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DNA-based detection and characterisation of strictly anaerobic beer-spoilage bacteria

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Academic Dissertation in Microbiology

Abstract

The *Sporomusa* sub-branch of the class *"Clostridia"* includes six strictly anaerobic Gram-stain-negative beer-spoilage bacteria: *Megasphaera cerevisiae*, *Pectinatus cerevisiiphilus*, *Pectinatus frisingensis*, *Selenomonas lacticifex*, *Zymophilus paucivorans* and *Zymophilus raffinosivorans*. They spoil beer by producing foul-smelling compounds and turbidity. These species have only been isolated from the beer production chain. They are difficult to detect and identify in breweries, since they do not tolerate oxygen and may cause spoilage at very low levels. Traditional cultivation methods provide information about the product contamination only after 1–6 weeks of incubation. Moreover, cultivation methods do not allow reliable species identification. Hence, more rapid and specific detection and identification tools are needed for strictly anaerobic beer spoilers. The main aim of this study was to utilise DNA-based techniques in order to improve detection and identification of the *Sporomusa* sub-branch beer-spoilage bacteria and to increase understanding of their biodiversity, evolutionary relationships and natural sources.

Specific polymerase chain reaction (PCR) tests combined with a colorimetric microplate hybridisation assay as a PCR read-out were established for the most common beer-spoilage anaerobes, i.e. for *M. cerevisiae* and the genus *Pectinatus*. The microplate assay facilitated result interpretation and improved work safety, but was more laborious and time-consuming compared to gel electrophoresis as the PCR read-out. We also designed group-specific end-point and real-time PCR tests to detect all absolute and potential beer-spoilage species of the *Sporomusa* sub-branch in a single reaction. The real-time PCR format saved work and time. The group-specific PCR tests provide a cost-effective tool for routine monitoring of brewery samples for strictly anaerobic beer spoilers. In addition, genus identity and spoilage potential of the detected bacteria could be determined

by restriction fragment length polymorphism and melting curve analysis of the PCR products, respectively.

PCR analysis of the spoilage bacteria in brewery samples is hampered by the presence of inhibitors and the high sensitivity and simplicity required. We established easy, rapid (1–2.5 h) and inexpensive (0.5–4 €) procedures to prepare PCR-ready DNA from the target group bacteria in process samples containing high numbers of brewer's yeast cells and in finished beer. The contaminant detection in process samples was possible at a level of 10^1–10^3 cfu against 10^7–10^8 brewer's yeast cells. The detection of low levels of viable cells in beer was achieved by incorporating a culture enrichment step into the PCR tests. Using the established procedures, a few contaminating cells ($\leq$ 10 cfu 100 ml^{-1}) were usually detected in 1–3 d. PCR analysis took 2–8 h depending on the applied PCR format and the sample type. The developed PCR assays allow the brewer to obtain information about the presence and identity of the *Sporomusa* sub-branch spoilage bacteria in the beer production chain more rapidly and specifically than the cultivation methods. This should ensure financial benefits to the industry.

Three new spoilage species, *Megasphaera paucivorans*, *Megasphaera sueciensis* and *Pectinatus haikarae*, were described from the beer production chain using a polyphasic approach. They could be detected and isolated from brewery samples using the same cultivation methods as for *M. cerevisiae*, *P. cerevisiiphilus* and *P. frisingensis*. Ribotyping and comparative 16S rRNA gene sequence analysis were shown to be suitable tools to distinguish the new species from other brewery-related bacteria. Diagnostic characteristics useful for their phenotypic identification were also established.

This study provided new insight into the phylogenetic relationships and ecology of the *Sporomusa* sub-branch bacteria. In the 16S rRNA gene sequence based tree, the brewery-related *Megasphaera* spp. formed a distinct sub-group. No sequences in this sub-group derived from other sources, suggesting that *M. cerevisiae*, *M. paucivorans* and *M. sueciensis* may be uniquely adapted to the brewery ecosystem. The finding that *M. cerevisiae* is able to survive long periods in various hostile conditions encountered in the beer production could partly explain its establishment as a brewery contaminant. The genus *Selenomonas* was found to be polyphyletic and will require reclassification. Moreover, it was shown that *Z. paucivorans* and *Z. raffinosivorans* are in fact members of the genus *Propionispira*. Their close relatedness to the tree-inhabiting *Propionispira arboris* indicates that they could have found their way to breweries with plant material.

Tiivistelmä

Nykyisin bakteerisukupuun *Sporomusa*-alahaaraan klostridien luokkaan kuuluu kuusi anaerobista oluenpilaajalajia: *Megasphaera cerevisiae*, *Pectinatus cerevisiiphilus*, *Pectinatus frisingensis*, *Selenomonas lacticifex*, *Zymophilus paucivorans* ja *Zymophilus raffinosivorans*. Ne aiheuttavat pakatussa oluessa samennusta sekä virhehajuja ja -makuja. Näitä lajeja on toistaiseksi löydetty vain oluen tuotantoketjusta. Ne ovat hankalia osoittaa, koska ne eivät siedä happea, ja jo yksi elävä solu voi johtaa pilaantumiseen. Siksi panimoissa on jouduttu turvautumaan rikastusviljelyyn, jolla tulos saadaan vasta 1–6 viikossa. Tuote on tällöin usein jo myynnissä tai kuluttajalla. Menetelmä ei myöskään mahdollista kontaminantin tunnistusta. Tämän tutkimuksen tavoitteena oli parantaa *Sporomusa*-alahaaran oluenpilaajien osoittamista ja tunnistamista sekä tuoda uutta tietoa näiden bakteerien monimuotoisuudesta, sukulaisuussuhteista ja lähteistä DNA-pohjaisia tekniikoita hyödyntämällä.

M. cerevisiae -lajille ja *Pectinatus*-suvulle kehitettiin PCR-pohjainen kuoppalevytesti, joka perustui DNA-hybridisaatioon ja entsymaattiseen värireaktioon. Kuoppalevytesti helpotti PCR-tulosten tulkintaa ja vähensi haitallisten reagenssien tarvetta, mutta oli toisaalta työläämpi ja hitaampi elektroforeettiseen lopputuoteosoitukseen verrattuna. Lisäksi tutkimuksessa kehitettiin *Sporomusa*-alahaaran oluenpilaajabakteerien ryhmäspesifiseksi osoittamiseksi reaaliaikaiseen PCR:ään sekä lopputuoteosoitukseen perustuvat testit. Reaaliaikainen PCR vähensi työtä ja lyhensi analyysiaikaa. Ryhmäspesifiset PCR-testit tarjoavat kustannustehokkaan työkalun anaerobisten oluenpilaajabakteerien monitorointiin panimonäytteistä. Lisäksi ryhmäspesifisten PCR-tuotteiden restriktiofragmenttianalyysi ja sulamislämpötilan määritys mahdollistavat kontaminantin sukutason tunnistuksen ja haitallisuusasteen arvioinnin.

PCR-inhibiittorit sekä laadunvalvontatestiltä vaadittava helppous ja äärimmäinen herkkyys vaikeuttavat PCR:n soveltamista panimonäytteille. Tässä tut-

kimuksessa kehitettiin nopeita (1–2,5 h), edullisia (0,5–4 €) ja helppoja mene-
telmiä panimohiivaa sisältävien prosessinäytteiden sekä valmiin oluen esikäsitte-
lyyn. Kehitetyllä menetelmällä prosessinäytteistä voitiin osoittaa 10^1–10^3 kon-
taminanttia 10^7–10^8 panimohiivasolun joukosta. Olutnäytteiden analyysiä varten
PCR-testeihin liitettiin rikastuskasvatusvaihe, jolloin pieni määrä eläviä soluja
(≤ 10 pmy 100 ml^{-1}) voitiin yleensä havaita 1–3 vrk:ssa. PCR-tulos saatiin 2–8
tunnissa näytetyypistä ja PCR-sovelluksesta riippuen. Kehitetyt PCR-
menetelmät mahdollistivat kohdebakteerien osoittamisen spesifisemmin ja useita
päiviä nopeammin kuin viljelymenetelmät. Kontaminaation sattuessa korjaaviin
toimenpiteisiin voidaan ryhtyä entistä varhaisemmassa vaiheessa ja toimenpiteet
voidaan kohdentaa entistä tarkemmin, mikä auttaa vähentämään kontaminaatios-
ta aiheutuvia teollisuuden tappioita.

Genotyyppiseen ja fenotyyppiseen karakterisointiin perustuen tutkimuksessa
kuvattiin oluen tuotantoketjusta kolme uutta ehdottoman anaerobista oluenpilaa-
jalajia: *Megasphaera paucivorans*, *Megasphaera sueciensis* ja *Pectinatus haika-
rae*. Panimomikrobiologiassa vakiintuneiden viljelymenetelmien havaittiin so-
veltuvan myös uusien lajien osoittamiseen ja eristämiseen panimonäytteistä.
Lisäksi osoitettiin, että nämä bakteerit voidaan tunnistaa ribotyypityksen ja 16S
rRNA geenisekvenssivertailun avulla. Uusien lajien tunnistamiseksi löydettiin
myös fenotyyppiin perustuvat erottelevat testit.

Tutkimus toi uutta tietoa *Sporomusa*-alahaaran pilaajabakteerien sukulaisuus-
suhteista ja ekologiasta. 16S rRNA geenisekvenssien vertailuun perustuvassa
sukupuussa panimoista tavatut *Megasphaera*-lajit muodostivat muista elinpii-
reistä löydettyjen bakteerien sekvensseistä erillisen alaryhmän. Tulos viittaa
siihen, että *M. cerevisiae*, *M. paucivorans* ja *M. sueciensis* ovat sopeutuneet
elämään yksinomaan panimoekosysteemissä. Lisäksi tutkimuksessa osoitettiin,
että *M. cerevisiae* voi säilyä erilaisissa panimoprosessia jäljittelevissä stressiolo-
suhteissa pitkiä aikoja elinkykyisenä. Tämä piirre voi osittain selittää lajin etab-
loitumisen panimoihin. *Selenomonas*-suku todettiin polyfyleettiseksi ja vaativan
uudelleenluokittelua. Lisäksi *Zymophilus*-lajien osoitettiin itse asiassa kuuluvan
Propionispira-sukuun, jonka ainoaa lajia on tavattu vettyneestä sydänpuusta.
Mahdollisesti myös *Zymophilus*-lajit ovat peräisin kasvimateriaalista, jonka mu-
kana ne ovat kulkeutuneet panimoihin.

Academic dissertation

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Preface

I am indebted to Technology Directors of VTT Biotechnology, Prof. Juha Ahvenainen (2002–2007) and Prof. Anu Kaukovirta-Norja (2007–), and Technology Manager Dr. Niklas von Weymarn for providing excellent working facilities and the possibility to finalise this dissertation. I also thank Niklas for the opportunity to take the cover picture, which for the first time shows flagellation of *Pectinatus haikarae*.

I express my sincere gratitudes to the pre-examiners Prof. Barry Ziola and Doc. Peter Hackman for showing interest in my thesis despite the tight schedule, and for their constructive criticism that was instrumental in improving the manuscript. I warmly thank Prof. Per Saris for allowing me to finish my PhD studies and for all his help during the eventful final stages of this project.

I am deeply grateful to my supervisor, Doc. Auli Haikara, the mother of *Pectinatus*, for introducing me to the exciting world of strictly anaerobic beer-spoilage bacteria. I am also indebted to Auli for her efforts to make me a better microbiologist and for her support over the years. I also thank Auli for sharing with me many memorable, often rip-roaring, events and the passion for good food. My very special thanks are due to my mentor, Dr. Arja Laitila. I thank Arja with open arms for her excellent advice and endless support and encouragement, without which I could not have finished this project. Thank you for always being

List of original publications

This thesis is based on the following original publications, referred to in the text by their Roman numerals (I–V). In addition, some unpublished data is presented.

I   Satokari, R., Juvonen, R., Mallison, K., von Wright, A. and Haikara, A. 1998. Detection of beer spoilage bacteria *Megasphaera* and *Pectinatus* by polymerase chain reaction and colorimetric microplate hybridization. Int. J. Food Microbiol. 45 (2), 119–127.

II   Juvonen, R., Satokari, R., Mallison, K. and Haikara, A. 1999. Detection of spoilage bacteria in beer by PCR. J. Am. Soc. Brew. Chem. 57 (3), 99–103.

