under a carbon dioxide atmosphere such as in fermentation tanks and lines (Banner 1995). The agents quickly lose their activity in a basic environment, making careful rinsing after alkaline cleaning essential. Peracetic

* Readily biodegradable = disinfectant degraded within 28 days to 60% of BOD/COD or to 70% of DOC (OECD-test No. 301 A-F) (Orth 1998).

acid- and hydrogen peroxide-based disinfectants also perform well in the presence of organic soil, but they are markedly less effective when the temperature is decreased from ambient (20°C) to 4°C (Donhauser *et al.* 1991). At low temperatures, such as in the fermentation cellar, higher concentrations are needed to obtain a good result.

Table 5. Disinfectants and their use in the brewery (according to Banner 1995 and Orth 1998).

Disinfectant type	Use
Hydrogen peroxide – peracetic acid a) peracetic acid (2.5–15%) b) with organic or inorganic acids and surfactants	Brewhouse Fermentation, storage, pressure tanks Pipelines Filler Bottle washing Dialysis/ reverse osmosis
Halogenes a) alkaline chlorine b) acid iodophores	Malthouse Brewhouse Tanks and pipelines Hoses, fittings, filtration Bottle washing (rinse water)
Surface active agents a) quaternary ammonium compounds, pH 4–9 b) amphoterics	General plant cleaning Malthouse, hoses, fittings Mixing machines, filler
Halogenated carbonic acids – chlorine/ iodine/ bromine with inorganic acids	Tanks and pipelines
Alkylamines (foam disinfection)	Filler Environmental hygiene
Biguanides Aldehydes – formaldehyde/ glutaraldehyde	Soaking of small utensils and instruments Air sanitation by fogging (bottling hall) Water treatment systems (glutaraldehyde): cooling, pasteurizer, can/ bottle warmer
Chlorine dioxide	Bottle washing (rinse water)

Chlorine- and iodine-based disinfectants rapidly destroy cell proteins and they also perform well at low temperature. However, these disinfectants are inactivated by proteins, which reduce their effectivity in the presence of wort or beer residues (Donhauser *et al.* 1991, Banner 1995). Chlorine is often used as hypochlorite solutions under alkaline conditions, whereas iodine disinfectants are most active around pH 2–3, making the latter more suitable for use in brewery CIP (Banner 1995). Chlorine dioxide, like chlorine, is a powerful oxidising agent. However, the generation of chlorine dioxide from the stabilised chemical and the activating acid is laborious and quite hazardous, which has limited its use (Banner 1995). The bactericidal activity of chlorine dioxide against *E. coli* was strongly influenced by the state of the cells during the course of the treatment (Foschino *et al.* 1998).

Quaternary ammonium compounds (QAC) adsorb to cation-active sites of the cell surface, causing changes in the permeability and leaking of cellular substances. Because of their low surface tension they have good penetrating ability but are also difficult to rinse, which may have an adverse effect on the products (Donhauser *et al.* 1991, Gingell and Bruce 1998). Acidic QACs are effective against a wide spectrum of microorganisms, especially yeasts. (Gingell and Bruce 1998). Residual films of QACs may reduce the foam level of beer and can also interfere with the growth and metabolism of brewery yeast. Because of this and also due to foaming and rinsing problems, QAC products are not used in CIP operations but mainly for soaking purposes (Gingell and Bruce 1998, Banner 1995).

When heat is used for disinfection, moist heat is far more effective than dry heat. *L. brevis*, the most common beer spoilage bacterium in lager breweries (Back 1994a), has been shown to withstand more than 60 min at 80°C in dry conditions (Donhauser *et al.* 1991). In the process, such dry conditions may occur if microorganisms are located between metal surfaces, between a seal and a stainless steel surface or in microscopically small cracks in the process materials.

2.4.5 Assessment of process hygiene

Total quality management (TQM) can be divided into three quality processes: quality control, quality assurance and quality improvement. The Hazard Analysis Critical Control Point (HACCP) system is a safety tool and it can be incorporated into TQM programmes for the following reasons: to improve the efficacy of the operations and the quality of the products, to satisfy the requirements of the customers and purchasers, to prove a due diligence defence in legal actions, or to keep up with the competitors (Vanne *et al.* 1996). The HACCP system replaces traditional end-point quality control with a more systematic approach based on preventive quality assurance (Kennedy and Hargreaves 1998). The health risks involved in beer production are mostly of chemical or physical origin due to the fact that pathogens do not grow in beer (Urban and Natter 1999). However, in addition to complying with legislation to assure consumer safety, a well designed HACCP system can be used to manage and optimise the analysis of product quality parameters throughout the production process (Kennedy and Hargreaves 1998).

Microbiological methods are not always necessary to control microbial hazards. They are slow and the results are available only after a certain delay. The control of microbiological hazards deals with the prevention or limitation of growth, survival or contamination. Growth and survival depend on parameters such as temperature, time, disinfectants and other microbicidal compounds, pH, available nutrients, moisture etc. Contamination depends largely on the efficiency of cleaning, which itself depends partly on the same parameters (de Boer and Beumer 1998). These can be monitored by measuring physical parameters such as (Hammond 1996):

- cycle times
- solution temperatures
- flow rates

or by chemical analyses such as:

- detergent concentrations (conductivity)
- alkalinity (in-line or off-line)

- specific chemical activities (e.g. sequestrant concentration)
- pH
- soil load of detergent solution (by measuring colour, suspended solids, tendency to foam etc.).

However, despite quality assurance of the CIP procedures, there is also a need to ensure that the cleaning process actually worked. This can be done by (Hammond 1996):

- visual inspection
- swab samples
- final rinse water sampling
- analysis of the next batch.

Methods employed for sampling and enumeration of surface-attached microorganisms include swabbing, rinsing, agar flooding and agar contact methods (Table 6). However, there are some limitations associated with these methods. When numbers of attached bacteria are determined by removal of cells, a serious deficiency is that it is extremely difficult to remove attached cells quantitatively (Wirtanen 1995). Techniques such as swabbing, agar contact methods, sponges etc. remove only the top of the biofilm. Another obstacle is caused by the fact that some microorganisms are likely to be in a non-culturable form as a consequence of nutrient gradients found in thick biofilms, the irregular inputs of nutrients and the stress caused by desiccation, cleaning and disinfection. Using direct epifluorescence microscopy it is not possible to enumerate bacteria when they aggregate in microcolonies or form biofilms with more than one bacterial layer (Carpentier and Cerf 1993, Wirtanen 1995).

For enclosed production equipment, the assessment of surface hygiene levels is particularly difficult (Holah 1992). Grooves, crevices, dead ends, corrosion patches, etc. are areas where biofilms typically accumulate and are hard to access (Wong and Cerf 1995). On-line sensors, which could detect films and deposits on the surfaces of liquid handling processing equipment (e.g. pipes, bends, plate heat exchangers), would be particularly useful. On-line monitoring of biofilms has been achieved by measuring heat transfer resistance, dissolved

oxygen and pH (Ludensky 1998). These techniques provided quantitative information on biofilm accumulation, removal and biofilm microbial activity. This demonstrated the possibility to detect and record, in real time, the impact of biocide treatment on biofilm growth. However, to date these methods have not been adopted in the brewing industry.

Table 6. Sampling of microorganisms from surfaces (according to Wong and Cerf 1995).

Method	Surface	Advantages	Disadvantages
Agar contact method	Smooth and flat or slightly bent	Simple and easy portable.	Quantitates low cell numbers only. The colony forming units may be underestimated due to clusters of cells. Variable reproducibility. The proportion of microbes detached is unknown. The agar and incubation conditions are selective, and the proportion of injured and non-culturable cells is unknown.
Agar flooding method	Internal surfaces of equipment (tubing, valves, pumps etc.)	*In situ* growth is monitored.	The cfu may be underestimated due to clusters of cells. The agar and incubation conditions are selective, and the proportion of injured and non-culturable cells is unknown.
Rinse solution method	Internal surfaces of containers, tanks and pipelines	Large areas can be sampled. Different tests can be performed.	The proportion of microbes detached is unknown. When plate count is applied, the same disadvantages as for the agar methods are valid.
Swab or sponge method	Any surface, including irregular or confined spaces	Versatile. Different tests can be performed. Quantitates high cell numbers.	Variable reproducibility. The proportion of microbes detached is unknown. When plate count is applied, the same disadvantages as for the agar methods are valid.

Cultivation methods have been used for microbiological analysis for about a century and they rely on specific microbiological media to isolate and enumerate viable bacteria, yeasts and moulds. If the right medium and cultivation conditions are chosen, the method is sensitive (theoretically one single cell can be detected from the sample) and gives both qualitative and quantitative information. A further advantage is that a sample can be simultaneously tested for the presence of various microorganisms simply by including several types of selective media in the analysis. However, biofilms in industrial environments are subjected to various stresses such as starvation, chemicals, heat, cold and desiccation, which injure the cells and may render them non-culturable. The proportion of culturable cells in industrial food processing premises is unknown, but in most natural environments only a small percentage of the living microbial population consists of culturable cells (Carpentier and Cerf 1993).

Alternative microbiological detection methods based on different direct or indirect measurement principles are continuously being developed for the quality control of foods and drinks. Most of these methods were originally intended for the detection of food pathogens before being applied to beer and other beverages (Table 7). Unfortunately, many of these new 'rapid' techniques need a pre-enrichment step to increase the sensitivity of the method. Thus they are still dependent on cultivation. Another obstacle may be interfering background in the samples, which makes extensive sample pre-treatment necessary (de Boer and Beumer 1998, Storgårds *et al.* 1998). However, the PCR methods developed are very promising and will probably soon be applied in the breweries (DiMichele and Lewis 1993, Stewart and Dowhanick 1996, Sami *et al.* 1997, Satokari *et al.* 1997, 1998, Vogeser and Geiger 1998, Juvonen *et al.* 1999). The ATP bioluminescence method is already in use in many breweries both in hygiene monitoring and in product quality control.

There is a range of chemical methods available for assessing swab and final rinse samples, such as specific tests for detecting detergent or disinfectant residues, beer residues or microbial residues (Hammond 1996). The ATP bioluminescence system can be used to monitor total ATP derived from both microbes and soil, or only microbial ATP. Generally, bacteria contain about one femtogram (1 fg = 10^{-15} g) ATP per cell. The range of variation is reported to be between 0.1 and 5.5 fg per cell (Stanley 1989). Yeast cells have about 10–100 times more ATP than bacterial cells. The ATP concentration varies through the

microbial growth cycle and is also dependent on growth conditions (Stanley 1989). Under practical conditions the sensitivity is about 1000fg (10^{-12} g), which corresponds to about 1000 bacterial cells or 10 yeast cells (Stanley 1989, Hammond 1996).