III   Juvonen, R., Koivula, T. and Haikara, A. 2008. Group-specific PCR-RFLP and real-time PCR methods for detection and tentative discrimination of strictly anaerobic beer-spoilage bacteria of the class *Clostridia*. Int. J. Food Microbiol. 125 (2), 162–169.

IV   Juvonen, R. and Haikara, A. 2009. Amplification facilitators and pre-processing methods for PCR detection of strictly anaerobic beer-spoilage bacteria of the class *Clostridia* in brewery samples. J. Inst. Brew. 115 (3), 167–176.

V   Juvonen, R. and Suihko, M.-L. 2006. *Megasphaera paucivorans* sp. nov., *Megasphaera sueciensis* sp. nov. and *Pectinatus haikarae* sp. nov., isolated from brewery samples, and emended description of the genus *Pectinatus*. Int. J. Syst. Evol. Microbiol. 56 (4), 695–702.

Author's contribution

I   Riikka Juvonen with Reetta Satokari planned the research, designed and supervised the experiments, interpreted the results and wrote the paper. Riikka Juvonen was mainly responsible of the inhibition studies, which she also performed. Kirstie Mallison carried out most of the PCR experiments.

II   Riikka Juvonen is the corresponding author. She was responsible for the planning the research and the design of the experiments, the interpretation of the results and writing the paper with Reetta Satokari.

III   Riikka Juvonen is the corresponding author. Riikka Juvonen planned the research, designed the experiments and was partly responsible for the experimental work. She interpreted the results and wrote the article.

IV   Riikka Juvonen is the corresponding author. Riikka Juvonen planned the research, designed the experiments and was mainly responsible for the experimental work. She interpreted the results and wrote the article.

V   Riikka Juvonen is the corresponding author. Riikka Juvonen planned the research, designed the experiments and executed part of the experimental work. She interpreted the results and wrote the article. Maija-Liisa Suihko supervised the work.

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Appendices

Papers I–V

***Appendices of this publication are not included in the PDF version.
Please order the printed version to get the complete publication
(http://www.vtt.fi/publications/index.jsp).***

List of abbreviations

ARB	A software environment for sequence data
ARDRA	Amplified ribosomal DNA restriction analysis
ATNC	Apparent total n-nitroso compounds
BLAST	Basic local alignment search tool
BOX	Invertedly repeated element
BSA	Bovine serum albumine
C_p	Crossing point value in real-time PCR
CFA	Cellular fatty acid
CFU	Colony forming unit
C-MRS	Concentrated de Man Rogosa Sharpe medium
D_{oxy}	Decimal reduction time in oxygenic conditions
DEFT	Direct epifluorescence filter technique
DDH	DNA-DNA hybridisation
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
DMS	Dimethyl sulphide
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EBC	European Brewery Convention
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EC-MRS	Extra-concentrated MRS medium
ERIC-PCR	Enterobacterial repetitive intergenic consensus PCR
FISH	Fluorescence *in situ* hybridisation
FRET	Fluorescence resonance energy transfer

GC mol%	Molar DNA guanine and cytosine content
GTG_5	Polytrinucleotide
LAB	Lactic acid bacteria
LAMP	Loop-mediated isothermal amplification
MCA	Melting curve analysis
MRS	de Man Rogosa Sharpe medium
NaOH	Sodium hydroxide
NBB-C	Nachweismedium für Bierschädliche Bakterien
NTU	Nephelometric turbidity unit
OD	Optical density
ºP	Plato degree
PCR	Polymerase chain reaction
PYF	Peptone yeast extract fructose medium
PVP	Polyvinylpyrrolidone
QC	Quality control
qPCR	Real-time PCR
RDP II	Ribosomal Database Project II
rRNA	Ribosomal ribonucleic acid
RAPD-PCR	Randomly amplifed polymorphic DNA PCR
REP	Repetitive extragenic palindromic sequence
Rep-PCR	Repetitive element PCR
RFLP	Restriction fragment length polymorphism
SDS	Sodium dodecyl sulphate
SMMP	Selective medium for *Megasphaera* and *Pectinatus*
STPP	Sodium tripolyphosphate
tuf	Translation elongation factor
T_m	Melting point temperature
VDK	Vicinal diketones
VTT	Technical Research Centre of Finland
Å	Ångström
2-MRSF	Double-concentrated MRS-fructose medium

1. Introduction

Beer is one of the world's oldest alcoholic beverages, produced already before 4000 BC (Campbell, 2003). It is a major biotechnological product. In 2008, the volume of the global beer market reached 145 billion liters with a value of 405.9 billion dollars (www.researchandmarkets.com/reports/53577). Louis Pasteur in 1876 (as quoted by Van Vuuren and Priest, 2003) was the first to demonstrate that bacteria can cause beer spoilage. As we know today, the range of beer-spoilage microbes is rather narrow due to the natural antimicrobial properties of beer and good hygienic practices of modern breweries (Storgårds *et al*., 2006). However, technical improvements in the production process and changes in the beer market continuously modify this ecosystem. Gram-stain-negative strictly anaerobic bacteria are the most recently discovered beer-spoilage organisms. They are phylogenetically affiliated to the *Sporomusa* sub-branch of the class "*Clostridia*" in the phylum *Firmicutes* (Willems and Collins, 1995). They are thought to represent an intermediate in the development of Gram-positive bacteria from their Gram-negative ancestors (Stackebrandt *et al*., 1985). The emergence of strictly anaerobic bacteria in breweries in the late 1970s has been linked with decrease in the oxygen content of packaged beer and with the concomitant increase in the production of unpasteurised products. Hitherto, six species have been described in the beer production chain (Schleifer *et al*., 1990; Haikara and Helander, 2006).

Today, the need to extend the distribution chains and shelf-lives of beer due to globalisation of the market and the growing trend to produce minimally processed low-alcohol products are creating more opportunities for microbial spoilage. Economic losses due to a spoilage incidence can be considerable, since beer production is usually done in large units (batch sizes of 6,000–10,000 hl). Hence, there is an increased pressure worldwide to safeguard the microbiological quality and safety of beer. The ideal way to control microbial contaminations

is through prevention. Quality control (QC) is needed for verifying adequacy of these preventive actions.

In brewery QC, strictly anaerobic beer-spoilage bacteria are the most difficult contaminants to detect and identify due to their oxygen sensitivity and the requirement to detect even a single viable cell in a package of beer. In practice, culture enrichment is the only widely available method for their detection (Haikara and Helander, 2006). However, this method does not allow early warning of the contamination, since it takes 1–6 weeks to obtain the results. Moreover, it does not provide strain or species level identification needed for assessing contamination sources and planning effective countermeasures. Hence, more rapid and informative QC methods are needed for these contaminants.

DNA-based techniques have revolutionized how bacteria are classified, identified and detected by facilitating analysis of the hereditary material of the cells, that ultimately defines all the properties of an organism. Polymerase chain reaction (PCR) PCR is an *in vitro* deoxyribonucleic acid (DNA) amplification technique that enables rapid (< 0.5–3 h) and easy generation of measurable amounts of almost any DNA fragment from a few cells (Mullis *et al*., 1986). Therefore, PCR has attracted considerable interest for rapid, specific and sensitive detection of microbes in food and beverages (McKillip and Drake, 2004; Storgårds *et al*., 2006). However, several bottlenecks still exist for wide application of PCR in brewery QC (Brandl and Geiger, 2003). Moreover, technical advances in DNA analysis necessitate constant application development (Mackay *et al*., 2007).

The DNA-based techniques, particularly comparative 16S rRNA gene sequence analysis, have greatly expanded our knowledge of prokaryotic diversity by unravelling phenotypically cryptic and yet-uncultured species. They have also indicated that many still undescribed species exist in the beer production chain (Sakamoto *et al*., 1997; Nakakita *et al*., 1998; Suihko and Haikara, 2001; Timke *et al*., 2005c). The number of 16S rRNA gene sequences deposited in public databases has increased markedly during recent years (Yarza *et al*., 2008). The current 16S rRNA gene sequence databases, with more than 500,000 sequences, provide a vast resource for discovery, phylogenetic studies and identification of brewery-related bacteria for better understanding of their ecology, biodiversity and relevance.

1.1 The brewing process

The process of beer brewing largely relies on the ability of brewer's yeast to transform malted barley (malt), hops and water into an alcoholic beverage. A great variety of beer styles and types exists. The following presents a general outline of the process. For a more detailed description see Lewis and Young (1995) and Campbell (2003).

The brewing process starts with the production of wort from the malted barley, hops and water. The malt is milled and mixed with water to degrade and solubilise the malt macromolecules in a process called mashing. During the 1–2 h process, temperature is varied to activate different enzymes. Coarse non-soluble malt components, spent grains, are removed by filtration and the resulting liquid, wort, is boiled with hops to modify its flavour and colour, to inactivate enzymes and microbes from the raw materials and to precipitate haze precursors. Brewery adjuncts to supplement part of the malt starch may be added at this point. Boiling also converts hop acids into more soluble isomers, which give beer its typical bitterness and inhibit many Gram-stain-positive bacteria. Wort is a highly nutritious medium and should be used immediately. Before inoculation of the brewer's yeast (pitching), the wort is clarified, cooled and oxygenated to enhance the yeast growth. The typical pitching rate is 10–20 millions cells per ml.

During fermentation, the yeast converts fermentable sugars of the wort to carbon dioxide, ethanol and flavour compounds and anaerobiosis develops along with a drop of pH from 5.0–5.2 to 3.8–4.0. This stage takes at least 2–7 d. The brewer's yeast strains are traditionally divided into lager/bottom-fermenting (*Saccharomyces pastorianus*) and ale/top-fermenting (*Saccharomyces cerevisiae*) types. Towards the end of the fermentation, the lager strains sediment on the cone of the tanks, whereas the ale strains form a head foam. The lager fermentations are carried out at 10–15°C, whereas ales are produced at a higher temperature. Flocculated yeast cells are collected for reuse in subsequent fermentations. The fermented wort, so-called green beer, still requires a maturation of 7–21 d at 1–14°C to develop typical aroma, flavour and carbonation level. Microbes are generally not able to grow at this stage, but contaminants may survive. The residual yeast cells are normally removed from the cool-stabilised beer by filtration, which also reduces the number of possible contaminants. To ensure biological stability, the finished beer is sterile-filtered or pasteurised before or after the packaging. Flash pasteurisation is normally conducted by heating the bulk product at 73–75°C for 1–2 min. Bottle pasteurisation involves more severe

heating in a package (70–72°C, 20–25 min). When properly performed, the latter process ensures microbiological stability. However, unwanted microbial metabolites produced at earlier stages may remain in the product. Avoidance of bottle pasteurisation requires the maintenance of good hygienic conditions during packaging in order to avoid post-pasteurisation contamination.

The finished beer has a combination of antimicrobial factors. It is acidic (pH 3.8–4.7) and contains ethanol (0.5–10%, w/w), hop bitter compounds (*ca*. 17–55 ppm iso-α-acids) and sulphur dioxide, has low levels of oxygen (< 0.1 ppm) and nutrients as well as a high content of carbon dioxide (0.5%, w/w) (Sakamoto and Konings, 2003).

1.2 Bacteriology of beer spoilage

Every beer production stage is prone to microbial contamination from various sources. In breweries, the microbial contaminations are typically divided into primary, originating from the production area, and secondary contaminations, originating from the filling area (Back, 2005). Most contaminations of unpasteurised beer are secondary in nature and typically affect only some packages, whereas the primary contamination may lead to spoilage of the whole production batch (Back *et al*., 1988; Back, 1994).

The types and the range of possible spoilage microbes vary during the beer production process, when nutritious, oxygenated wort is converted into beer which is a nutrient-poor anaerobic medium rich in natural antimicrobials. Only few Gram-stain-negative or -positive bacteria are able to grow in the brewing process, and even fewer in the finished beer (Table 1). Worldwide, hop-resistant *Lactobacillus* and *Pediococcus* strains cause most spoilage cases (Back, 2005; Suzuki *et al*., 2008). The effects of the spoilage bacteria range from relatively minor changes in beer flavour and in fermentation performance to gross off-flavours and aroma defects, turbidity problems, abnormal attenuation rates and reduced yeast crops.

Beer has been considered to be a microbiologically safe beverage, as standard beer does not support the growth of food pathogens. Recently, Haakensen and Ziola (2008) reported spoilage of home-brewed beers by the species *Bacillus cereus* and *Bacillus licheniformis*, which include strains able to cause food poisoning. The isolated strains were also able grow in commercial beers with a rather high pH (pH 4.8–5.2, alc. 4–5 vol-%). Some spoilage bacteria may also produce harmful metabolites, such as N-nitrosamines or biogenic amines, during

the brewing process or in the finished beer (Priest, 2003a; Van Vuuren and Priest, 2003).

Table 1. Overview of types, occurrence and effects of beer-spoilage bacteria in the beer production process (Narendranath *et al.*, 1997; Priest, 2003a, Storgårds *et al.*, 2006).

Group or genus	Effects on fermentation	Effects in finished beer		
		Turbidity	Ropiness	Off-flavours
Acetobacter [1]		+	+	Acetic acid
Bacillus, *Brevibacillus*		–	+	–
Clostridium		–	–	Butyric, caproic, propionic and valeric acids
Gluconobacter [1]		+	+	Acetic acid
Enterobacteria	Decreased fermentation rate, formation of ATNC [4]	–	–	Acetaldehyde, acetic acid, DMS [5], fusel alcohols [6], phenolic compounds and VDK [7]
Lactobacillus	Reduced ethanol production	+	+	Acetic and lactic acids, acetoin, diacetyl, phenolic compounds
Megasphaera		+	–	H_2S, acetic, butyric, caproic, isobutyric, isovaleric and valeric acids
Pectinatus		+	–	Acetoin, H_2S, dimethyl trisulphide, methyl mercaptane, acetic, lactic, propionic and succinic acids
Pediococcus	Decreased fermentation rate	+	+	Diacetyl, lactic acid
Selenomonas		+	–	Acetic, lactic and propionic acids
Zymomonas [2]		+	–	Acetaldehyde, H_2S
Zymophilus [3]		+	–	Acetic and propionic acids

[1] In the presence of oxygen, [2] In primed beer, [3] At elevated pH (5–6), [4] Apparent total n-nitroso compounds, [5] Dimethyl sulphide, [6] N-propanol, iso-butanol, iso-pentanol and iso-amylalcohol, [7] Vicinal diketones.

The following review will focus on the beer-spoilage bacteria of the *Sporomusa* sub-branch, the target organisms of this thesis. This subject was recently reviewed by Haikara and Helander (2006), Haikara and Juvonen (2009) and Marchandin *et al.* (2009).

1.3 Beer-spoilage bacteria of the *Sporomusa* sub-branch of the class "*Clostridia*"

Gram-stain-negative, strictly anaerobic bacteria are the most recently discovered beer-spoilage microbes. The first strains were isolated from spoiled beer in the late 1970s, but there are indirect evidence of their existence in breweries starting from 1946 (Haikara, 1984). It has been postulated that improvements in the filling technology for reducing the oxygen level in packaged beer coupled with avoidance of bottle pasteurisation made the growth of these bacteria in beer eventually possible (Haikara and Helander, 2006). The brewery contaminants in this group include both potential and absolute beer spoilers. By definition, an absolute spoiler is able to grow in beer without a long adaptation period and to cause obvious quality defects, whereas a potential spoiler does not grow in standard beers under normal conditions and does not always cause obvious quality defects or requires a long adaptation (Back, 2005).

1.3.1 Phylogeny and classification

The Gram-stain-negative, strictly anaerobic beer-spoilage bacteria are currently assigned to the genus *Megasphaera* Rogosa 1971a, 187[AL] emend. Engelmann and Weiss 1985; emend. Marchandin, Jumas-Bilak, Gay, Teyssier, Jean-Pierre, Siméon de Buochberg 2003, *Pectinatus* Lee, Mabee and Jangaard 1978, 582[AL]; emend. Schleifer, Leuteritz, Weiss, Ludwig, Kirchhof and Seidel-Rüfer 1990, *Selenomonas* Von Prowazek 1913, 36[AL] (as quoted by Shouche *et al.*, 2009), or *Zymophilus* Schleifer, Leuteritz, Weiss, Ludwig, Kirchhof and Seidel-Rüfer 1990, 26[VP]. Phylogenetic analyses, supported by chemotaxonomic markers, placed these bacteria in the *Sporomusa* sub-branch of the class "*Clostridia*" in the phylum *Firmicutes* (Schleifer *et al.*, 1990; Doyle *et al.*, 1995; Willems and Collins, 1995; Strömpl *et al.*, 1999; Marchandin *et al.*, 2003; Helander *et al.*, 2004) (Fig. 1). This sub-branch has also been known as clostridial cluster IX (Willems and Collins, 1995), *Sporomusa-Pectinatus-Selenomonas* phyletic group (Strömpl *et al.*, 1999) and the family "*Acidaminococcaceae*" (Garrity *et*

al., 2007). In the latest edition of Bergey's Manual of Systematic Bacteriology, all *Sporomusa* sub-branch members were classified in the family *Veillonellaceae* (Rainey, 2009) that formerly only included the coccus-shaped species (Rogosa, 1971b). The bacteria in this family are morphologically diverse Gram-stain-negative anaerobes that have significantly lower DNA G+C content than the members of *Clostridium sensu stricto*. They have been thought to represent an intermediate in the development of Gram-stain-positive bacteria from their Gram-stain-negative ancestors (Stackebrandt *et al.*, 1985). In this thesis, the term *Sporomusa* sub-branch will be used to describe this group.
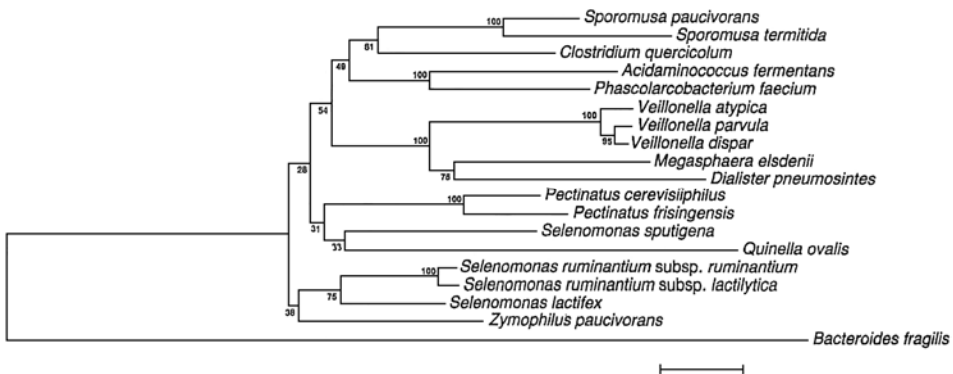
Figure 1. Phylogenetic position of strictly anaerobic beer-spoilage bacteria in the *Sporomusa* sub-branch of the class "*Clostridia*" in the phylum *Firmicutes* (Willems and Collins, 1995). The bar represents a 2% sequence difference.

The genus *Pectinatus* and the species *Pectinatus cerevisiiphilus* were described to accommodate an unusual strictly anaerobic isolate from spoiled beer in the USA (Lee *et al.*, 1978). The taxonomic study of Schleifer *et al.* (1990) on 47 strains of strictly anaerobic rods from breweries led to an emended description of *P. cerevisiiphilus* and to the description of another species, *Pectinatus frisingensis*, first isolated from Finnish beer (Haikara, 1984). The third species, *Pectinatus portalensis*, was characterised from winery wastewater (Gonzalez *et al.*, 2005). However, it will be probably rejected, since no isolate is available (Vereecke and Arahal, 2008). Recent genetic studies have suggested that some brewery isolates phenotypically identified as *P. cerevisiiphilus* could represent a fourth new species (Suihko and Haikara, 2001). However, further phenotypic and genetic characterisation will be required to prove their species status.

Based on comparative 16S rRNA gene sequence analysis, *P. frisingensis* was related to *P. cerevisiiphilus* with 95% similarity (Willems and Collins, 1995). The nearest neighbours to these species were three *Selenomonas* species (*S. lacticifex, S. ruminantium*, *S. sputigena*) (88.4–89.4%) and *Zymophilus paucivorans* (88.2–88.4%). However, some of the relations were not statistically supported (Fig. 1). *P. portalensis* was the most closely related to *P. frisingensis* (Gonzalez *et al.*, 2005). The sequence of long 16S-23S spacer and the order and types of tRNA genes in this region suggested that *P. cerevisiiphilus* and *P. frisingensis* are more closely related to *S. lacticifex* than to *Zymophilus*, whereas short 16S–23S spacer showed equal distances to both (Motoyama and Ogata, 2000a). Ribotyping and flagellin gene analyses indicated that *P. frisingensis* is genetically more diverse than *P. cerevisiiphilus* (Motoyama *et al.*, 1998; Suihko and Haikara, 2001; Chaban *et al.*, 2005). The antibody binding assay to flagellar proteins suggested that *P. frisingensis* is the older of the two species (Chaban *et al.*, 2005).

The genus *Megasphaera* currently comprises the beer-spoilage organism *Megasphaera cerevisiae* together with the ruminal organism *Megasphaera elsdenii* and the clinical species *Megasphaera micronuciformis* (Marchandin *et al.*, 2009). Phylogenetic 16S rRNA gene sequence analysis showed *M. cerevisiae* to be related to *M. elsdenii* and *M. micronuciformis* with 92% and 94.5% similarity (Marchandin *et al.*, 2003). The absolute branching order between these species remained uncertain. The nearest other relatives were *Allisonella histaminiformans*, *Anaeroglobus geminatus* and *Dialister* spp. (Carlier *et al.*, 2002; Marchandin *et al.*, 2003). The use of DNA-based techniques has revealed that biodiversity within the genus *Megasphaera* is still underestimated (Suihko and Haikara, 2001; Zozaya-Hinchliffe *et al.*, 2008).

The genus *Zymophilus* comprises two species, *Z. paucivorans* and *Zymophilus raffinosivorans*. They were closely related to each other at the genomic level as measured by DNA-DNA hybridisation (DDH, 44–48%) and comparative sequence analysis of 16S-23S spacer regions (Schleifer *et al.*, 1990; Motoyama and Ogata, 2000a). The long 16S-23S spacer of *Z. raffinosivorans* contained isoleucine and alanine tRNA genes, whereas that of *Z. paucivorans* had only the latter one. Based on 16S rRNA gene sequence analysis, the closest relatives to *Z. paucivorans* were *Pectinatus* and *Selenomonas* spp. (Fig. 1). Collins *et al.* (1994) considered *Z. paucivorans* to be phylogenetically intermixed with *Selenomonas* species. However, the branching order in their phylogenetic tree was not statistically supported at the corresponding nodes.

S. lacticifex is the only brewery-related species in its genus (Schleifer *et al.,* 1990), whereas the other eight species mainly inhabit oral or ruminal environments (Shouche *et al.*, 2009). In the 16S rRNA gene sequence analysis, the closest relatives to *S. lacticifex* were *S. ruminantium* (93.9%), *Z. paucivorans* (91.9%), *S. sputigena* (90%) and *Pectinatus* spp. (88.4–89%) (Fig. 1). Only the relation to *S. ruminantium* was statistically significant. The analysis of the long 16S-23S spacer sequences suggested that *S. lacticifex* is closer to *Pectinatus* than to *Zymophilus*, whereas the reverse was true in the case of the short 16S-23S spacer (Motoyama and Ogata, 2000a). Hence, phylogenetic relations between the brewery-related *Pectinatus*, *Selenomonas* and *Zymophilus* species are not yet resolved.

The study of Timke *et al*. (2005c) on biofilms from beer bottling plants indicated that nearly half of 78 samples harboured strictly anaerobic bacteria. *Pectinatus* was only detected on a few occasions. It appears that many yet-uncultured strictly anaerobic beer-spoilage bacteria may still exist in this environment.

1.3.2 Occurrence

All the brewery-related species of the *Sporomusa* sub-branch have hitherto been isolated from the beer production process or from spoiled beer. Their natural sources are unknown.