Table 7. Microbiological detection methods in process and hygiene control of brewery applications (Storgårds et al. *1998).*

Method	Principle	Applications	Detection limits
Cultivation methods (traditional method)	Cultivation in solid or liquid media, incubation for 1 to several days	Process and product samples Hygiene control	Theoretically 1 cfu [1)] per sample
Direct epifluorescence filter technique (DEFT)	Fluorescence staining of cells, direct microscopy	Process and product samples	200–250 yeast cells/membrane ~1000 bacterial cells/membrane
Microcolony method	Fluorescence staining of microcolonies, direct microscopy	Process and product samples	1–5 cfu/membrane 1 cfu/membrane
ATP bioluminescence	Detection of total or microbial ATP	Process and product samples Hygiene control	Bacteria:~1000 cfu/sample Yeasts: 1–20 cfu/sample Wort or beer: <1 µl
Direct impedimetry	Detection of electrical changes in the growth medium	Process and product samples Hygiene control	Pitching yeast: 100 bacterial cfu/ml Rinse water: 20 cfu/ 100 ml
Polymerase chain reaction (PCR)	Detection of microbial nucleic acids (DNA or RNA)	Process and product samples	Beer: 1 cfu/ 250 ml 20 cfu/ml Pitching yeast: 1 cfu/ 10^8 yeast cells
Flow cytometry	Detection and sorting of stained cells moving in a fluid stream	Process and product samples	Soft drink: $50 – 3 \cdot 10^4$ yeast cells/ml

1) cfu; colony forming units

The concentration of process or product samples has always been a crucial step in the detection of very low numbers of contaminants in beer. Filtration of beer for the recovery of microorganisms can be improved by increasing the temperature (to 30°C) and by the use of top pressure. Filtration did not have a major effect on cellular ATP contents of *L. brevis* or *S. cerevisiae* even when using top pressure up to 1.7 bar (Hammond *et al.* 1998). A bypass-membrane filter device was developed which makes it possible to increase the sample volume up to 40 fold (Back and Pöschl 1998). In this application, the beer is continuously pumped from the product line over a bypass line and filtered through a membrane of suitable pore size (0.2–0.65 µm). After filtration, the beer is led back to the main product line. The device is recommended to be installed after the filter or flash pasteuriser and/or before the filling department. The membrane is subsequently analysed by cultivation in appropriate broth or on agar, or alternatively analysed after 1 day of enrichment by the PCR method (Back and Pöschl 1998), or assayed by the ATP bioluminescence technique (Hammond *et al.* 1998).

Chemical characterisation of spoilage processes can be valuable in trouble shooting, i.e. establishing the causes of spoilage (Dainty 1996). *Pectinatus* spp. can be identified based on large quantities of propionic acid and hydrogen sulphide in beer and correspondingly, *M. cerevisiae* based on butyric, valeric and caproic acids in beer (Haikara 1992a). Chemical analysis of metabolised products is especially useful in the case of older samples in which the bacteria are dead or non-culturable.

3. Aims of the study

The objective of brewery process hygiene is to ensure product quality and minimise contamination risks. The optimisation of cleaning procedures is important to improve economic efficiency and to reduce damage to the environment. Rapid and reliable methods should be used for hygiene control.

The principal aim of the present study was to provide solutions to some microbiological problems arising during beer production and dispensing. Another important issue was to broaden understanding of the biofilm phenomen in the brewing process, eventually resulting in improved preventive measures and control methods.

The specific aims were

- to investigate the cleanability and recontamination rate of beer dispense systems and to evaluate the applicability of the ATP bioluminescence method for hygiene monitoring of the dispensing equipment (I)
- to determine the biofilm formation ability of microbial contaminants isolated from beer production and dispensing (II)
- to examine the susceptibility to biofilm formation of process surface materials and of immobilised yeast reactors used in beer production (II, III, IV)
- to study the effects of ageing on the cleanability of materials used in gaskets (IV)
- to compare methods used for biofilm detection, with particular reference to hygiene assessment (I, II, III, IV, V)
- to propose a reliable detection method for *L. lindneri* to be used in breweries and to identify and characterise new isolates of this species (VI).

4. Materials and methods

A brief summary of the materials and methods used is presented here. Details are given in the original papers I – VI.

4.1 Microorganisms

The microorganims used in the study were obtained from the VTT Culture Collection (Suihko 1999) and they were originally isolated from brewery or beer dispensing samples, with the exception of *Bacillus thuringiensis* (III, IV), *Lactobacillus delbrueckii* (VI), *L. fructivorans* (VI) and *Pseudomonas fragi* (III, IV). The bacterial species are listed in Table 8 and the yeast species in Table 9. The microorganisms were used as pure cultures (II), or as mixed cultures of *B. thuringiensis* and *P. fragi* (III, IV), of *Enterobacter* sp. and *P. damnosus* (III, IV), or of *Enterobacter* sp., *L. lindneri* and *Dekkera anomala* (V).

The microorganisms were cultivated according to standard laboratory practices on media recommended by Suihko (1994, 1999). *Lactobacillus* spp., *Megasphaera* sp. and *Pectinatus* spp. were incubated in an anaerobic work station containing a gas mixture of 80% nitrogen, 10% carbon dioxide and 10% hydrogen, *Pediococcus* spp. in carbon dioxide atmosphere (Merck Anaerocult® C) and the other species in aerobic conditions at 25–30°C depending on the species.

Table 8. Bacterial strains used in the study.

Species	VTT code	Application
Acetobacter aceti [1)]	E-82044	Attachment and biofilm formation (II)
Acetobacter pastorianus	E-74002	Attachment and biofilm formation (II)
Bacillus thuringiensis	E-86245^{T}	Biofilm formation and removal (III, IV)
Brevibacillus parabrevi [2)]	E-83171	Attachment and biofilm formation (II)
Clostridium acetobutylicum	E-93498	Attachment and biofilm formation (II)
Enterobacter cloacae [3)]	E-86247	Attachment and biofilm formation (II)
Enterobacter sp. [4)]	E-86263	Biofilm formation and removal (III, IV), hygiene monitoring methods (V)
Enterococcus faecium	E-90381	Attachment and biofilm formation (II)
Gluconobacter oxydans	E-89365^{T}	Attachment and biofilm formation (II)
Lactobacillus brevis	E-88338	Attachment and biofilm formation (II),
	E-89348	detection and identification (VI)
	E-91457	
	E-91458^{T}	
Lactobacillus delbrueckii subsp. *delbrueckii*	E-79097^{T}	Detection and identification (VI)
L. delbrueckii subsp. *bulgaricus*	E-96662^{T}	Detection and identification (VI)
Lactobacillus fructivorans	E-91473^{T}	Detection and identification (VI)
Lactobacillus lindneri	E-92006	Attachment and biofilm formation (II),
	E-92007	hygiene monitoring methods (V),
	E-82166	detection and identification (VI)
	E-89362	
	E-90380	
	E-91454	
	E-91460^{T}	
	E-94546	
	E-95588	
	E-95589	
Lactobacillus paracasei	E-90377	Attachment and biofilm formation (II)
Lactobacillus sp.	E-88324	Attachment and biofilm formation (II)
Megasphaera cerevisiae	E-84195	Attachment and biofilm formation (II)
Obesumbacterium proteus	E-78073^{T}	Attachment and biofilm formation (II)
Pectinatus cerevisiiphilus	E-88329	Attachment and biofilm formation (II)
Pectinatus frisingensis	E-79100^{T}	Attachment and biofilm formation (II)
	E-91471	
Pediococcus damnosus [5)]	E-76067	Biofilm formation and removal (III,
	E-88309	IV), attachment and biofilm formation
	E-93441	(II), hygiene monitoring methods (V)
Pseudomonas fragi	E-84200^{T}	Biofilm formation and removal (III, IV)

At the time of the studies known as (Suihko 1994):
1) Corynebacterium sp., 2) *Bacillus* sp., 3) *Enterobacter intermedius,* 4) *Pantoea agglomerans,* 5) *Pediococcus inopinatus* (E-76067), T; type strain of the species

Table 9. Yeast strains used in the study.

Species	VTT code	Application
Dekkera anomala	C-75001[T] C-91183	Attachment and biofilm formation (II), hygiene monitoring methods (V)
Issatchenkia orientalis [1)]	C-89178	Attachment and biofilm formation (II)
Pichia anomala	C-94191	Attachment and biofilm formation (II)
Pichia membranaefaciens	C-86170 C-94192	Attachment and biofilm formation (II)
Rhodotorula mucilaginosa	C-89179	Attachment and biofilm formation (II)
Saccharomyces cerevisiae (ex. *diastaticus*)	C-68059	Attachment and biofilm formation (II)

1) At the time of the studies known as *Candida crusei* (Suihko 1994). T; type strain of the species.

4.2 Attachment and biofilm formation

The surfaces used for attachment or biofilm formation in semistatic conditions were stainless steel (AISI 304, 2B) (II, III, IV, V), EPDM (ethylene propylene diene monomer rubber) (III, IV), NBR (nitrile butyl rubber, also called Buna-N) (III, IV), Viton (fluoroelastomer) (III, IV) and PTFE (polytetrafluoroethylene, Teflon) (III, IV). New (unused) materials were used (II, III, V) as well as materials exposed to prolonged alkali-acid treatments simulating repeated CIP cycles (IV). In addition, materials aged in industrial processes were examined (IV). In dynamic flow conditions simulating secondary fermentation immobilised yeast reactors, DEAE-cellulose and ceramic glass beads were tested for biofilm formation of *L. lindneri* (II).

The media used in biofilm formation experiments were fermented heat-treated (90°C, 7 min) beer (II), autoclaved unfiltered beer from maturation (II), wort sucrose broth (II, V), or a rich nutrient broth described by Wirtanen and Mattila-Sandholm (1993) (III, IV). In semistatic conditions, the biofilm was allowed to develop for 2–10 days at 25°C with moderate agitation (60–80 rpm) and the medium was replaced every second day with fresh sterile medium in order to

leave only the sessile organisms and to provide fresh nutrients. Obligate anaerobic bacteria were studied in an anaerobic workstation without agitation. In dynamic flow conditions, secondary fermentation with immobilised yeast was simulated.