P. cerevisiiphilus and *P. frisingensis* have occurred worldwide – in Finland, Germany, Japan, Norway, the Netherlands, Spain, Sweden and the USA (Lee *et al*., 1978; Schleifer *et al*., 1990; Hage and Wold, 2003; Haikara and Helander, 2006). *P. frisingensis* is the dominant species isolated from the beer production process (Motoyama *et al*., 1998; Suihko and Haikara, 2001; Suiker *et al*., 2007). Most *Pectinatus* isolates originate from unpasteurised packaged beer. Documented spoilage cases due to secondary *Pectinatus* contaminations peaked during the 1980s and the early 1990s, but since have been decreasing. During the time period 1988–2004, the mean contamination rate was 4% in Germany (Back, 2005). The *Pectinatus* bacteria appear to be common inhabitants in brewery bottling hall deposits. In biofilms on bottling machines, *Pectinatus* species were regarded as occasional invaders flourishing on favourable niches rather than permanent biofilm members (Timke *et al*., 2005c). Characterisation of environmental isolates has indicated that several sources may exist in a single brewery (Hakalehto, 2000; Suiker *et al*., 2007). Moreover, *Pectinatus* bacteria have supposedly been detected in pitching yeast and in malt steeping water (Haikara and

Helander, 2006). Interestingly, the discovery of *P. portalensis* from winery effluent water also links this species to the production of alcoholic beverages. Gonzalez *et al*. (2005) suggested that *Pectinatus* bacteria have reservoirs in anaerobic, organic-matter rich habitats related to disposal of alcoholic beverages.

M. cerevisiae appears to be geographically less widespread than *Pectinatus*. Contaminations have been reported in Australia, Finland, Germany, Norway and Sweden (Hage and Wold, 2003; Haikara and Helander, 2006). *M. cerevisiae* shares its ecological niche with *Pectinatus*. It has mainly been found from spoiled beer and from brewery bottling hall deposits. Sporadic findings were reported in pitching yeast and in a brewery CO_2 line (Haikara and Helander, 2006). In Germany, *M. cerevisiae* was responsible for 4.6% and 9.3% of secondary contaminations in 1987 and 1994 (Back *et al*., 1988; Back, 1994).

S. lacticifex, *Z. paucivorans* and *Z. raffinosivorans* have been isolated from pitching yeast in Germany and in Finland. Moreover, brewery waste and drainage system were reported as sources of *Z. raffinosivorans* (Haikara, 1989; Schleifer *et al*., 1990; Seidel-Rüfer, 1990). Seidel-Rüfer (1990) examined more than 3000 yeast samples from German breweries at the end of the 1980s. Of these samples 0–0.03% were contaminated with *S. lacticifex* and 0.12–0.7 % with *Zymophilus*.

Table 2. Discriminatory characteristics of Gram-stain-negative bacteria of the *Sporomusa* sub-branch isolated from the beer production chain. [1,2]

Characteristic	*Megasphaera cerevisiae*	*Pectinatus cerevisiiphilus*	*Pectinatus frisingensis*	*Selenomonas lacticifex*	*Zymophilus paucivorans*	*Zymophilus raffinosivorans*
Curved rods	–	+	+	+	+	+
Cocci	+	–	–	–	–	–
Acid from:						
N-acetyl-glucosamine	ND	–	+	ND	–	+
Cellobiose	–	–	+	+	+	+
Inositol	ND	–	+	–	–	+
Lactose	–	–	–	+	–	+
Maltose	–	–	–	d	+	+
Mannitol	–	+	+	–	+	+
Melibiose	ND	+	–	+	–	+
Ribose	–	+	+	+	+	+
Raffinose	–	–	–	+	–	+
Rhamnose	–	+	+	–	–	+
Sorbitol	–	+	+	–	+	+
Sucrose	–	–	–	+	+	+
Xylitol	ND	–	d	–	–	+
Xylose	–	+	–	+	–	+
Acetoin production	–	+	+	ND	–	–
Succinate production	–	+	+	–	–	–
Lactic acid as the main metabolite	–	–	–	+	–	–
G+C content (mol%)	42–45	38–41	38–41	51–52	39–41	38–41

[1] Symbols: +, positive reaction; –, negative reaction; d, variable reaction; ND, not done, [2] Adapted from Schleifer *et al*. (1990) and Haikara and Helander (2006).

1.3.3 General phenotypic properties

The phenotypic traits useful for distinction between the *Sporomusa* sub-branch brewery contaminants are shown in Table 2. All species stain Gram-negative (Schleifer *et al*., 1990; Haikara and Helander, 2006). *M. cerevisiae*, *P. cerevisiiphilus* and *P. frisingensis* possess a thick peptidoglycan layer reminescent of Gram-stain-positive bacteria and an outer membrane typical of

Gram-stain-negative bacteria (Haikara, 1981; Haikara and Lounatmaa, 1987; Helander *et al*., 2004). *S. lacticifex*, *Z. paucivorans* and *Z. raffinosivorans* use a peptidoglycan structure similar to that of *Pectinatus* (Schleifer *et al*., 1990; Ziola *et al*., 1999). *Pectinatus* cells have a unique comb-like flagellation on only one side of the cell which leads to the formation of an X-pattern during movement (Fig. 2; Lee *et al*., 1978; Haikara *et al*., 1981). Flagellation of *S. lacticifex* or *Zymophilus* species has not been studied. *M. cerevisiae* is a non-motile coccus (Fig. 3).

Figure 2. Electron micrograph of *Pectinatus frisingensis* (Haikara *et al.*, 1981).

Figure 3. Electron micrograph of *Megasphaera cerevisiae* (Haikara and Lounatmaa, 1987).

1.3.4 Beer-spoilage properties

P. cerevisiiphilus and *P. frisingensis* are absolute beer-spoilage bacteria. It is currently not known to what extent beer-spoilage ability varies between the strains. *Pectinatus* mainly spoils unpasteurised packaged beers by producing turbidity and large quantities of propionic acid (> 1000 mg l^{-1}), some acetic acid and sulphur compounds (dimethyl trisulphide, H_2S, methyl mercaptane,). The spoiled product has a smell of rotten eggs that makes it unfit for consumption (Haikara and Helander, 2006).

Many factors control the growth of *Pectinatus* species in beer, including oxygen and ethanol level and acidity. *P. frisingensis* is better adapted to this environment than *P. cerevisiiphilus*, which probably explains its dominance in the breweries. *P. frisingensis* was more oxygen-tolerant (D_{oxy} > 55 h in saturated air, 32°C) than *P. cerevisiiphilus* (D_{oxy} = 3.3 h). Both species were able to grow at the oxygen levels present in packaged beer (0.4–0.8 mg l^{-1}) (Chowdhury *et al*., 1995). *P. frisingensis* grew well in commercial beers with 3.7–4.4% (w/v) alcohol, but not in strong beers (≥ 5.2%, w/v) (Haikara, 1984). In culture medium up to 5.8–8% (w/v) alcohol was tolerated (Tholozan *et al*., 1997). *Pectinatus* species are the most acid-tolerant beer spoilers in the *Sporomusa* sub-branch. *P. frisingensis* showed growth retardation at pH 4.1. The optimum pH for growth was lower than for *P. cerevisiiphilus* (Tholozan *et al*., 1997). Both species tolerate hop bitter acids at levels normally found in beer (Haikara and Helander, 2006).

Pectinatus species are mesophiles that grow at 15–40°C with an optimum at 30–32°C (Lee *et al*., 1978; Schleifer *et al*., 1990). In co-culture with *S. cerevisiae* in wort, *P. cerevisiiphilus* grew to a limited extent even at 8°C. At 15°C, its metabolites started to disturb the yeast growth (Chowdhury *et al*., 1997). Hence, the entry of *Pectinatus* bacteria into the brewing process, e.g. through pitching yeast, could potentially lead to fermentation problems. *P. frisingensis* survived cooling from 30°C to 2°C. It rapidly restored the disturbed cellular homeostasis at 30°C when provided with a carbon source (Chihib and Tholozan, 1999). Temperature influenced the oxygen tolerance of *P. cerevisiiphilus*, which increased at low temperature (Chowdhury *et al*., 1995). Heat resistance studies indicated that the treatments applied in the brewing process are sufficient to inactivate *Pectinatus* cells (Watier *et al*., 1995). However, thermal adaptation may markedly increase the heat-resistance (Flahaut *et al*., 2000).

Pectinatus species are fermentative organisms. They thrived well on glucose, fructose and lactate. *P. frisingensis* used a wider range of carbohydrates than *P. cerevisiiphilus* (Schleifer *et al*., 1990). Ethanol, maltose and the main amino acids of beer were not metabolised (Schleifer *et al*., 1990, Tholozan *et al*., 1996). However, most *P. frisingensis* isolates from Japanese breweries were able to grow on maltose (Motoyama *et al*., 1998).

There is less data about spoilage properties of the other species. *M. cerevisiae* is an absolute spoilage species that mainly affects unpasteurised low-alcohol products. It produces copious amount of butyric acid with minor amounts of C5 and C6 fatty acids and H_2S, which cause a very unpleasant smell (Haikara and Lounatmaa, 1987). The contaminated beer normally becomes turbid in 4–6 weeks. The growth rate of *M. cerevisiae* was reduced above 2.1% (w/v) of alcohol and was completely inhibited at 4.2% (w/v) (Haikara and Lounatmaa, 1987). *M. cerevisiae* is rather acid-sensitive. The spoilage rate was reduced at the pH of normal beer and no gowth was detected at pH 4.0–4.1 (Haikara and Helander, 2006). The growth temperature in culture medium ranged from 15 to 37°C (Haikara, 1989). In simulated wort fermentation, limited growth was detected at 8°C (Watier *et al*., 1996). In the case of heavy primary contamination, flash pasteurisation might not eliminate the risk of spoilage (Watier *et al*., 1996). *M. cerevisiae* strains formed a uniform group in terms of utilised carbon sources that included arabinose, fructose, lactate and pyruvate (Engelmann and Weiss, 1985).

S. lacticifex was considered by Seidel-Rüfer (1990) to be an absolute spoilage species, since it grew in beer at pH 4.3–4.6. Since no beer spoilage incidents caused by this species have been reported, it could be considered a potential spoiler. In a culture medium, *S. lacticifex* was more sensitive to low pH than *M. cerevisiae* or *Pectinatus*. *S. lacticifex* still grew at the low temperature of yeast storage (10°C), although its optimum was around 30°C. It utilised a wide range of carbon sources, including arabinose, cellobiose, glucose, lactate and maltose (Schleifer *et al*., 1990).

Z. raffinosivorans was considered to be a potential spoiler owing to its ability to grow in beer at pH 5.0, but not at pH 4.6. *Z. paucivorans* was able to grow in beer at pH 6.0, but not at pH 5.0, and it was considered to be a rather harmless brewery contaminant (Seidel-Rüfer, 1990). Both species grew optimally at 30°C. Growth did not occur at 37°C. *Z. raffinosivorans* was able to utilise a greater variety of carbon substrates than *Z. paucivorans* (Schleifer *et al*., 1990).

1.3.5 Traditional detection and identification techniques

Every beer production stage needs at least occasional microbiological monitoring. Nowadays, the main emphasis of microbiological quality assurance is on preventive measures (Back *et al.*, 1988; Back, 2005). Product analysis plays a role in verifying the microbiological quality of unpasteurised beer that is most prone to contamination during the filling stage. In addition to the absolute and potential spoilage organisms, the detection of indicator microbes can be useful as it provides an early indication of a process failure or development of poor hygienic conditions (Back, 2005).

Official microbiological specifications for brewery samples do not exist. It is generally regarded that even a low level of spoilage microbes constitutes a risk because of the long process time and shelf-life of beer (Jespersen and Jakobsen, 1996). The microbiological guidelines suggested for unpasteurised beer range from zero to 50 cfu in 100–250 ml^{-1} (Jespersen and Jakobsen, 1996; Back, 2005). In pitching yeast and in process samples before beer filtration, a single spoilage organism amongst 10^6–10^8 cultivation yeast cells or per ml should be detected. These guidelines are based on the detection limit of cultivation methods rather than on data about harmful levels.

Breweries mainly rely on classical cultivation methods combined with basic phenotypic characterisation of isolates. How the sample is processed depends on the organism sought, the production stage, and available facilities and resources.