In the preliminary biofilm studies (II), the amount of viable cells attached, metabolic activity estimated by the ATP bioluminescence method and the area covered by biofilm were rated as presented in Table 10.

Table 10. Rates of viable cells attached, metabolic activity as estimated by ATP bioluminescence and biofilm coverage in preliminary biofilm studies (II).

Rating	Viable cells attached, cfu / test coupon	Metabolic activity, rlu / test coupon	Biofilm formed, % of area covered
+	10^3–10^4	100–500	1–5
++	10^4–10^5	500–5000	5–30
+++	>10^5	>5000	>30

4.3 Cleaning trials

4.3.1 Cleaning-in-place (CIP)

An experimental test rig (Tetra Pak Oy, Finland) constructed according to European Hygienic Equipment Design Group norms (EHEDG 1993a) was used in the simulation of closed cleaning procedures (Fig. 2/III). The volume of the closed system was 30 l and the diameter of the pipes was 51 mm in the transfer section and 63 mm in the test section. The test coupons were placed in a rack in the vertical part of the system. The temperatures used in the experiments were 10–70°C and the flow rates 0.8 and 2.0 m/s.

4.3.2 Foam cleaning

A pilot-scale multipressure cleaner was used in the foam cleaning experiments (V). The chemicals and their concentration, the pressure, the flow rate and angle and the rinsing temperature are variables that can be altered in foam cleaning experiments.

4.4 Methods used for detachment of microorganisms from surfaces

Swabbing of surfaces was used in combination with the plate count method (I, II, III, IV, V) and in combination with the ATP bioluminescence method (I). Rinse water analysis was used in combination with the cultivation method and with the ATP method in evaluation of cleaning results of beer dispensing systems (I).

Surface-active agents were used in sampling solutions in combination with Hygicult® TPC contact agar slides (Orion Diagnostica, Finland) for hygiene assessment in a brewery (V). The sampling solutions consisted of detergents approved for use in the food industry, a viscous substance to aid in sampling from non-horizontal surfaces and orange colour to visualise the moistened points.

Ultrasonication was used in detachment of biofilm from stainless steel surfaces (V). An ultrasound pen (U 200 H Ikasonic, Germany) was used in combination with a prototype sampling chamber developed at VTT Electronics (Finland). The sampling area of the chamber was 32 mm^2 and the ultrasound pen was operated at 100 or 150 W for 30 seconds.

4.5 Detection methods

4.5.1 Cultivation methods

The plate count method was used for enumeration of viable, culturable bacteria using the spread plate technique (I–VI) or the membrane filter technique (I).

4.5.2 ATP bioluminescence

The ATP bioluminescence technique was used to analyse swab and rinse water samples from dispensing systems (I), to estimate the metabolic activity of surface attached cells (II, IV) and to estimate organic residues after CIP or after foam cleaning (IV, V). The ATP was either measured from the sample

suspension by a portable luminometer (Bio-Orbit 1253, Finland) (I), or directly from the surface by a BioProbe luminometer (Hughes Whitlock Ltd., UK) (II, IV, V).

4.5.3 Protein detection

Protein detection based on colour reactions was tested in laboratory scale using Swab'N'Check (Konica, Japan) and Check Pro (DiverseyLever, UK) kits (V). Samples of microbial suspensions or wort solutions were dried onto stainless steel coupons and analysed according to the manufacturer's instructions.

4.5.4 Epifluorescence microscopy

Epifluorescence microscopy and image analysis was used to estimate the area covered by biofilm on test surfaces stained with acridine orange (II, III, IV, V). Image analysis was performed by the CUE-2 planomorphometry program (Galai Production Ltd., Israel) in a microcomputer system connected to an Olympus BH-2 fluorescence microscope (Japan). Fifty fields of each sample were analysed in order to obtain a mean value.

4.5.5 Impedance measurement

Bacterial growth on surfaces prior to and after CIP was monitored by impedance measurements (III, IV, V). The test surfaces were placed in the measuring cells of a BacTrac 4100 instrument and changes in capacitance (E-value) were monitored at 30°C for 48 h.

4.5.6 Scanning electron microscopy

Samples for scanning electron microscopy were fixed in 2% glutaraldehyde at 4°C for 1–2 hours, flushed in phosphate buffer and dehydrated in an alcohol series. The samples were dried in air, fixed on brass stubs, evaporated with carbon in a TB500 Temcarb carbon coater or coated with gold in a Jeol JFC-

1100E ion sputter or both. The samples were then examined and photographed in a Jeol JSM-820 scanning electron microscope at VTT Building Technology.

4.6 Identification and characterisation methods

4.6.1 API strips

Carbohydrate fermentation tests were carried out using API 50 CHL strips (BioMérieux S.A., France) (I, VI). Incubations were carried out at 30°C in anaerobic conditions for up to 18 days until acid formation was detected. Identifications were performed by comparing the fermentation profiles with the APILAB Plus database, version 4.0.

4.6.2 SDS-PAGE

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of whole-cell protein extracts and subsequent identification of strains was carried out at the BCCM / LMG Culture Collection as described by Pot *et al.* (1994) (VI). The protein patterns were scanned by laser densitometry, normalised and compared by the Pearson product moment correlation coefficient (*r*). Grouping of patterns (cluster analysis) was performed by the unweighted-pair group method (UPGMA) using the software package GelCompar (Pot *et al.* 1994) and represented as a dendrogram. The protein patterns were also compared with a reference database of SDS-PAGE protein patterns of lactic acid bacteria (Pot and Janssens 1993, Pot *et al.* 1994).

4.6.3 Ribotyping

Ribotyping was carried out using the automated RiboPrinter™ Microbial Characterisation System (Qualicon, USA) at CCFRA (VI). The software compared ribogroups of single patterns or composite patterns of the strains with ribogroups of reference strains. Identification was performed by comparing the ribopatterns of the unknown strains with relevant reference strains.

5. Results and discussion

5.1 Biofilm formation in beer production and dispense (I, II, III, IV)

In preliminary experiments, the sessile growth of microorganisms originally isolated in connection with microbial contaminations in breweries was examined (II). The assumption was that the microbiological problems experienced could have been a consequence of biofilm formation in some part of the process equipment. Stainless steel was used as substratum, as this is the most common material in the process equipment. Autoclaved beer from the maturation stage was used as medium because of its nutritious yeast content and because surfaces are frequently exposed to this medium for prolonged times in beer production. Yeast strains were also tested for biofilm formation in wort sucrose broth, which had already been shown to enhance biofilm formation by some brewery organisms (Storgårds *et al.* 1996). In cleanability studies, biofilm was grown from a mixed culture of *Enterobacter* sp. and *P. damnosus* on stainless steel and on different gasket materials in a rich nutrient broth (III, IV), and from a mixed culture of *L. lindneri*, *Enterobacter* sp. and *D. anomala* on stainless steel in wort sucrose broth (VI).

In the brewery environment, biofilms could be produced in either static or dynamic flow conditions. In this study, biofilm was allowed to form in semistatic conditions by replacing the medium every second day. The biofilm formation ability of a *L. lindneri* strain was also studied in dynamic flow conditions in secondary fermentation immobilised yeast reactors with cellulose or glass beads as carrier materials (II). In semistatic conditions the incubation time was 2, 4 or 10 days to cover different situations in the production. However, a relatively high temperature (25°C) compared to actual process temperatures was used in order to speed up the development. In the immobilised yeast reactors a lower temperature (15°C) and correspondingly a longer incubation time (8 weeks) was used.

All the yeasts tested (8 strains) attached to stainless steel, producing some amounts of biofilm in unfiltered beer as observed by epifluorescence microscopy and image analysis (Table 3/II). The area covered by biofilm after 10 days was 1.7–87.7% depending on the strain. A *D. anomala* strain and a *S. cerevisiae* (ex.

diastaticus) strain produced significant amounts of biofilm already in 2 days, covering 62.6 and 31.3% of the area examined, respectively. However, biofilm production by the yeast strains was generally stronger and also more rapid in wort sucrose broth than in unfiltered beer. The area covered ranged from 1.8 to 88.4% in 2 days and from 3.6 to 100% in 10 days in wort sucrose broth.

Biofilm production on stainless steel by the bacterial brewery contaminants in unfiltered beer was much less intense than by the yeast contaminants (Table 2/II). Of the 20 bacterial strains tested, 6 strains produced some amounts of biofilm in the test conditions used, but the area covered in 10 days in these cases ranged only from 4 to 15%. Additionally, 5 strains attached to the stainless steel surface without signs of biofilm formation (less than 0.5% covered). The bacteria found to produce biofilm were acetic acid bacteria belonging to the species *Acetobacter aceti* (known as *Corynebacterium* sp. at the time of the experiments, but recently reidentified at DSMZ), *A. pastorianus* and *G. oxydans*, lactic acid bacteria belonging to the species *L. lindneri* and an *Enterobacter cloacae* strain (previously identified as *E. intermedius*). *L. lindneri* was found to grow on yeast and carrier materials in immobilised yeast reactors simulating continuous secondary fermentation, with significant production of lactic acid (Fig. 3/II).

Biofilm production was found to be strain dependent rather than species dependent. A *D. anomala* strain isolated from lager beer was found to be a strong biofilm producer in the test conditions resembling the lager beer process, whereas the other *D. anomala* strain tested, originally isolated from stout, produced much less biofilm (Table 3/II). Similarly, the *L. brevis* strain isolated from a draught beer sample did not attach to stainless steel (Table 2/II), whereas the *L. brevis* strain previously tested (Storgårds *et al.* 1996) was found to be a strong biofilm producer. Of the three *L. lindneri* strains tested, the two isolated from bottled or draught beer attached readily to stainless steel and produced biofilm (Table 2/II). However, the *L. lindneri* strain attaching to the immobilised yeast reactors showed only poor attachment and no biofilm production on stainless steel (Fig. 3, Table 2/II).

Biofilm production in dispense systems was not directly identified in this study. However, poor hygiene of the dispensing equipment was clearly shown to be the cause of microbiological contamination of draught beer (I). Aerobic bacteria that

do not normally grow in beer were encountered in high amounts in draught beer samples from the tap. Presumably these bacteria were able to survive and multiply in the biofilm accumulating in the dispensing devices. In addition, beer spoilage organisms such as yeasts and lactic acid bacteria, among them *L. brevis*, were frequently isolated from beer samples from the tap (I). The dispensing system was in many cases recontaminated within one week after cleaning (Tables 5, 6, Fig. 3/I). In some cases the level of contamination was already high the day after cleaning, indicating that the biofouling was not properly removed in the first place. Later, true biofilm formation was repeatedly observed on working dispense lines (Fig. 3).