The detection of microbial contaminants in beer usually involves collecting cells from the samples (100–500 ml) by membrane filtration followed by plating on selective or non-selective media. This procedure has been shown to reduce drastically the viability of the oxygen-sensitive *Megasphaera* and *Pectinatus* bacteria (Haikara, 1985a). Therefore, the only reliable cultivation methods available in most breweries are a shelf-life test and a "forcing test" in which packaged beer is incubated without and with a concentrated medium with subsequent monitoring of haze formation (Haikara and Helander, 2006). The shelf-life test samples are incubated at an ambient temperature for up to six weeks. Forcing with nutrients at an elevated temperature (27–30°C) allows shortening of the incubation time to two weeks. Concentrated MRS (de Man Rogosa Sharpe) and NBB-C (Nachweismedium für Bierschädliche Bakterien) media are recommended for the forcing test in EBC *Analytica Microbiologica* (Hage *et al.*, 2005). The selectivity of NBB-C medium can be modified towards absolute, potential or indirect spoilage bacteria by varying the ratio of the medium and

water added to the beer (Back, 2005). *M. cerevisiae* and *Pectinatus* bacteria can be selectively enriched in beer using SMMP (Selective Medium for *Megasphaera* and *Pectinatus*) medium (Lee, 1994). For SMMP enrichment 14 d incubation at 28–30°C is advised (Hage *et al*., 2005). *M. cerevisiae* turns the medium colour from purple/violet to yellow (Anon., 1998). The detection of the strict anaerobes in turbid process samples relies on microscopic examination after a fixed enrichment period. The *Sporomusa* sub-branch beer-spoilage bacteria are easy to cultivate in an anaerobic glove box using several common media, such as MRS, NBB, and PYF (peptone yeast extract fructose) media. *M. cerevisiae* does not utilise glucose and commercial media (e.g., MRS medium) need to be supplemented with 1% fructose or lactate to support good growth (Haikara and Helander, 2006).

Sensory evaluation and microscopy are used to confirm and to characterise microbial growth in the positive samples. The presence of the strictly anaerobic beer spoilers is suspected when Gram-stain-negative cells with a typical morphology and foul-smelling compounds are detected. These characteristics may be misleading, as the cell morphology may vary with age of the culture (Lee *et al*., 1978; Haikara and Helander, 2006) and the typical smell may be masked due to a mixed contamination. The breweries have rarely experience or facilities to cultivate anaerobic bacteria. Hence, further identification and characterisation are usually outsourced.

The major limitation of cultivation methods is their long incubation time. The results are not available early enough to make decisions i.e. about the reuse of brewer's yeast, cleaning of equipment or beer dispatch. Furthermore, the cultivation methods do not allow identification or tracing of the contaminant. Moreover, culture enrichment is poorly applicable to the analysis of naturally turbid process samples. Upon exposure to harsh conditions, bacteria may also enter into a viable but non-culturable state that cannot be detected on the standard media but may be able to grow in beer (Amann *et al*., 1995; Suzuki *et al*., 2006a). Moreover, unknown species that have never been cultivated due to lack of suitable methods abound in nature (Amann *et al*., 1995). Despite these drawbacks, the cultivation methods are simple and sensitive and allow distinction between viable and dead cells. They are valuable for following trends, giving an indication of emerging problems. The shelf-life test is currently the most reliable method for predicting beer spoilage.

The standard phenotypic identification of anaerobic bacteria involves characterisation of pure culture isolates using physiological, morphological and bio-

chemical tests (Holdeman *et al.*, 1977, Jousimies-Somer *et al.*, 2002). It is a slow and laborious process (1–2 weeks) that often leads to unclear or incorrect results due to intraspecific variation in many phenotypic traits and the impact of culture condition on the phenotype. Moreover, the number of standard tests is often inadequate to account for existing biodiversity. Hence, new and known species can remain hidden (Suihko and Haikara, 2001; Marchandin *et al.*, 2003). To facilitate and speed up the phenotypic identification, various tests have been miniaturised or automated (Priest, 2003b). With the exception of the BIOLOG system (www.biolog.com), their identification databases do not include the anaerobic beer-spoilage bacteria. However, they offer a standardised, convenient and rapid tool for isolate characterisation (Haikara and Lounatmaa, 1987; Marchandin *et al.*, 2003).

1.3.6 Alternative detection and identification techniques

Numerous alternative detection and identification methods have been developed with the aim of improving microbiological testing in terms of speed, specificity, throughput and convenience. They are based on the visualisation of cells or microcolonies or on the analysis of cellular metabolites or constituents (for reviews see Priest, 2003b; Russell and Stewart, 2003; Storgårds *et al.*, 2006).

Fluorescence microscopy has been applied to monitor cells or microcolonies on membrane filters after beer filtration. In direct epifluorescence filter technique (DEFT), collected cells are stained with fluorescent viability dyes for visualisation under a microscope (Storgårds *et al.*, 2006). Beer samples require culture enrichment, since the practical detection limit of the technique is *ca.* 10^3 cells (Storgårds *et al.*, 2006). Using DEFT, time savings of 1–3 days were obtained in the detection of low levels of *M. cerevisiae* and *Pectinatus* (Haikara, 1985b). Due to the limited information content of the analysis and the tediousness of microscopy, DEFT has been superseded by other methods in brewing microbiology. Microcolonies of beer-spoilage bacteria have been visualised using ATP-driven bioluminescence before they are visible to the naked eye. An automated image analysis system was able to detect microcolonies of *P. frisingensis* after 2 days of cultivation (Nakakita *et al.*, 2002).

Metabolites of *M. cerevisiae* and *Pectinatus* species are unique among the typical beer-spoilage bacteria (Table 1). Metabolite analysis by gas chromatography has proved useful for identification of the growth of *Megasphaera* and *Pectinatus* in culture media and in beer, especially when cells are not anymore

culturable (Haikara and Helander, 2006). Gas chromatographic analysis of cellular fatty acids (CFA) has also been applied to the detection and identification of *Megasphaera* and *Pectinatus* in spoiled beer (Helander *et al*., 2004). Timke *et al*. (2005c) used the main CFA of the *Pectinatus* spp., 13:1(3OH), as their biomarker in brewery biofilms. Moreover, CFA analysis was found to be a useful taxonomic tool for the *Sporomusa* sub-branch bacteria (Moore *et al*., 1994). A commercial system based on the technique is available for general identification of bacteria (Priest, 2003b). The CFA analysis requires rigorous standardisation of culture conditions for reliable results.

Immunoassays are based on the specific antibody-antigen interaction. Several assay formats have been developed to detect this reaction (Russell and Stewart, 2003). The immunological characteristics of *M. cerevisiae*, *P. cerevisiiphilus* and *P. frisingensis* cells have been studied for their detection, identification and typing using polyclonal or monoclonal antibodies or synthetic peptides (Gares *et al.*, 1993; Ziola *et al*., 1999 and 2000; Hakalehto, 2000; Haikara and Helander, 2006). A specific immunofluorescence filter assay for *P. cerevisiiphilus* was able to detect 2–4 cells in 10 ml of beer in less than 3 h (Gares *et al*., 1993). Compared to nucleic acid probes, the production and selection of specific antibodies is still difficult and expensive (Priest, 2003b). Microscopy-based immunoassays are also rather tedious when manually performed. To overcome this drawback, various automated imaging systems are now available (Storgårds *et al*., 2006).

1.4 DNA-based techniques for studying prokaryotic diversity in brewery samples

DNA-based techniques have revolutionized our view of prokaryotic diversity by allowing us to study the hereditary material of cells (Amann *et al*., 1995; Achtman and Wagner, 2008). The following literature review deals with the DNA-based techniques applied for the detection, identification and characterisation of beer-spoilage bacteria, with emphasis on the *Sporomusa* sub-branch group. Some techniques not yet applied in brewing microbiology will also be presented.

1.4.1 Prokaryotic genome as a target for DNA analysis

The known prokaryotic genomes range in size from 0.16 to 10 Mb, the largest one being twice as big as the smallest eukaryotic genome (Medini *et al*., 2008).

M. elsdenii and *M. micronuciformis* (the close relatives of *M. cerevisiae*) have genomes of 1.8 Mb and 2.6 Mb, respectively (Marchandin *et al*., 2003). Genetic forces including nucleotide substitutions, gene duplications, horizontal gene transfer, gene losses and chromosomal rearrangements modify prokaryotic genomes continuously (Coenye *et al*., 2005). The rates at which these changes occur vary between different genomic parts and between different species. The prokaryotic genomes are basically composed of conserved core sequences for essential cell functions (replication, transcription and translation) and of dispensable sequences for environmental adaptation (Medini *et al*., 2008). Only a few core genes are highly conserved, a much larger set are moderately conserved and an even greater number are narrowly distributed (Medini *et al*., 2008). A substantial part of the dispensable material comprises selfish, horizontally mobile elements, such as bacteriophages, plasmids and transposons with associated genes. They may carry genetic information over the species borders (Maiden, 2006; Medini *et al*., 2008). Horizontal elements do not share a recent ancestor and are not applicable to phylogenetic studies (Ludwig, 2007). The genomes also contain dispersed repetitive elements that may promote genomic plasticity in bacteria.

Ribosomal ribonucleic acids (rRNA), i.e. 5S (~120 bp), 16S (~1500 bp) and 23S (~3000 bp), are structural and functional components of ribosomes (Bouchet *et al*., 2008). These rRNA species are essential for efficient functioning of cellular protein synthesis machinery. The conserved core genes encoding for rRNA are usually organized into a single co-transcibed *rrn* operon in the order 16S-23S-5S. Intergenic spacer regions of variable length separate the genes from each other (Garcia-Martinez *et al*., 1999). Their length differences mainly derive from presence-absence of functional units, such as tRNA genes. The other parts are non-essential, more rapidly evolving sequences (Garcia-Martinez *et al*., 1999). Different species have 1–15 *rrn* operons that may show substantial intraspecific divergence. *M. elsdenii* has seven and *M. micronuciformis* four operons (Marchandin *et al*., 2003). *P. cerevisiiphilus*, *P. frisingensis*, *S. lacticifex* and *Zymophilus* carry at least two different-sized operons. The longer one has 1–2 tRNA genes (Motoyama and Ogata, 2000a). The copy number within a species is usually constant (Bouchet *et al*., 2008).

The 16S rRNA gene is the most widely used target in general identification and systematics of prokaryotes. It fulfills well the basic requirements of a universal phylogenetic marker, i.e. ubiquitous distribution among bacteria, conserved function, presence of both conserved and highly variable regions, suffi-

cient information content and low frequency of recombination and horizontal gene transfer. It covers evolutionary history from species up to domain level; the fastest evolving sites were estimated to substitute at rates more than 1000 times faster than the slowest ones (Van de Peer *et al*., 1996). Moreover, a vast public database of more than 500,000 sequences is currently available in the Internet for data extraction (Amann and Fuchs, 2008).

1.4.2 Overview of techniques

DNA-based techniques applied for studying brewery-related bacteria are shown in Fig. 4. They can be divided into direct approaches that target (probe methods) or determine (comparative sequence analysis) specific sequence stretches, and indirect procedures (pattern techniques, whole genome similarity and composition) that provide differentiating data without the need to know the primary nucleotide order (Ludwig, 2007). Depending on the technique, the diversity can be studied directly from the sample material (culture-idenpendent) or after cultivation (culture-dependent).

   DNA-based techniques differ from phenotypic methods in many ways. They often provide more reliable identification, being independent of the influence of single mutable phenotypic traits and culture conditions on the results. They also allow discovery and identification of bacteria that lack a distinctive phenotype. Moreover, more rapid and reliable detection and identification of fastidious, slow-growing or rare species, even unculturable ones, is possible (Amann *et al*., 1995; Suihko and Haikara, 2001; Marchandin *et al*., 2003; Clarridge, 2004). DNA analysis also allows for elucidating evolutionary relationships and building of more natural taxonomic classification systems (Ludwig, 2007; Achtman and Wagner, 2008).