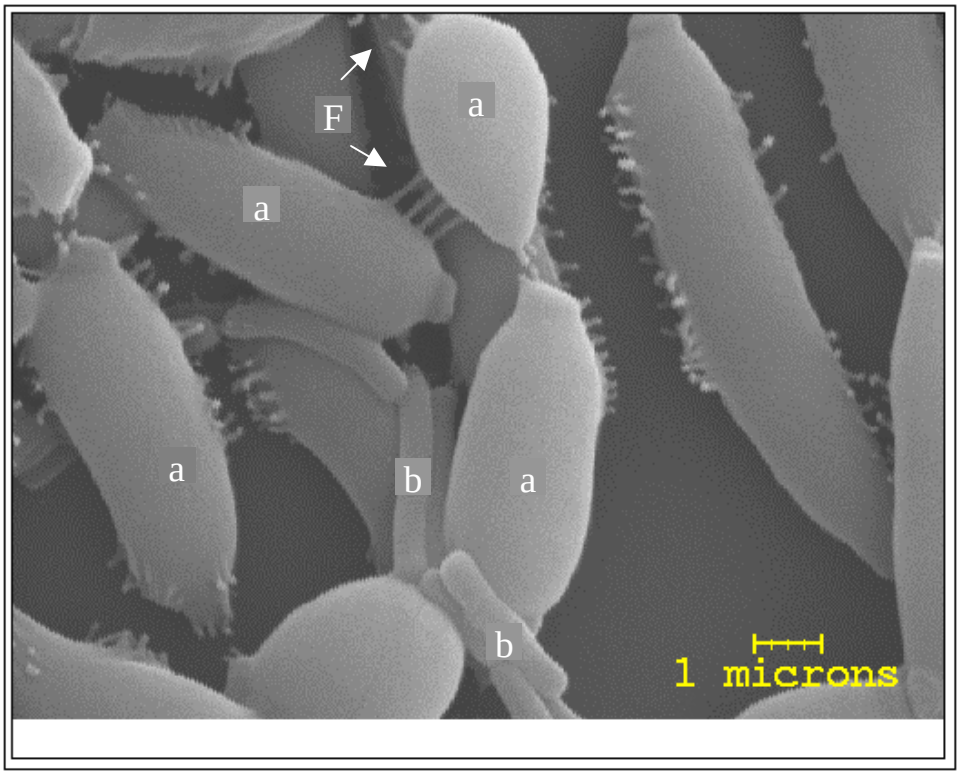


Fig. 3. Scanning electron micrograph of a biofilm on the inner surface of a dispensing line. Legend: a → Yeast cells. Note protruding fimbriae (F). b → Bacteria.

In the brewery environment, reported biofilm findings are sparse. Banner (1994) isolated a wide variety of microorganisms associated with biofilms in the brewery filling area, including genera which may have a detrimental impact on the product, such as *Lactobacillus* and *Saccharomyces*. Furthermore, biofilms have repeatedly been observed in beer dispensing system lines (Harper 1981, Casson 1985, Thomas and Whitham 1997). In these cases, both beer spoilage organisms and non-spoilage organisms have been associated with the biofilms. Thomas and Whitham (1997) found *Pediococcus* spp. and acetic acid bacteria as well as brewing and wild yeasts adhered to the dispensing lines.

According to Zottola and Sasahara (1994) classical biofilms, which require several days to several weeks to develop, have not been identified and reported in the food processing industry due to the prevailing conditions that seldom allow the growth of microorganisms for this length of time. By contrast, the majority of experimental data generated represents the attachment of bacteria to food contact surfaces under simulated conditions (Kumar and Anand 1998). However, Holah *et al.* (1989) and later Gibson *et al.* (1995) introduced stainless steel coupons into various food processing environments to investigate biofilm formation under real conditions. Attached microorganisms were detected by epifluorescence microscopy in the range of 10^3 to $>10^7$ cells/cm^2 at sites adjacent to the product flow in plants processing baked beans, egg glaze, fish and butter (Holah *et al.* 1989). On most of the surfaces studied by these authors, only individual organisms were detected (71.6%), microcolonies being detected on 20.8% of the coupons (Holah and Gibson 1999). Extensive biofilms were found on 6.6% of the coupons that were exposed for 1.5 to 120 hours near to, or as part of, food contact surfaces and in these cases biofilm covered 3.6–98.4% of the area.

Czechowski and Banner (1992) showed that *L. brevis*, *E. agglomerans* and *Acetobacter* sp. isolated from brewery environments did form biofilm on stainless steel, Buna-N and Teflon. In previous studies, Storgårds *et al.* (1996) reported bottom fermenting brewer's yeast and brewery isolates of *L. brevis*, *Enterobacter* sp. and *Acetobacter* sp. to produce biofilm on stainless steel in autoclaved wort (11% w/w), in wort sucrose broth and in a rich nutrient broth. Biofilm formation of the studied strains was most rapid in wort sucrose broth. The area covered by biofilm of *Enterobacter* sp. and *Acetobacter* sp. in 2 days was 82–96% of the surface as analysed by epifluorescence microscopy and

image analysis. Biofilm coverage produced in wort sucrose broth by *L. brevis* was >90% in 6 days and by brewer's yeast 60% in 12 days.

The adhesive properties of bacteria depend on their genetic capabilities and on their metabolic state. Laboratory culture can result in significant changes in the adhesiveness of natural isolates with time – possibly due to selection of less adhesive strains by repeated transfer in liquid suspensions (Fletcher 1992b). In this study, some of the tested strains had been isolated from brewery samples more than 20 years ago and they still had not lost their ability to grow as biofilms. Some of the strains that did not produce biofilm in the chosen test conditions might have been able to grow as biofilm in different test conditions or in the same conditions at the time of initial isolation. The results show that the environmental conditions strongly affect biofilm production and that there appear to be strains within a single species with abilities to attach to surfaces at different stages in the process. When choosing suitable strains for cleaning and disinfection testing, attention should be paid to biofilm producing properties in the particular test conditions.

5.2 Significance of surface hygiene

There is considerable evidence that foodborne pathogens and spoilage organisms attach to surfaces such as stainless steel, aluminium, glass, rubber materials, Teflon and nylon materials typically found in food processing environments (Holah *et al.* 1988, Mafu *et al.* 1990, Mattila *et al.* 1990, Czechowski and Banner 1992, Banner 1994, Hood and Zottola 1995, Wong and Cerf 1995, Blackmann and Frank 1996, Hood and Zottola 1997, Chumkhunthod *et al.* 1998). Development of a biofilm is a result of both adherence and subsequent growth (Blackman and Frank 1996). While true biofilms have only seldom been reported on food contact surfaces, individual adherent microorganisms may be as significant as well developed biofilms. The biotransfer potential is defined as the ability of microorganisms associated with an equipment surface to cause contamination of the process (Hood and Zottola 1995). The biotransfer potential is related to the strength of microbial attachment to surfaces.

5.2.1 Susceptibility of surfaces to biofilm formation (III, IV)

Process materials such as stainless steel, EPDM (ethylene propylene diene monomer rubber), NBR (nitrile butyl rubber), Viton (a fluoroelastomer) and PTFE (Teflon) were found to differ in their susceptibilities to biofilm formation (III, IV). Biofilm formed readily on EPDM, Viton and PTFE (Fig. 3/III). NBR was found to inhibit biofilm development when new, but the material became significantly more susceptible with increasing age, simultaneously with reduced hydrophobicity (Fig. 2/IV). The bacteria involved in the biofilm influenced the amount of biofilm formed. The inhibitory effect of NBR was more pronounced on a mixed culture biofilm of *Enterobacter* sp. and *P. damnosus* than on a mixed culture of *B. thuringiensis* and *P. fragi* (Fig. 3/III). On the other hand, especially PTFE but also Viton was less susceptible to biofilm formation by *B. thuringiensis* and *P. fragi* than by *Enterobacter* sp. and *P. damnosus* (Fig. 3a/III).

In immobilized systems, the carrier materials also differ in their susceptibility to attachment of contaminating organisms. In immobilized yeast reactors used for secondary fermentation, the attachment of *L. lindneri* to DEAE cellulose carrier material was faster than to porous glass beads, and consequently the lactic acid concentration in the beer outflow exceeded the taste threshold (0.4 g l^{-1}) 11 days sooner in the reactor with cellulose than in the reactor with glass beads (Fig. 4/II). In the case of the cellulose carrier, yeast cells attach only to the surface of the material, whereas in the porous glass beads yeast cells are partly bound to the surface and partly inside the beads (Kronlöf 1994). Thus the microenvironment inside the beads could be less favourable for contaminating bacteria in competition with yeast cells. Another factor influencing attachment could be the positively charged diethylaminoethyl groups of the DEAE cellulose, which probably have a strong affinity for negatively charged cell wall components (Kronlöf 1994).

Complex media have been shown to support extensive biofilm formation, whereas a chemically defined minimal medium that supported planktonic growth did not support biofilm formation (Blackman and Frank 1996). In this study, the test surfaces were soiled with beer from the prefilter process before biofilm development and removal experiments (III, IV). In experiments simulating dairy conditions, milk substances were used instead of beer. Soiling of the surfaces

was found to promote biofilm formation on PTFE and on EPDM rubber by a mixed culture of *Enterobacter* sp. and *P. damnosus*, and on PTFE, NBR and Viton by a mixed culture of *B. thuringiensis* and *P. fragi* (Fig. 3/III).

Mixed population biofilms have been observed to be thicker and stronger than monospecies biofilms (Skillman *et al.* 1997). In this study, the mixed culture of *Enterobacter* sp. and *P. damnosus* produced biofilm covering 93–100% of the examined area on clean and presoiled surfaces of stainless steel and PTFE in 4–5 days at 25°C (Fig. 3b/III). However, the mixed culture of *B. thuringiensis* and *P. fragi* only produced biofilm covering 5–20% of the area on PTFE in 5 days, depending on whether the surface had been presoiled or not (Fig. 3a/III). Blackman and Frank (1996) studied a range of hydrophobic and hydrophilic surfaces including stainless steel, Teflon (PTFE), nylon and polyester floor sealant. They found that the materials supported biofilm formation by *Listeria monocytogenes* in 1–30 days depending on medium and temperature. The biofilm coverage by the pure culture was 33% on stainless steel and 22% on Teflon after 7 days at 21°C. Thus the susceptibility of a surface to biofilm formation by different microorganisms varies greatly. When the hygienic properties of process equipment are evaluated, the choice of suitable test microorganisms is important and working solely with pure cultures may result in false conclusions.