   No single perfect technique for studying microbial diversity in brewery samples exists. The choice depends on the application, available facilities and resources and the target organism. Different techniques usually complement each other. Ease of use and low costs of design and synthesis have made the probe methods based on short synthetic DNA stretches preferred tools for the specific detection of bacteria. There are two major types of probe techniques, i.e. amplification and hybridisation.
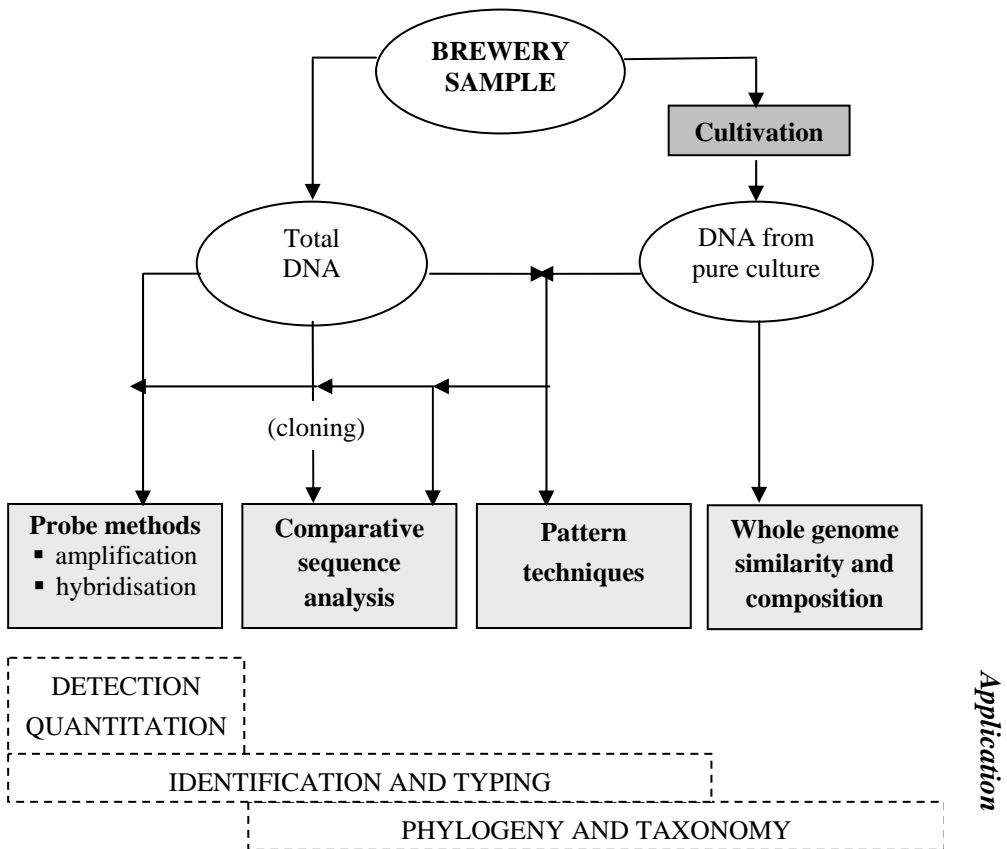
Figure 4. Overview of DNA-based techniques for studying bacterial diversity in brewery samples.

1.5 Amplification-based probe techniques for beer-spoilage bacteria

1.5.1 PCR principle

Although invented as long ago as 1985 (Mullis *et al.*, 1986), PCR is still one of the most powerful and widely used technique for the detection, characterisation and identification of bacteria; either used as a stand-alone method or as a prepa-rative tool for increasing assay sensitivity or for delineating target locus.

PCR is essentially an exponential DNA synthesis reaction in a test tube, by which a particular DNA fragment can specifically be amplified (Mullis *et al.*, 1986). The initiation points of the new strands are flanked by a pair of short

oligonucleotide probes (usually 15–25 bp), called primers, each of which hybrid-ises in a 5' to 3'direction to its complementary site on the opposite strands. New DNA is synthesised in a 3-step thermocyclic process in order to provide the op-timum temperature for each step. First, double-stranded DNA (dsDNA) is dena-tured at a high temperature (above 90°). Second, the primers are annealed at the design-specific temperature. Third, a thermostable DNA polymerase extends the primed region at around 70°C. In reality, the various steps are partly overlap-ping. Due to the wide thermal range of activity of the DNA polymerases, anneal-ing and extension steps can also be perfomed at one temperature (Mackay *et al.*, 2007). The number of PCR cycles varies from 25 to 40. In an ideal case, the target fragment is doubled in each cycle, i.e. the PCR efficiency is two. A 10^{12}-fold amplification of the target may be reached in less than one hour. Hence, PCR is potentially a highly specific, sensitive and rapid technique for the detec-tion of low levels of specific microbes.

Basic PCR components include a thermostable DNA polymerase, a suitable buffer containing Mg^{2+} (cofactor), deoxyribonucleotides and one or more prim-ers with a desired specificity (Saiki, 1990). The efficacy of PCR is determined by its efficiency, fidelity and specificity, which are in turn influenced by many factors including target length and sequence, primer design, DNA polymerase, buffer composition and sample impurities. A number of modifications of the basic reaction conditions and technique have been developed to enhance the efficacy, such as the use of hot start, proof-reading enzymes, competitor probes, PCR enhancers and nested and touch-down PCR techniques (Don *et al.*, 1991; Rådström *et al.*, 2004; Wolffs *et al.*, 2004; Mothershed and Whitney, 2006). For a review of the primer design and the PCR optimisation see Innis and Gelfand (1990). Today, several bioinformatic tools for primer design are freely accessi-ble on the Internet (Albuquerque *et al.*, 2009). When using PCR, one should bear in mind that it can create sequence artifacts due to the formation of chimera or heteroduplexes or due to polymerase errors. PCR may also skew the template to product ratios in a multitemplate PCR due to the unequal amplification efficien-cies of different templates (Acinas *et al.*, 2005).

1.5.2 PCR detection formats

PCR detection formats can be categorised to end-point and real-time PCR ac-cording to the time point of the product detection. In end-point PCR, the DNA is first amplified and the PCR products are examined after the reaction has been

completed. The detection usually involves size separation of the products using agarose or polyacrylamide gel electrophoresis followed by band visualisation with DNA-specific fluorescent dyes, such as ethidium bromide or SYBR Green I (Mackay *et al*., 2007). Using capillary electrophoresis, even a single-base size difference can be detected. There are also several techniques to detect and verify the products by their sequence. In solution hybridisation or PCR-ELISA (Enzyme-Linked Immunosorbent Assay), PCR products are hybridised to a complementary DNA probe while immobilised on microtitre plate wells (Mothershed and Whitney, 2006). The resulting hybrids can be visualised and quantified using an enzyme-labelled reporter molecule and a signal-producing substrate. Several variations of the procedure exist. The PCR-ELISA has potential to improve the end-point detection in many ways (Soumet *et al*., 1995; Koskiniemi *et al*., 1997). It can be more sensitive and objective than agarose gel electrophoresis and is easier and faster than Southern hybridisation. Moreover, the procedure is amenable to automation, allows high sample throughput and does not require toxic reagents.

The real-time PCR (also called kinetic, quantitative, online, and homogenous PCR) was introduced in 1993 (for a review see Mackay *et al*., 2007). It integrates amplification, detection and quantification of the target fragments into a single closed-tube assay. The whole assay can be completed in 0.5–2 h. The amount of the PCR products is measured cycle by cycle using fluorescent DNA labels and a thermocycler with an integrated fluorimeter. The time point at which fluorescence exceeds the background noise level (10^{10}–10^{11} product copies) is called the crossing point value (C_p) or threshold cycle. It is proportional to the initial template concentration allowing quantification. The fewer templates the reaction vessel contains, the longer it takes to reach this value.

The real-time PCR fluorescence signal can be generated using dsDNA-associating dyes or fluorogenic probes or both (Mackay *et al*., 2007). The dyes provide an inexpensive and easy approach to monitor real-time accumulation of any PCR product. Most dyes bind to the minor grooves of dsDNA, which leads to enhancement of their fluorescence. Different products, including primer dimers, can usually be discriminated by measuring their melting point temperature (T_m, the temperature at which 50% of dsDNA is denatured). T_m depends on the size and on the nucleotide distribution and content of the products (Ririe *et al*., 1997). The melting data can be acquired after PCR in a few minutes by continuous measurement of the reaction fluorescence while temperature is increased. SYBR Green I (emission 495 nm, excitation 537 nm) is the most widely applied

dye for the real-time PCR detection of microbes in food (McKillip and Drake, 2004). However, it may inhibit PCR and interfere with DNA melting as well as preferentially bind to GC-rich areas. Furthermore, it has low stability in dilute solutions. Several alternatives have been proposed, but they have not yet found wide usage (Gudnason *et al*., 2007). Most commercial PCR reagent mixtures rely on SYBR Green I.

Fluorogenic probes allow confirmation of the PCR product identity by sequence and multiplexing by colour. Detection is based on measurement of the change in fluorescence due to the probe hybridisation or hydrolysis, or due to dye incorporation into the product. Many fluorogenic probe chemistries rely on fluorescence resonance energy transfer (FRET) between two closely situated (1–10 Å) fluorophores or a fluorophore and a non-fluorescent quencher with an overlapping emission and absorption spectrum (Mackay *et al*., 2007). This permits hybrid quantitation without removing unbound probe. The widely used hydrolysis probes (also known as TaqMan, dual-labelled and 5'-nuclease probes) carry a reporter fluorophore and a quencher at the opposite ends. When attached to the probe, fluorescence from the reporter is absorbed by the quencher. The 5'-nuclease activity of DNA polymerase hydrolyses the probe during the extension phase, releasing fluorescence from the reporter. Hybridisation probe systems are composed of a pair of fluorogenic probes that hybridise adjacent to each other, leading to excitation of the reporter by the donor fluorophore via FRET. They allow product characterisation by melting curve analysis (MCA). Fluorogenic probes, especially less common designs, are more expensive than DNA-associating dyes. They may also lead to false negative results from target strains that exhibit a few nucleotide mismatches in the probe-binding region.

The first real-time PCR instrument was launched in 1996. A large selection of systems is currently available (Espy *et al*., 2006). They are normally composed of a fluorescence measuring thermocycler, a computer and a software for operation and data analysis. A LightCycler® was the first instrument based on rapid-cycle PCR. It has the capability to run 30 cycles in 10–15 min (Wittwer *et al*., 1997). The rapid cycling rate (20°C s⁻¹) is achieved by using glass-plastic composite capillaries with a high surface to volume ratio and an air-heated fan. The reaction is kinetic rather than step-wise in that the temperature may always be changing (Wittwer *et al*., 1997). Most other instruments use thermoelectrically heated metal blocks holding plastic tubes with a high thermal mass. They generally need longer cycling times (Espy *et al*., 2006). Options for MCA and 3–4 fluorescence channels are standard features in modern instruments. The configu-

ration with 384-well blocks essentially enables a low-density array set-up. Nanoplate systems accommodate up to 3,072 reactions in a device with the size of a standard microscope slide (Brenan *et al*., 2009). Downscaling of a PCR thermocycler on a microchip shortened the run time to a few minutes (Pipper *et al*., 2008).

1.5.3 PCR primer and probe systems for beer-spoilage bacteria

Primers mainly define the PCR specificity. They can be designed to amplify almost any fragment of a structural or functional gene. The *rrn* operon, especially the 16S rRNA gene and 16S-23S spacer region, has been the most widely used target for developing PCR tests for beer-spoilage bacteria in various taxonomic ranks (Tables 3 and 4). Primer sets have been published for the most common beer-spoilage species or genera. Furthermore, PCR applications exist for a few bacterial groups relevant to the brewing industry. Haakensen *et al*. (2008b) developed a PCR test to detect *Firmicutes* in brewery samples. This phylum includes at least 40 potential beer-spoilage species. Bischoff *et al.* (2001) applied a universal PCR together with restriction fragment length polymorphism (RFLP) to detect and identify beer contaminants. The assay was evaluated with three lactic acid bacteria (LAB). Several primer sets have also been designed for the group-specific detection of LAB in brewery samples, in wine, in food or in the gut (Stewart and Dowhanick, 1996; Walter *et al*., 2001; Heilig *et al*., 2002; Lopez *et al*., 2003; Neeley *et al*., 2005; Renouf *et al*., 2006).

   Beer-spoilage ability of LAB is a strain-specific feature that is only broadly associated with species status. In contrast, beer-spoilage ability has been found to correlate well with an organism's ability to resist hop bitter acids (Suzuki e*t al*., 2006b). Improved understanding of the genetic basis of hop resistance has enabled the design of PCR tests to disinguish between potential spoilage and non-spoilage LAB strains (Table 4). However, many spoilage strains lacking the known genetic markers of hop resistance still exist. When assaying for functional genes, it must also be remembered that their presence only shows a genetic potential – the genes may be non-functional or may not be expressed.