The substratum plays a major role in biofilm processes during the early stages of biofilm accumulation and may influence the rate of cell accumulation as well as the initial cell population distribution (Characklis and Marshall 1990). Physicochemical characteristics of the substratum that influence adhesion are electrostatic charge, surface free energy and hydrophobicity (Fletcher 1992b). High surface energy materials, which are hydrophilic, frequently negatively charged and usually inorganic materials such as glass, metals, or minerals readily adsorb dissolved solutes and atmospheric contaminants. Low energy materials, which are relatively hydrophobic and low in surface charge, generally organic polymers such as plastics, are not contaminated as quickly as high-energy surfaces (Fletcher 1992b). The adsorption of certain proteins to surfaces as well as the pH and temperature of the contact surface also affect the degree of adhesion of microorganisms (Kumar and Anand 1998).

Rubber gaskets or sealings have been found to accumulate biofilm, and the number of bacteria recovered from these gaskets increases with the length of time the gaskets are in use (Czechowski 1990, Mattila-Sandholm and Wirtanen 1992, Wong and Cerf 1995). However, gasket materials are in some cases less susceptible to biofilm formation than stainless steel. NBR has been found to inhibit the growth of a range of bacteria (Ronner and Wong 1993, Wirtanen 1995, Chumkhunthod *et al.* 1998), and EPDM has also been found to be bacteriostatic towards some bacteria (Ronner and Wong 1993). There is no strong evidence that hydrophobicity should predict microbial adherence to solid surfaces, but it may be one of many factors involved in the initial adherence (Hood and Zottola 1995). A general trend of decreasing colonisation density with increase in substratum hydrophobicity was observed for *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* (Eginton *et al.* 1995b).

The susceptibility of process surface materials to biofilm formation is dependent on many factors. The medium which produced the highest observed level of adherent cells on stainless steel was found to vary for different food-borne microorganisms (Hood and Zottola 1997). Thus the nature of the processed products probably has a major influence on biofilm accumulation in food and beverage production environments. Experiments related to the attachment of microorganisms in food processing environments should be carried out under conditions similar to those existing in such environments (Kumar and Anand 1998).

5.2.2 Cleanability (III, IV, V)

Hydrophobic properties of materials are probably also involved in biofilm removal and detachment of cells from surfaces. PTFE (Teflon) was the most hydrophobic material tested in this study, followed by EPDM, Viton and NBR (Table 5/IV). Correspondingly, PTFE was also the most easily cleanable of the gasket materials studied, especially when hot alkaline washes were used (Figs. 5a/III, 6/IV). NBR was easily cleanable when new, but cleanability was reduced with increasing age and decreasing hydrophobicity (Figs. 5, 6/IV). Thus decreased cleanability may be connected with decreased hydrophobicity partly also as a result of increased colonisation, as discussed previously in 5.2.1. Eginton *et al.* (1995b) found that the ease of removal of *S. epidermidis* increased

as substratum hydrophobicity increased. However, inert hydrophobic particles such as bacterial spores have been found to attach more firmly to hydrophobic materials such as PTFE, resulting in reduced cleanability (Rönner *et al.* 1990).

Physical deterioration was observed both on experimentally aged rubber materials and on materials aged in industrial processes (IV). Microscopical examination revealed marked changes in the surface structure of EPDM, NBR and Viton (Fig. 1/IV), but PTFE was not affected in the same way. The cleanability of the deteriorated materials was reduced as indicated by residual biofilm, viable bacteria and increased amounts of ATP found on the surfaces after CIP (Figs. 3, 4, 5, 6/IV). Viton and NBR appeared to deteriorate faster than EPDM, and PTFE was least affected by ageing. The EPDM gaskets that had been installed in a brewery process for 3–4 years also showed reduced cleanability (Fig. 7/IV). The frequency of use of the valve as well as the temperature and chemical nature of the matrix apparently affected the changes in cleanability of the gaskets (Table 6/IV). However, even the cleanability of gaskets that had been installed in the same valve varied. This could have been due to different gaskets being differently exposed to pressure in the valve.

The cleanability of a particular surface is dependent on the size and type of surface irregularity. Pores, crevices and jagged edges retain bacteria after cleaning (Taylor and Holah 1996). Abraded domestic sink materials gave rise to surface damage, which affected the cleanability compared with the non-abraded material (Holah and Thorpe 1990). Differences in cleanability can also be due to chemical surface composition changes induced by cleaning detergent actions (Leclercq-Perlat and Lalande 1994, Leclercq-Perlat *et al.* 1994).

The surface topography of the aged rubber materials was not measured due to the highly irregular and bent surface structure, making such measurements unreliable. However, visual assessment and measurement of surface roughness alone is not sufficient to indicate the probable cleanability performance of materials. The surface topography can change significantly without influencing the parameters commonly used for describing surface topography (R_a, R_z, I_0). Thus, surfaces with similar R_a values may have different topographies and exhibit different extents of bacterial adhesion (Eginton *et al.* 1995b, Taylor and Holah 1996).

The temperature of the detergent solutions had a clear effect on the cleaning results in this study. Higher amounts of residual biofilm were found on stainless steel and on PTFE after cold CIP compared to hot CIP (Fig. 5/III). Viable bacteria were also more often detected on the test surfaces after cold CIP (Table 4/III). Even a moderate increase in temperature may be decisive. Increasing the temperature from ambient to 40°C improved the efficiency in foam cleaning (Fig. 4/V). An increase of the temperature used in cleaning and/or disinfection has been found to improve the performance of such operations markedly (Czechowski and Banner 1992, Wirtanen *et al.* 1996, Blanchard *et al.* 1998).

The flow velocity was found to be important for the cleaning result in CIP (III). Reduced flow velocity in combination with cold CIP resulted in reduced cleanability as observed by a higher incidence of viable bacteria after cleaning (Table 5/III). The amount of residual biofilm was also higher with lower flow velocity on PTFE and Viton after cold CIP and on EPDM after hot CIP (Fig. 5/III). Mechanical force induced by sufficient flow velocity is essential in the cleaning of pipes (Wirtanen *et al.* 1996), and increasing flow velocity was also found to improve disinfection efficiency (Blanchard *et al.* 1998).

Attached viable cells have been found remaining on surfaces after CIP procedures (Mattila *et al.* 1990, Hood and Zottola 1995). The resistance of microorganisms to cleaning was dependent on the surface to which the organisms were attached, with stainless steel being more easily cleaned and sanitised than polyester, polyester/polyurethane or NBR (Krysinski *et al.* 1992, Ronner and Wong 1993). The strength of attachment to surfaces was independent of the numbers of organisms initially attached but differed between organisms, between different surface materials and changed with the age of the developed biofilm (Eginton *et al.* 1995a). As the rank of order of adhesiveness of test surfaces differs between organisms, this could lead to a selective concentration, with time, of one organism relative to another and to an enrichment of particular organisms on the surface (Eginton *et al.* 1995b).

The current methods used to determine the biocidal activity of cleaning and disinfection operations on biofilms or bacteria attached to surfaces are laborious and hard to standardise due to the difficulty of contaminating and recovering viable organisms from a surface in a repeatable and reproducible way (Wirtanen and Mattila-Sandholm 1992, Bloomfield *et al.* 1994, Foschino *et al.* 1998).

When reporting cleanability results from laboratory experiments conclusions should be made cautiously, because simulating plant conditions is very difficult. In the plant, a milieu of organisms exists in accordance with the nature of the substrate, the frequency and adequacy of cleaning, and endogenous and exogenous sources of microorganisms. Furthermore, the processing plant has a variety of product contact surfaces of various ages (Krysinski *et al.* 1992).

5.3 Detection of biofilms with particular reference to hygiene assessment (I, II, III, IV, V)

Several methods have been used in the study of surface-attached microorganisms and biofilms (Wirtanen *et al.* 1999). However, the problems associated with repeatability and reproducibility in studying microbial growth on surfaces are well known. The threshold of detection of adhering microorganisms can vary according to the enumeration technique employed and some techniques underestimate the number of microorganisms on a surface (Holah *et al.* 1990, Carpentier and Cerf 1993, Boulangé-Petermann 1996). Another difficulty especially in hygiene assessment is associated with sampling. Areas where biofilms mostly grow are those which are the most difficult to rinse, clean and disinfect and are also more difficult to sample, regardless of the method used (Wong and Cerf 1995). Appropriate sampling methods should be chosen depending on whether the purpose is routine hygiene assessment, process development or troubleshooting (Holah 1999).

5.3.1 Sampling methods (I, V)

The swab method was used in hygiene assessment of critical parts of working dispense systems (I). The samples were either analysed at the site or analysed later in series in the laboratory. The same sample could be analysed both by cultivation and by the ATP method, which was an advantage in comparing the two methods. In this application, the technique was found to be sufficiently accurate to detect gross changes in surface hygiene and hence the performance of the cleaning. The swabbing method was also used in combination with plate counting to estimate the number of living bacteria in biofilms before and after

CIP (III, IV). In this application the method was not considered sufficiently reliable, as some of the bacteria could easily remain in the cotton matrix.

Final rinse water samples were analysed to evaluate the cleaning result achieved in working dispense systems (I). In this application the sampling method proved to be quite informative in evaluating the cleaning result immediately by the ATP method. However, when using the method to monitor the efficiency of CIP operations, possible disinfectants used in the final rinse should be neutralised to avoid false negative results (Hammond 1996).

Surface-active agents can be used to improve detachment of microorganisms in sampling. These agents break the biofilm without affecting the survival rate of the microorganisms detached and they can be applied either by spraying onto the surface or by moistening the swab before sampling (Salo *et al.* 1999). In this study, sampling solutions containing non-toxic detergents were used in combination with the agar contact method for hygiene assessment in a brewery bottling hall. Detachment of biofilms or attached bacteria was found to be improved by these surface-active agents without harming the culturability of the detached microorganisms (Fig. 2/V). However, when detergents are used for improved sampling in combination with the ATP bioluminescence method, it should be noted that the detergents may decrease or increase the reaction rate and internal standardisation should therefore be used in the assay (Simpson and Hammond 1991).

Ultrasonication was used to improve detachment of biofilms from surfaces (V). The technique is based on the scrubbing action caused by microscopic bubbles that implode or collapse under the pressure of agitation, producing shock waves which loosen particles and microbes from the surface (Jeng *et al.* 1990). In combination with a surface-active agent, this method detached bacteria and yeast cells from a mixed culture biofilm 10 to 100 times more effectively than conventional swabbing (Fig. 3/V). Ultrasonication has been used successfully in the removal and elimination of biofilms (Zips *et al.* 1990, Mott *et al.* 1998, Pagan *et al.* 1999) and in detaching surface-attached microorganisms for enumeration (Jeng *et al.* 1990, Lindsay and von Holy 1997). A portable ultrasonic apparatus was developed for dislodgement of biofilms from food processing equipment in order to assess the effectiveness of cleaning protocols (Lagsir-Oulahal *et al.* 1999).

Removal of attached bacteria for analysis is a critical step in quantification of microorganisms from surfaces because of strong adherence of biofilms to the surfaces. The strength of adhesion and thus the resistance to removal varies depending on the organisms, the surfaces, the length of time the cells have been attached and the type and amount of associated inorganic deposits and organic adhesives. A number of methods have been applied to remove attached bacteria, ranging from relatively ineffective swabbing to treatment with ion sequestering agents or detergents, to sonication and vortexing. It is impossible to recommend any one procedure and optimum procedures should be determined in individual cases (Fletcher 1992b). At present a method for reliable detachment of microorganisms from process surfaces is still lacking (Wirtanen *et al.* 1999).

Routine hygiene assessment is largely based on contact agar plates or on the swabbing method in conjunction with cultivation or ATP (Hammond 1996). The swab method in combination with cultivation has been shown to be unreliable in the estimation of surface populations below $3 \cdot 10^5$ cfu/cm^2 (Holah *et al.* 1988) and to cause enormous scatter and variation in the results (Wirtanen *et al.* 1995). Furthermore, the swab method does not quantitatively detach microbes growing as biofilms, as was observed by direct microscopy (Storgårds *et al.* 1996). The advantage of the swab method is that it provides good access in confined spaces of process areas. Contact plates can only be used for flat surfaces. Both methods are simple, user friendly and accurate enough to detect any gross changes in surface hygiene over a manufacturing time period. In both methods the area sampled is relatively small compared e.g. to the inner surface of a fermentation tank. Analysis of the final rinse water is one way of obtaining information about larger part of process equipment after cleaning. The major advantage of rinse water sampling is that access to the process area is not required (Hammond 1996). However, the disadvantage with all three methods is that the actual proportion of microbes detached remains unknown.

Although detachment of microorganisms from surfaces can be improved by the methods described above, some residual biofilm on the test surface was detected even after ultrasonication. This supports the idea that *in situ* approaches to study surface-bound microorganisms should be used whenever possible, as they avoid the inaccuracies caused by incomplete cell removal. Such methods are direct microscopy techniques (II, III, IV, V), ATP measurement directly from the

surface of interest (II, IV, V) and impedance measurement from test coupons (III, IV, V). These methods will be discussed in more detail in the next section.

5.3.2 Detection methods (I, II, III, IV, V)

The poor reliability of cultivation methods in estimating the number of microorganisms on surfaces is well known (Holah *et al.* 1988, Carpentier and Cerf 1993, Yu and McFeters 1994, Wirtanen 1995, Storgårds *et al.* 1998). Established culture media often underestimate microbial populations in many systems (Yu and McFeters 1994) and furthermore, a large proportion of the microbial cells are possibly in a non-culturable state caused by environmental stress (Carpentier and Cerf 1993). Additionally, biofilm cells may be aggregated in large clumps after removal from surfaces. These may not be dispersed when preparing dilutions, leading to inaccurate counts of the total number of cells (Hood and Zottola 1995). Finally, most of the biofilm is often composed of extracellular polysaccharides and glycoproteins, which means that enumeration of culturable microorganisms does not reveal the true surface hygiene in process control applications (Storgårds *et al.* 1998). Cultivation methods are also too slow to enable rapid countermeasures in the case of failure in hygiene management. However, the cultivation method is still a widely used enumeration technique and as such it was used as a reference method throughout these studies.

Epifluorescence microscopy was used in combination with image analysis to estimate the proportion of surface covered by biofilm (II, III, IV, V). Both living and dead cells as well as biofilm residues on a surface were detected by epifluorescence microscopy. However, bacterial growth on surfaces is characteristically discontinuous and 'patchy' (Characklis and Marshall 1990), comprising of microcolonies and EPS in addition to single cells. This often resulted in high variability in the microscopic fields of the same test coupons and also in replicate coupons reducing the reliability of image analysis. Acridine orange may bind to some materials including plastics and detritus in samples from natural environments, causing background fluorescence (Fletcher 1992b). Disturbing background fluorescence was observed when analysing biofilms on rubber materials and another complication in microscopic analysis of flexible materials such as rubbers is the difficulty of focusing on the object (III, IV).

Nevertheless, epifluorescence microscopy was the most informative of the methods used here to study microbial growth on surfaces and it is recommended as a reference method in hygiene research whenever possible. An important advantage of direct microscopy techniques is that cells on a surface are studied rather than cells that have been detached by some method.

Microscopic techniques have been extensively used in biofilm studies (Wirtanen *et al.* 1999). Epifluorescence microscopy, utilising fluorescent dyes, has been invaluable for assessing bacterial attachment to surfaces (Fletcher 1992b). Scanning electron microscopy (SEM) has been useful in confirming biofilm formation and in visualising biofilm structures, although it does not give quantitative results (Chumkhunthod *et al.* 1998). Direct epifluorescence microscopy (DEM) has been shown to estimate surface populations of attached bacteria (*A. calcoaceticus*) in the range of $3 \cdot 10^3$ to $5 \cdot 10^7$ colonies/cm^2 (Holah *et al.* 1988). DEM was found to be applicable to a range of bacteria (*A. calcoaceticus, S. epidermidis, Bacillus licheniformis, L. mesenteroides, Streptococcus lactis* and *P. fragi*) and food grade surface materials (stainless steel 316 2B, high-density nylon, polyvinyl chloride (PVC) and polypropylene) (Holah *et al.* 1989). In the present study, DEM was applied to pure and mixed cultures of a range of bacteria and yeasts mainly isolated from brewery samples (see Tables 8 and 9 for further details). The applicability of DEM to stainless steel and PTFE was found to be better than to the rubber materials studied (III, IV).

Impedimetry was used in cleanability studies to detect viable microorganisms from test surfaces (III, IV, V). The method detected microorganisms hidden in the crevices of the materials and not removed by swabbing or detected by epifluorescence microscopy, which was an advantage especially when examining deteriorated surfaces (IV). However, the cultivation conditions used in impedance measurements, such as medium and temperature, influence the results obtained by this method because optimum growth conditions vary between different organisms. This can be a problem when studying mixed culture populations, and thus the growth conditions used in this study were obviously not optimal for *P. damnosus* (III, IV). Furthermore, sublethally injured or stressed cells may prolong the detection time and result in underestimation of the number of living cells (Johansen *et al.* 1997, Ayres *et al.* 1998).

Impedance microbiology is based on the monitoring of electrical changes caused by the growth of microorganisms. Nutrient macromolecules are broken down into smaller high-charged units as a result of microbial metabolism and the resulting conductivity change of the medium is measured. The threshold concentration necessary to cause a detectable increase in impedance is approximately 10^6–10^7 bacteria or 10^4–10^5 yeasts per ml. The time to reach this threshold concentration is a function of both initial concentration and the growth kinetics of the organism in a particular medium (Dowhanick 1994). Impedimetry allows the assessment of bacterial viability to be undertaken while the bacteria remain surface-bound. In this way the physiological conditions of the bacteria are maintained and the problems associated with bacterial removal from surfaces are avoided (Holah *et al.* 1990).

The ATP analysis of swab samples and final rinse waters from working dispense installations provided a rapid and simple method for the hygiene monitoring of dispense systems. The method showed 87% agreement with the plate count method for swab samples and 74% agreement for rinse water samples (Tables 3, 4/I). The ATP method was later introduced in Germany for hygiene assessment of dispensing installations and was found to be a suitable method for rapid analysis of swab and beer samples from the tap (Schwill-Miedaner *et al.* 1997, Schwill-Miedaner and Eichert 1998). However, it should be noted that the chemicals used for cleaning and disinfection have been shown to affect the ATP bioluminescence reaction even at relatively low concentrations (Velazquez and Fiertag 1997, Green *et al.* 1998, 1999). The reaction may be partially quenched or even enhanced by chemical residues left on surfaces, thus causing aberrant results. In hygiene monitoring the users require the ATP assay to be as simple as possible, which means that no internal standard normally is used (Lappalainen *et al.* 1999). This was also the case in this study (I). However, addition of ATP standard to the reaction mixture to ensure proper function of the reagents would provide an indication of possible detergent effects.

When attachment of brewery isolates of bacteria and yeasts to stainless steel was studied, a good agreement between direct ATP analysis of the surface and the plate count method was found (Fig. 1/II). An arbitrary detection limit of 100 RLU (relative light units) for this assay could be set based on detection of viable cells by cultivation. Direct ATP analysis was also used to study cleanability of different process materials in CIP and of stainless steel in foam cleaning (IV, V).

Detectable levels of ATP were observed on aged materials after CIP, supporting the findings of image analysis for the same materials (Fig. 4/IV). An advantage of direct ATP measurements especially in the examination of deteriorated surfaces is that microorganisms hidden in crevices can be detected. ATP analysis also demonstrated that elevated rinsing temperatures in foam cleaning had a beneficial effect on the cleaning result, which was verified in the corresponding plate count results (V). However, in another study assessing the cleanliness of surfaces after CIP, the ATP bioluminescence method was found to be insensitive (Wirtanen *et al.* 1996). This could be due to the low numbers of bacteria normally left on surfaces after CIP and to the decrease in ATP concentrations observed in viable but non-culturable cells (Federighi *et al.* 1998).