Table 3. DNA amplification techniques for *Sporomusa* sub-branch beer-spoilage bacteria.

Target organism	Target gene	Product size, bp	Tech-nique	Application	Pre-PCR processing	Assay sensi-tivity and time	Comments	Ref.
Pectinatus	16S rRNA	815	EP-PCR	Beer	Filtration through PCM, enzymatic lysis, phenol-chloroform extraction, ethanol precipitation.	10 h: 2×10^3 cfu 100 ml^{-1}		1
M. cerevisiae *Pectinatus* DSM 20764 *P. cerevisiiphilus* *P. frisingensis*	16S rRNA	385 393 443 74	EP-PCR	Pure culture	No data.	No data	Universal primer set as an internal positive control.	2
P. cerevisiiphilus *P. frisingensis*	16S rRNA and 16S-23S spacer	~600 ~1000+ 1200	EP-PCR	Pure culture	Enzymatic lysis, CTAB treatment, phenol-chloroform extraction, acetate and alcohol precipitations.	No data		3
Pectinatus	16S rRNA	NA	LAMP	Pure culture	Prepman Ultra Sample preparation reagent.	No data	Detection by real-time turbidimeter.	4
P. cerevisiiphilus *P. frisingensis*	16S rRNA and 16S-23S spacer	621 701+883	Multiplex EP-PCR	Pure culture	"	No data	Artificial positive control DNA.	5
M. cerevisiae *Pediococcus claussenii* *Pediococcus damnosus* *Pediococcus inopinatus*	16S rRNA	452 462 566 566	Multiplex EP-PCR	Pure culture	"	1×10^2 cfu ml^{-1}	"	5
P. cerevisiiphilus *Pectinatus haikarae* *P. frisingensis*	16S rRNA and 16S-23S spacer	621 508 701+883	Multiplex EP-PCR	Pure culture	"	No data	Modified from ref. 5.	5
M. cerevisiae *Megasphaera paucivorans/ Megasphaera sueciensis*	16S rRNA	452 155	Multiplex EP-PCR	Pure culture	"	No data	Modified from ref. 5.	6

[1]Satokari *et al.* (1997), [2]Sakamoto *et al.* (1997), [3]Motoyama and Ogata (2000b), [4]Tsuchiya *et al.* (2007), [5]Asano *et al.* (2008), [6]Iijima *et al.* (2008), Symbols: NA, not applicable; EP-PCR, end-point PCR with agarose gel electrophoresis; LAMP, loop-mediated isothermal amplification; PCM, polycarbonate membrane; CTAB, cetyltrimethylammonium bromide.

Table 4.DNA amplification techniques for other beer-spoilage bacteria.

Target organism	Target gene	Product size, bp	Technique	Application	Pre-PCR processing	Assay sensitivity and time	Comments	Ref.
Lactobacillus brevis	5S rRNA	117	EP-PCR	Beer	Filtration through Durapore, recovery of cells in ethanol with evaporation, enzymatic cell lysis, phenol extraction and ethanol precipitation.	11 h: 3x10^1 cfu 250 ml^{-1}	Primers found later to be unspecific.	1
L. brevis	5S rRNA	117	EP-PCR	Beer	As above but filtration through Isopore, sonication, addition of coprecipitant and *Pfu* polymerase instead of *Taq* polymerase.	11 h: 1 cfu 250 ml^{-1}		2
L. brevis *Lactobacillus casei* *Lactobacillus plantarum*	16S rRNA	739 316 590	EP-PCR	Beer	Filtration through PCM, membrane dissolution in chloroform, cell recovery in water phase, centrifugation, water wash.	6 h: 2x10^1 cells ml^{-1}	Multiplexing gave anomalous products, PCR inhibition.	3
L. brevis *Lactobacillus lindneri*	16S rRNA	~900 ~900	EP-PCR	Pure culture	No data.	No data	-	4
LAB	16S rRNA	764	Nested EP-PCR	Yeast slurry	1 h: Centrifugation, InstaGene Matrix.	8 h: 1:2x10^4 (bacteria:yeast)	PCR inhibition.	5
Beer-spoilage lactobacilli	*horA*	342	EP-PCR	Pure culture	Enzymatic lysis, CTAB and chloroform extractions, acetate and alcohol precipitations.	1x10^5 cfu ml^{-1}	Good correlation with beer-spoilage ability.	6
L. lindneri	16S rRNA	~1000	EP-PCR	Beer	2.15 h: Filtration through Durapore, wash with 0.1M NaOH, 0.5% SDS and water, enzymatic lysis, phenol-chloroform extraction, ethanol precipitation.	7.5–8 h: 6x10^1 cfu 100 ml^{-1}	PCR inhibition.	7
L. brevis *L. casei* *Lactobacillus coryniformis* *L. plantarum*	16S rRNA	861 735 453 731	EP-PCR	Pure culture	No data.	No data		8
Eubacteria	16S-23S spacer	Varies	EP-PCR and RFLP	Beer	(30–40 h enrichment), centrifugation, Chelex-100 and proteinase K, Triton X-100, heating.	1x10^3 cfu 50 ml^{-1} 1 cfu in bottle (enrichment)	Identification by RFLP analysis.	9
Obesumbacterium proteus biotype II	16S rRNA	~435	EP-PCR	Wort and yeast slurry	Centrifugation (2500 x *g*), Promega DNA extraction kit.	1x10^4 cells ml^{-1} 1x10^7 cells ml^{-1}		10

Target organism	Target gene	Product size, bp	Technique	Application	Pre-PCR processing	Assay sensitivity and time	Comments	Ref.
O. proteus biotype I	16S rRNA	422, 481	EP-PCR and qPCR	Beer; Yeast slurry	Filtration through PCM, membrane dissolution in chloroform, cell recovery in water, heating. Centrifugation, heating.	$2 \times 10^2 – 2 \times 10^3$ cfu 100 ml^{-1}; $2 \times 10^3 – 2 \times 10^4$ cfu per 2×10^8 yeast cells		11
Lactobacillus paracollinoides	16S-23S spacer	270, 480	EP-PCR	Pure culture	As in ref. 6.	No data		12
Beer-spoilage LAB	*horB*, *horC*		EP-PCR	Pure culture	As in ref. 6.	5×10^1 cfu ml^{-1}	49/51 spoilers had *horB/horC*.	13
Beer-spoilage LAB	*horA*	543	qPCR	Pure culture	As in ref. 6.	1×10^2 cfu ml^{-1}		14
Beer-spoilage LAB	*horA*	198	qPCR	Beer	Filtration through Durapore, PureGene DNA purification.	$1 \times 10^2 – 2 \times 10^2$ cfu 100 ml^{-1}		15
Beer-spoilage LAB	*hitA*, *horA*, *horC*, ORF5, 16S rRNA	179, 210, 98, 117, 148	Multiplex EP-PCR	Pure culture	As in ref. 15.	No data	Only *horA* predicts beer-spoilage ability.	16
Firmicutes	16S rRNA		Multiplex qPCR	Beer	Filtration through Durapore membrane, (16 h enrichment in MRS), DNA isolation using Puregene DNA Purification System DNA.	$5 \times 10^1 – 1 \times 10^2$ cfu 100 ml^{-1} (*P. damnosus*) 3–10 cfu 341 ml^{-1} (with enrichment)	Multiplexed with universal eubacterial primers.	17
L. brevis *L. casei* *L. coryniformis* *L. lindneri* *L. paracollinoides* *L. plantarum*	16S rRNA	861, 854, 453, 850, 729, 490	Multiplex EP-PCR	Pure culture	"	1×10^3 cfu ml^{-1}	"	18
P. damnosus *L. brevis* *L. lindneri*	16S rRNA *gyrB* *gyrB*	NA	LAMP	Pure culture	Prepman Ultra Sample preparation reagent.	No data	Detection by real-time turbidimeter.	19

[1]Tsuchiya *et al.* (1992), [2]Tsuchiya *et al.* (1993), [3]DiMichele and Lewis (1993), [4]Taguchi *et al.* (1995), [5]Stewart and Dowhanick (1996), [6]Sami *et al.* (1997), [7]Yasui *et al.* (1997), [8]Sakamoto *et al.* (1997), [9]Bischoff *et al.* (2001), [10]Maugueret and Walker (2002), [11]Koivula *et al.* (2006), [12]Suzuki *et al.* (2004), [13]Suzuki *et al.* (2005), [14]Suzuki *et al.* (2006c), [15]Haakensen *et al.* (2007), [16]Haakensen *et al.* (2008a), [17]Haakensen *et al.* (2008b), [18]Asano *et al.* (2008), [19]Tsuchiya *et al.* (2007) Symbols: NaOH, sodium hydroxide; qPCR, real-time PCR; SDS; sodium dodecyl sulphate.

Until recently, PCR analysis of beer-spoilage bacteria involved the detection of a single species or genus at a time (Tables 3 and 4). Multiplex PCR is a variation of PCR in which several loci are amplified in a single reaction. Asano *et al.* (2008) designed multiplex PCR tests for the identification of beer-spoilage cocci, *Pectinatus* species and common beer-spoilage lactobacilli. The multiplex PCRs showed same sensitivities as their simplex counterparts. PCR performance was controlled using an artificial control DNA. The multiplex tests were recently modified also to detect the new *Megasphaera* and *Pectinatus* species (Iijima *et al.*, 2008). Sakamoto *et al.* (1997) multiplexed *M. cerevisiae* and *Pectinatus* specific primer sets with a universal primer set in order to detect possible PCR failure. However, the samples should always contain bacterial DNA for the internal standard to work. Primer sets for various hop-resistance genes were recently incorporated into a single assay, but only the presence of *hor*A correlated with beer-spoilage ability (Haakensen *et al.*, 2008a).

The non-commercial PCR applications for *Sporomusa* sub-branch beer-spoilage bacteria are currently based on end-point PCR and agarose gel electrophoresis for the product detection (Table 3). The use of other PCR detection formats, such as PCR-ELISA or real-time PCR, could facilitate the implementation of the PCR technique in brewery QC by providing a higher automation level and sample throughput, more objective data interpretation and better work safety.

Three suppliers currently provide PCR kits for beer-spoilage bacteria (Table 5). Kits are available both for screening brewery samples for a range of bacterial species and for the species identification. They all include internal amplification controls and a decontamination system for old PCR products. A Foodproof® Beer Screening kit uses a mixture of primers and hybridisation probes based on conserved and variable 16S-23S spacer regions. It allows concurrent detection of 25 common spoilage bacteria (Kiehne *et al.*, 2005). The bacteria can be further identified using MCA. The kits from Gen-ial mainly use SYBR Green I for the product detection. A new First Beer P1 HybProbe kit is based on hybridisation probes and detects both beer-spoilage bacteria and yeasts in a single test (Schönling *et al.*, 2007). PIKA GmbH offers hydrolysis probe-based identification and screening kits for enterobacteria, LAB, *Megasphaera*, *Pectinatus* and yeasts for use in a microtitre plate format. The reactions follow identical temperature programmes and thus can be run in parallel (Haikara *et al.*, 2003).

Table 5. Commercial DNA-based test kits for beer-spoilage bacteria.

Kit name	Target organism	Technique	Analysis time, h (no enrichment)	Sensitivity, cfu ml^{-1}	Manufac-turer
Beer-spoilage [1]	8 *Lactobacillus* 1 *Megasphaera* 3 *Pectinatus* 2 *Pediococcus*	qPCR, EP-PCR	2–4.5	*ca*. 10^1–10^2	Pika GmbH
foodproof® Beer Screening kit [1]	15 *Lactobacillus* 1 *Megasphaera* 3 *Pectinatus* 6 *Pediococcus*	qPCR (Light Cycler®)	2.5 [3]	*ca*. 10^1–10^3	Roche
Primermix P1 Screening [1]	1 *Acetobacter* 22 *Lactobacillus* 1 *Megasphaera* 1 *Pectinatus* 4 *Pediococcus* 1 *Selenomonas*	qPCR	3.5 [3]	$2x10^1$–$1x10^2$	Gen-ial
VIT-Bier Plus *L. brevis*	9 *Lactobacillus* *P. damnosus*	FISH [2]	3	Trace con-tamination after 48 h enrichment	Vermicon GmbH
VIT-Bier *Megas-phaera/ Pectinatus*	*M. cerevisiae* *Pectinatus*	FISH	3	No data	”
HybriScan-Beer [1]	Relevant beer spoilage *Lactobacillus, Megasphaera, Pectinatus* and *Pediococcus* spp.	RNA-based sandwich hybridisation	No data	No data	Scanbec GmbH (Sigma-Aldrich)

[1] Species identification kits are also available, [2] Fluorescence *in situ* hybridisation, [3] Homann *et al*. (2002).