The ATP bioluminescence method has been used in breweries to monitor surface hygiene by analysing swab samples and to evaluate the efficiency of cleaning and disinfection by analysing rinse water samples (Hammond 1996, Werlein 1998). The ATP assay is rapid, requiring only a few seconds in hygiene applications. A further advantage of the method in hygiene monitoring is its ability to detect product residues and soil in addition to viable microorganisms. The method was shown to detect as little as 1 µl wort or beer (Ehrenfeld *et al.* 1996), or 0.004% of beer in rinse water (Werlein 1998). The inability of the method to distinguish between living microbial cells and other organic material is of little significance, because neither should be present on a clean surface. In hygiene control the ATP method allows real-time estimation of the cleanliness of process surfaces, thus making recleaning possible if considered necessary.

Simple tests based on detection of protein on surfaces and developed for hygiene monitoring purposes have been found to give comparable results with the ATP bioluminescence method (Baumgart 1996). In this study, two protein detection kits were tested for detection of wort or brewery isolates of *Enterobacter* sp., *L. lindneri* and *D. anomala*. The tests could detect 10^5 or 10^7 yeast cells and 10^6 or 10^7 bacterial cells as counted in a Thoma chamber, or correspondingly 1 ml of 0.1%P or 10%P wort (Table 1/V). Thus the detection levels of the tests were distinctively higher than those reported for ATP assays. In the case of the less sensitive test only visible amounts of microorganims or wort could be detected, which obviously do not need specific testing.

5.4 Detection and characterisation of *Lactobacillus lindneri* (VI)

5.4.1 Detection of *L. lindneri*

The bacteria generally regarded as most common contaminants in modern breweries are lactic acid bacteria belonging to the genera *Lactobacillus* and *Pediococcus* (Back 1994a, Jespersen and Jakobsen 1996, Priest 1996). *L. lindneri* is a particularly harmful species because of its high resistance to hop bitter substances, to thermal treatment and to some disinfectants (Back 1981, Rinck and Wackerbauer 1987, Back *et al.* 1992). It was also shown to produce biofilm and to be a harmful contaminant of immobilised yeast reactors used for maturation (II). Among the media hitherto reported no single medium can be used to detect all members of beer spoilage lactic acid bacteria (Jespersen and Jakobsen 1996, Priest 1996). Some lactic acid bacteria are very fastidious and sluggish and are reluctant to grow outside the beer environment to which they are adapted, even on laboratory media (Taguchi *et al.* 1990, Jespersen and Jakobsen 1996). Contamination caused by *L. lindneri* may be very difficult to detect in brewery quality control due to weak growth on routine cultivation media.

L. lindneri strains isolated from beer samples in Finland and Japan failed to grow on media commonly used in brewery quality control, such as UBA (Universal Beer Agar) or SDA (Schwarz Differential Agar). Some strains also showed no or very poor growth on MRS (De Man-Rogosa-Sharpe medium) (Table III/VI). The best way to speed up detection using cultivation methods for these fastidious lactobacilli was to enrich beer samples with NBB-C medium (Fig. 1/VI). The 4:1 mixture of MRS broth and beer, used e.g. for hygiene assessment of the filler and crowner, also supported rapid growth of the six *L. lindneri* strains tested. In combination with the membrane filtration technique, NBB-A agar provided best growth of the media tested in this study (Table IV/VI).

In brewery microbiology, a range of rapid methods have been developed for the detection of beer spoilage organisms, reviewed by Barney and Kot (1992), Dowhanick (1994), Storgårds *et al.* (1998) and Quain (1999). However, these alternative methods seem not to be in use in the breweries as they often lack the

speed, sensitivity and specificity required or include the use of advanced, expensive equipment and reagents. At present, the use of selective media and incubation conditions still appears to be the method preferred by breweries, even though the detection of beer spoilage organisms by cultivation in laboratory media does not always provide the specificity and the sensitivity required (Jespersen and Jakobsen 1996). Recently, a more rapid detection method based on the PCR method in combination with pre-enrichment was developed (Juvonen *et al.* 1999). This method was able to detect low levels (≤10 cfu/100 ml beer) of *L. lindneri* in only 2–3 days. A further advantage of the PCR method is that the detection can be designed to be species-specific, which is not possible by cultivation methods.

5.4.2 Characterisation of *L. lindneri*

In industrial laboratories, identification is normally kept to a minimum. When identification is performed, it aims to be pragmatic, searching for key properties such as beer spoilage ability rather than for taxonomic details (Gutteridge and Priest 1996). Characterisation is a better term than identification to describe this activity. The characterisation of particular problem-causing strains is an important tool in the tracing of contamination sources. Identification and characterisation can be based on four levels of expression of genetic information (Gutteridge and Priest 1996):

- the genome
- proteins
- cell components
- morphology and behaviour.

Ribotyping is a method based on analysis of the genome, SDS-PAGE is based on analysis of cellular proteins and miniaturised systems of nutritional and biochemical tests, such as API strips, are based on morphology and behaviour. These methods were used in the characterisation of *L. lindneri* strains from brewery samples and they will be discussed below.

Characterisation by API 50 CHL

The German *L. lindneri* type strain and the identical strain obtained from Döhler GmbH were the only ones identified correctly by API 50 CHL. All proposed *L. lindneri* strains isolated from Finnish, Swedish or Japanese brewery samples resulted in uncertain identification by this system (Table VI/VI). The carbohydrates generally fermented were glucose, fructose, maltose and ribose. All the Finnish and Japanese brewery isolates were ribose positive. However, the fermentation of ribose is supposed to be negative for *L. lindneri* in the current APILAB Plus database. As the proposed *L. lindneri* strains were later identified with great certainty as *L. lindneri* by SDS-PAGE analysis and by ribotyping, the results suggest that the API database could be improved by including more strains in it.

Miniaturised commercial systems based on biochemical tests for identification purposes are generally regarded as more reproducible than conventional methods (Gutteridge and Priest 1996). A main problem in identification of *Lactobacillus* strains, however, is the high phenotypic similarity among species, which can be as much as 95% despite the strains being unrelated by criteria such as rRNA sequence or DNA homology (Priest 1996). The phenotypic homogeneity of the lactobacilli necessitates the use of at least 50 tests such as in the API 50 CHL system, and still the results may not be reliable. Plasmid loss may cause altered phenotypes in lactobacilli, as many plasmids code for carbohydrate utilisation pathways (Priest 1996).

An additional problem associated with some lactobacilli is the extremely slow growth on cultivation media such as the MRS used in API 50 CHL. For slow-growing *Lactobacillus* strains from brewery environments, prolonged incubation of API 50 CHL strips for up to 10 days was used by Funahashi *et al.* (1998). In this study, incubation for up to 18 days for *L. lindneri* was needed before acid formation could be detected (VI). Furthermore, beer spoilage *Lactobacillus* strains typically use only a few of the sugars available in the API 50 CHL strips (Table VI/VI, Funahashi *et al.* 1998), thus making identification by phenotypic tests unsatisfactory. Despite the obstacles described above, API strips are still a convenient method for preliminary characterisation of strains and for use in laboratories lacking more sophisticated methodology.

Characterisation by SDS-PAGE

The LMG Culture Collection in Belgium uses a large database for the identification of lactic acid bacteria, based on whole-cell protein fingerprinting by SDS-PAGE (Pot and Janssens 1993, Pot *et al.* 1994). In this study, the brewery isolates were compared to the LMG database covering over 2000 strains from all known species of lactic acid bacteria and were identified as *L. lindneri* (Fig. 2/VI). The strains could be readily separated from other *Lactobacillus* species, e.g. from *L. brevis*, *L. fructivorans* and *L. delbrueckii*. The *L. lindneri* strains formed a separate cluster at a correlation level of almost 83%, being more homogenous than *L. brevis*. Only the Japanese strain took a somewhat separate position (correlation of 76%), which was probably related to the aberrant phenotypic behaviour of this particular strain. The SDS-PAGE analysis was found to be a reproducible characterisation method for the lactobacilli studied.

A microbial cell expresses some 2000 different proteins, which can be used as a source of information in the identification and characterisation of micro-organisms. Polyacrylamide gel electrophoresis (PAGE) of cellular proteins yields complex banding patterns, which can be considered as highly specific fingerprints of the strain investigated. These electrophoregrams are highly reproducible and individual strains within a given taxon can often be recognised (Pot *et al.* 1994). In SDS-PAGE, proteins are solubilised by treatment with the denaturing agent sodium dodecyl sulphate (SDS). The solution is then applied to PAGE and stained to visualise the protein band patterns (Pot *et al.* 1994, Gutteridge and Priest 1996). Densitometric analysis and computer-assisted comparison of the patterns are necessary for the objective comparison of a large number of protein extracts (Pot *et al.* 1994). Electrophoretic patterns have been found to be discriminatory at the species, subspecies or biotype level. Another advantage of the method is that a large number of strains can be compared effectively. Protein electrophoresis is regarded as particularly suitable for identification of lactic acid bacteria (Gutteridge and Priest 1996).

Characterisation by ribotyping

The riboprint patterns of the proposed *L. lindneri* strains as well as of the type strain and the Döhler GmbH test strain all differed very clearly from other lactic acid bacterial species ribotyped (66 strains belonging to 24 species). The

patterns of the *L. lindneri* strains and those of the most relevant reference strains are shown in Fig. 3/VI. The tested strains formed two ribogroups, the extremely slow-growing Japanese strain being the only representative of one group and all the other strains belonging to the other group. The similarity to the type strain within the latter group was very high (90–97%). The strains of this ribogroup were indistinguishable by this method, probably due to digestion by *Eco*R1, which produced only 4 bands resulting in poor discrimination. Thus the method proved to be excellent for the identification of *L. linderi* by comparing to the type strain, but unsuitable for discrimination between strains. The discrimination power of the method could probably be improved by the use of other restriction enzymes for digestion.

Ribotyping is used for characterisation of the restriction fragment length polymorphism (RFLP) of the ribosomal RNA genes. The total chromosomal DNA is cut with restriction enzymes, separated by gel electrophoresis and hybridised to probes for the 16S and 23S ribosomal RNA genes. Ribotyping was previously found to be quite laborious and complicated and not to show very good discrimination between strains (Prest *et al.* 1994). However, in the automated RiboPrinter™ system (Qualicon, USA) the whole *E. coli* region encoding the rRNA 16S-23S genes is used as a probe, thereby increasing the discriminatory power of the method. Automation makes the system reproducible and easy to handle, enabling the analysis of 1–8 strains to be carried out in 8 hours. The RiboPrinter™ system was successfully applied to differentiation and characterisation of new beer-spoilage lactobacilli isolated from brewery samples (Funahashi *et al.* 1998), of *P. cerevisiiphilus*, *P. frisingensis*, *S. lacticifex, Z. raffinosivorans* and *Z. paucivorans* strains (Motoyama *et al.* 1998) and of *Pediococcus* strains (Satokari *et al.* submitted). The *Pediococcus* strain (VTT-E-76067) used in this study (III, IV), previously identified as *P. pentosaceus* (at BRI) and later as *P. inopinatus* (Suihko 1994), was finally identified as *P. damnosus* by ribotyping and SDS-PAGE (Satokari *et al.* submitted). Based on the encouraging results of automated ribotyping, a ribogroup pattern database comprising a wide range of lactobacilli and pediococci from brewery environments has been constructed at VTT.

6. Summary and conclusions

Biofilm formation may affect the performance of the brewing process and the quality of the final product, thus causing economical losses. However, research concerning surface-associated microorganisms in beer production and dispensing have only attracted little interest in the past. In the present work, the surface-attached growth of spoilage organisms and their detection, characterisation and elimination were studied.

Microbiological contamination of draught beer was found to be a consequence of poor hygiene of the dispensing systems (I). The microbiological quality of draught beer often decreased already one week after cleaning, simultaneously with decreasing hygienic status of the dispensing devices. The normal cleaning procedure was not sufficient for heavily contaminated dispensing equipment, resulting in rapid recontamination of the systems. ATP analysis of swab and rinse water samples from working dispense installations showed good agreement with the plate count method. The ATP bioluminescence technique was found to provide a rapid and simple method for the hygiene monitoring of dispense equipment.

Microorganisms involved in beer spoilage were shown to produce biofilm on stainless steel in conditions resembling those of the brewing process (II). The organisms producing biofilms were strains of acetic acid bacteria, lactic acid bacteria, enterobacteria and yeasts. The formation of biofilm was highly strain dependent and different strains within the same species behaved differently in this respect. Microbial growth was observed on different surfaces used in process equipment, but environmental conditions strongly affected attachment and biofilm formation. Thus a strain adapted to immobilised yeast reactors did not grow on stainless steel and the biofilms observed in beer dispensing installations probably differ from those found in the brewery.

Process surface materials were found to differ in their susceptibility to biofilm formation (III, IV). Biofilm was found to develop readily on stainless steel, PTFE, EPDM and Viton when *Enterobacter* sp. and *P. damnosus* isolated from brewery samples were used as test organisms in a mixed culture. NBR inhibited the growth of these organisms when new, but gradually lost this ability with increasing age. In the case of a mixed culture of *B. thuringiensis* and *P. fragi*,

PTFE, NBR and Viton were all less susceptible to biofilm formation than stainless steel. Carrier materials in immobilized yeast reactors used for secondary fermentation were also found to differ in their susceptibility to attachment of contaminating bacteria (II). Thus, the organic DEAE cellulose carrier promoted faster attachment of *L. lindneri* than inorganic porous glass beads.

A trend towards reduced cleanability with increasing age was observed for all gasket materials studied (IV). This was probably due to the physical deterioration observed both in experimentally aged rubber materials and in materials that had been aged in industrial processes. The surface structure of Viton, NBR and EPDM showed considerable changes with increasing age, but PTFE was not affected in the same way. Reduced cleanability was indicated by increasing biofilm residues and an increasing incidence of viable bacteria on aged materials after CIP. Gaskets that had been installed in valves in the process for 3–4 years showed marked reduction in cleanability. However, the cleanability varied depending on the operation history of the valve. Thus, gaskets should be checked on a regular basis depending on their use and replaced in time to avoid the risk of contamination.

In the detection of surface-attached microorganisms and biofilms, both the sampling method and the detection method are of significance. In this study, the use of surface-active agents and/or ultrasonication distinctly improved the detachment of microorganisms and biofilms from surfaces (V). However, *in situ* methods were still considered the most reliable and should be applied whenever possible. Such methods include impedimetry, ATP measurements directly from surfaces and methods based on microscopy.

Epifluorescence microscopy was found to be useful in estimating the amounts of biofilm on surfaces (II, III, IV, V). In cleanability studies the method was more sensitive than methods based on cultivation, as it also detected dead cells and biofilm residues. However, the variation in the results obtained by this method was considerable, apparently due to the uneven distribution of biofilm and microcolonies on the surfaces. Another disadvantage of the method was the background fluorescence observed in connection with rubber materials, and flexible materials were technically difficult to examine. The method is restricted

to flat test coupons, and was therefore not applied to process hygiene assessment.

Detection of viable bacteria from surfaces after cleaning operations is not an easy task, due to the low numbers expected. In this study, impedimetry and conventional swabbing and plate counting were used to quantify viable microorganisms on surfaces (III, IV, V). In impedimetry, the assessment of bacterial viability can be undertaken while the microorganisms remain surface-bound, which is an advantage especially when examining deteriorated surfaces. With the plate count method optimal growth conditions are more easily applied, which is an advantage when studying fastidious microorganisms or mixed cultures. However, when using methods based on cultivation, it is important to remember that a large proportion of the microbial cells may be in a non-culturable state induced by environmental stress.

Of the hygiene assessment methods tested, the ATP bioluminescence technique was found to show good agreement with plate count results in the analysis of working beer dispensing systems (I). In ATP analysis directly from surfaces, the inaccuracy caused by detachment of microorganisms and soil is avoided. A good agreement between direct ATP measurement and plate count results was obtained when biofilm formation was studied (II). Direct ATP analysis also supported the findings obtained by epifluorescence microscopy or the plate count method in cleanability studies (IV, V). In hygiene monitoring applications, the ATP method is attractive because it is rapid, easy to use and also detects product residues and soil. Currently, however, direct ATP analysis is only suitable for flat surfaces, which restricts the use of this application in hygiene assessment.

Hygiene monitoring kits based on protein detection were found to be less sensitive than the ATP method (V) and can therefore only be recommended in cases where significant amounts of proteins are expected to be found.

Detection of fastidious microorganisms from product or process intermediate samples is sometimes difficult due to slow and weak growth on cultivation media used in brewery quality control. For *L. lindneri*, detection by cultivation could be accelerated by enrichment of the sample with a nutritious medium (NBB-C) compared to cultivation of membrane-filtered samples on solid agar

(VI). When the membrane-filter technique was used, best growth of the newly isolated strains was achieved on NBB-A medium.

Correct identification of newly isolated *L. lindneri* strains was not possible using API 50 CHL tests and the APILAB Plus database version 4.0. The fermentation of ribose is supposed to be negative for *L. lindneri*, but all the Finnish strains and the Japanese strain studied were repeatedly shown to be ribose positive (VI).

Automated ribotyping and SDS-PAGE of whole-cell proteins were successfully applied to correctly identify the *L. lindneri* brewery isolates (VI). Both ribotyping and SDS-PAGE identified the isolates on the species level when well-known reference strains were used and the reproducibility of the methods was good. Using SDS-PAGE it was possible to discriminate between the *L. lindneri* strains, but ribotyping resulted in poor discrimination. However, the ribotyping method could probably be improved by the use of alternative restriction enzymes for digestion.

Successful assessment of surface hygiene in the brewing process results in many beneficial impacts of economical and environmental value. Knowledge acquired about surface-attached microorganisms and biofilms has made us realise and understand the limitations of conventional hygiene control methods. At the same time, the demands for rapid or instant methods are growing, together with increased process efficiency. Preventive measures based on accurate information on causal connections between materials, process matrices and microorganisms are in most cases superior in ensuring process efficiency and product quality. In acute contamination situations, specific characterisation of the contaminant is needed for effective tracing of the contamination source.

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Title

Process hygiene control in beer production and dispensing

Abstract

Process hygiene plays a major role in the production of high quality beer. 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. The present study examined the growth of surface-attached beer spoilage organisms and the detection and elimination of microbial biofilms. Moreover, the detection and characterisation of *Lactobacillus lindneri*, a fastidious contaminant, was studied.

Beer spoilage microorganisms, such as lactic acid and acetic acid bacteria, enterobacteria and yeasts were shown to produce biofilm on process surface materials in conditions resembling those of the brewing process. However, attachment and biofilm formation were highly strain dependent. In addition, the substrates present in the growth environment had an important role in biofilm formation.

Different surface materials used in the brewing process differed in their susceptibility to biofilm formation. PTFE (polytetrafluoroethylene), NBR (nitrile butyl rubber) and Viton were less susceptible to biofilm formation than stainless steel or EPDM (ethylene propylene diene monomer rubber). However, the susceptibility varied depending on the bacteria and the conditions used in the *in vitro* studies. Physical deterioration resulting in reduced cleanability was observed on the gasket materials with increasing age. DEAE (diethylaminoethyl) cellulose, one of the carrier materials used in immobilized yeast reactors for secondary fermentation, promoted faster attachment and growth of contaminating *L. lindneri* than ceramic glass beads. Beer dispensing systems in pubs and restaurants were found to be prone to biofouling, resulting eventually in microbial contamination of draught beer and cleanability problems of the dispensing equipment.

Detection of surface-attached microorganisms is crucial in process hygiene control. *In situ* methods such as epifluorescence microscopy, impedimetry and direct ATP (adenosine triphosphate) analysis were the most reliable when studying surface-attached growth of beer spoilage microbes. However, further improvement of these techniques is needed before they can be applied for routine hygiene assessment. At present hygiene assessment is still dependent on detachment of microorganisms and soil prior to analysis. Surface-active agents and/or ultrasonication improved the detachment of microorganisms from surfaces in the sampling stage. The ATP bioluminescence technique showed good agreement with the plate count method in the control of working dispensing installations. Hygiene monitoring kits based on protein detection were less sensitive than the ATP method in the detection of wort or surface-attached microorganisms.

Effective process control should also be able to detect and trace fastidious spoilage organisms. In this study, the detection of *L. lindneri* was notably improved by choosing suitable cultivation conditions. *L. lindneri* isolates, which could not be correctly identified by API 50 CHL, were identified to the species level by automated ribotyping and by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) when compared with well-known reference strains. SDS-PAGE was also able to discriminate between different strains, which is a useful feature in the tracing of contamination sources.

Keywords
beer, manufacture, processes, dispensers, hygiene control, decontamination, microorganisms, biofilms, detection, identification

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