1.5.4 Pre-PCR processing of brewery samples

The PCR method for routine QC in the brewing industry should ideally fulfill the following criteria (Haikara *et al*., 2003):

- low detection limit, i.e. 1–10 viable cells in a package of beer or against a background of 10^6–10^9 brewer's yeast cells,
- accurate, i.e. low false positivity and negativity rate,
- fast and robust,
- easy to perform (preferably automated), and
- reasonable investment and operative costs.

The sample types analysed in the breweries can be categorized into filterable samples, such as finished beer and process waters, and non-filterable samples taken from the process stages before removal of the brewer's yeast. Pre-PCR processing comprises all the steps prior to the detection of PCR products and is a crucial step in determining the PCR assay performance (Rådström *et al*., 2004). No universal pre-PCR processing method exists. Therefore, the procedures must generally be optimised for each case. In general, pre-PCR processing strategies can be divided into: 1) optimisation of the sampling method, 2) optimisation of sample preparation, 3) optimisation of PCR using alternative DNA polymerases and/or PCR facilitators, and 4) combination of the different strategies (Rådström *et al*., 2004).

PCR detection of the spoilage bacteria in brewery samples is mainly hampered by low level and uneven distribution of the cells and by the presence of un-known PCR inhibitors (DiMichele and Lewis, 1993; Stewart and Dowhanick, 1996; Satokari *et al*., 1997; Pecar *et al*., 1999). Hence, an optimal pre-PCR proc-essing method for the brewery samples would overcome the influence of the inhibitors, while concentrating the cells from a large volume sample into a small PCR-analysable aliquot.

PCR has proved to be a promising technique for detection of spoilage bacteria in finished beer (Tables 3 and 4). A common approach to generating PCR-ready DNA from beer involves collecting the cells by vacuum filtration on flat-bed membranes and subsequent DNA extraction and purification using traditional procedures or commercial kits. The PCR detection limits of these procedures have varied between 1 cfu 250 ml^{-1} and 20 cfu ml^{-1} and it has been possible to obtain the results within a single working day.

PCR has hitherto not been applied to the monitoring of *Sporomusa* sub-branch bacteria in brewery process samples. Direct detection of LAB in the yeast slur-ries was possible only at a ratio of bacteria to yeast of 1:1.6 x 10^{4}, due to the inhibiting effect of the brewer's yeast cells (Stewart and Dowhanick, 1996). A nested PCR in which a second set of primers amplifies an internal part of the first round product lowered the detection limit to 1 cell per 10^{8} yeast cells. This technique also allowed the product confirmation by sequence, but at the cost of increased carry-over contamination risk and labour. A differential centrifugation in which bacteria were separated from the samples before the DNA extraction was applied to the detection of *Obesumbacterium proteus* in yeast slurries, with variable success (Maugueret and Walker, 2002; Koivula *et al.*, 2006).

Many of the pre-PCR processing methods developed for brewery samples are rather time-consuming (6–11 h), insensitive or expensive for routine QC. Hence, more practical strategies are required. One practical solution could be to increase cell number by culture enrichment before a quick DNA extraction and removal or inactivation of inhibitors. The cultivation step could also ensure that the detected cells are viable and thus potentially harmful. The feasibility of the culture enrichment for increasing PCR assay sensitivity for beer-spoilage LAB has been shown. The presence of *L. brevis*, *L. lindneri* and *P. damnosus* in beer samples (50 ml) was detected after 30–40 h incubation in NBB-C broth (Bischoff *et al*., 2001). Low levels of *P. damnosus* were detected from membrane-filtered samples after 16 h incubation in MRS broth (Haakensen *et al*., 2008b). The culture enrichment of the *Sporomusa* sub-branch bacteria for PCR has not been studied.

Another option to improve the PCR detection limits for *Sporomusa* sub-branch bacteria could be to separate and concentrate cells from large sample volumes, thereby minimising the time to results. A bypass membrane filter device for online sampling allowed 40-fold increase in beer volume compared to standard membrane filtration (Back and Pöschl, 1998). Stettner *et al*. (2007) used granulated polyethylenimin-coated polymers for unspecific adsorption of cells from beer for the pre-PCR enrichment. The benefits of the system over the standard membrane filtration were not presented. Numerous techniques have been applied to the pre-PCR separation and concentration of bacteria from food, beverages and water, including paramagnetic beads coated with specific bioreactive molecules, buoyant density centrifugation, flotation, chromatography and ultrafiltration (Benoit and Donahue, 2003; Rådström *et al*., 2004; Wolffs *et al*., 2005; Polaczyk *et al*., 2008). The paramagnetic beads allow easy handling and are amenable to automation. Eger *et al*. (1995) demonstrated the feasibility of immunomagnetic beads for concentrating LAB from beer for flow cytometry. Rudi *et al*. (2004) used successfully universal bacteria- and DNA-binding beads (Bugs'n Beads™) for the processing of faecal samples for PCR. This system might also be suitable for detecting beer-spoilage bacteria in complex brewery samples.

The PCR kits mainly use physical cell lysis methods or chemical extraction combined with paramagnetic DNA purification for pre-PCR processing (Table 5). The ShortPrep foodproof kit III procedure is a single tube extraction based on bead-beating and heating (Homann *et al*., 2002). It can also be used in combination with an automated DNA extraction and PCR setup system that provides the results in 2 h (Methner *et al*., 2004). PIKA Gmbh offers bead-beating and heat-

ing based procedures for rapid DNA extraction. The First-beer magnetic kit from Gen-ial uses paramagnetic beads to purify DNA from chemically lysed cells (Homann *et al*., 2002). The PCR detection limits of the kits vary from 10^1 to 10^3 cfu ml^{-1} (Homann *et al*., 2002; Methner *et al*., 2004; R. Juvonen, unpubl. results).

One of the limitations of the PCR technique is that inactivation of the cells does not normally destroy their DNA (Keer and Birch, 2003). Hence, PCR amplification of DNA from inactivated cells can lead to false interpretations concerning a spoilage risk or safety. Several approaches have been presented to minimise the influence of dead cells on PCR results. RNA is usually a less stable molecule than DNA. RNA can be PCR-amplified after its transcription to its DNA complement using reverse transcription PCR (Keer and Birch, 2003). The outcome of this approach depends on the target organism and sequence, on the inactivation procedure and post-inactivation conditions. A recent study indicated that amplification of elongation factor (*tuf*) messenger RNA or 16S rRNA may not be a suitable approach for live-dead distinction of beer-spoilage LAB due to the possible long-term stability of these molecules in the inactivated cells (Juvonen *et al*., 2009, submitted for publication). An enzymatic treatment of cells with externally added DNases was also proposed to reduce noise signals from dead cells (Nogva *et al.*, 2000). Using *Campylobacter jejuni* as a model organism, promising results were obtained. Most recently, DNA-blocking viability stains based on differential membrane permeability were applied for the live-dead distinction in PCR (Nocker *et al*., 2007).

1.5.5 Implementation of PCR in breweries

PCR is being increasingly implemented in breweries (Homann *et al*., 2002; Braune and Eidtmann, 2003; Kiehne *et al*., 2005; Wold *et al*., 2005). During the time period 2001–2003, four major European breweries evaluated the feasibility of the PCR technique for microbiological QC in an EU-funded project ("Development and demonstration of PCR-based methods for quality control in the brewing industry") (Braune and Eidtmann, 2003; Brandl and Geiger, 2003; Hage and Wold, 2003; Haikara *et al*., 2003; Juvonen *et al*., 2003; Taidi *et al*., 2003). This project showed that PCR is easily adopted by breweries even without previous experience in DNA techniques. Carry-over contaminations were not detected during the test periods. The real-time PCR format was considered more suitable for routine use than the gel-based end-point PCR systems. Specificity

and rapidity of the results were found to be the major benefits of PCR compared to the routine brewery methods. The major criticism against PCR compared to the cultivation was the complexity of the protocols and high costs. Troubleshooting and random checks to complement cultivation methods were considered to be the most interesting applications of PCR. Wold *et al*. (2005) later showed that real-time PCR is a more sensitive method than plate cultivation for the detection of LAB in brewery process samples. The foodproof® Beer Screening kit was evaluated in a ring trial by four breweries (Kiehne *et al*., 2005). Of 60 samples analysed, 56 were correctly identified. Misinterpretations only occurred in one brewery. The PCR technique was recently incorporated as a recommended method into EBC *Analytica Microbiologica* (Hage *et al*., 2005).

1.5.6 Other amplification techniques

After the invention of PCR, many other nucleic acid amplification techniques have been developed to circumvent existing patents and to overcome some of the PCR drawbacks (Mothershed and Whitney, 2006). Of these techniques only loop-mediated isothermal amplification (LAMP) has been applied in the brewing field (Tables 3 and 4). It is an isothermal reaction requiring at least four specifically designed primers that recognize a total of six distinct sequences on the target DNA and resulting in a visible precipitate. It should be able to amplify a few target copies and be less sensitive to nontarget DNA than PCR. A LAMP-based application for the identification of *L. brevis*, *L. lindneri*, *P. damnosus* and *Pectinatus* from isolated colonies in 1.5 h has been developed (Tsuchiya *et al*., 2007).

Multiple displacement amplification is a whole genome amplification technique that can be used to renew DNA from a low number of genomic copies; even from a single cell (Raghunathan *et al*., 2005). The use of this technique as a pre-PCR procedure suffers from the same drawback as nested PCR; it increases carry-over contamination risk and sample processing time. Multiple displacement amplification has not been applied to beer contaminant detection.

1.6 Hybridisation-based probe techniques for beer-spoilage bacteria

Fluorescence *in situ* hybridisation (FISH) involves hybridisation of fluorescent-labelled oligoprobes to their complementary nucleic acid targets, usually 16 rRNA, within fixed cells and their detection using flow cytometry, laser scan-

ning or microscopy (Amann and Fuchs, 2008). It enables the detection, identification, enumeration and localisation of single cells in the original sample.

FISH probes have been designed for various taxonomic ranks. A vast database is available online (Loy *et al.*, 2007). The *Firmicutes*-specific probe of Meier *et al.* (1999) allows the detection of all *Sporomusa* sub-branch beer spoilers. Yasuhara *et al.* (2001) first applied FISH for beer samples. The *P. cerevisiiphilus*- and *P. frisingensis*-specific assays detected 10^3 active cells in 100 ml of beer in 5 h. FISH kits are currently available for LAB, *M. cerevisiae* and *Pectinatus* (Table 5). Thelen *et al.* (2002) evaluated a "VIT-Bier plus *L. brevis*" kit for the detection of LAB in brewery samples. It reliably detected low levels of contaminants after 48 h culture enrichment. Dead cells did not give a detectable signal due to their low rRNA content. The major drawbacks of FISH for routine QC include tediousness of the procedures, high costs and the need for culture enrichment. Asahi breweries now market an image analysis system for automating the microscopy step. FISH has also been used as a research tool for studying the microbial ecology of brewery bottling halls (Timke, 2005a, b). The general benefits and drawbacks of the technique were discussed by Amann and Fuchs (2008).

Huhtamella *et al.* (2007) recently applied an RNA-based sandwich hybridisation to the group-specific detection of beer-spoilage LAB in brewery process samples. The 16S rRNA molecules were sandwiched between two probes and bound on magnetic beads for the detection by fluorescent ELISA or by redox recycling on a microchip. Total analysis time was 3 h with a detection limit of 10^5–10^6 cells per assay. Comparison of the method to the standard plate count showed 85% specificity and 81% sensitivity after 16–24 h culture enrichment. Test kits are now available based on this technology (Table 5).

PCR multiplexing capabilities are limited to fewer than *ca.* 10 primer sets. An increasing number of techniques allow parallel detection and identification of multiple target organisms or DNA fragments in a single miniaturised test amenable to high throughput analysis. DNA microarrays are an example of a technique in this category. They are arrays of hundreds or thousands of DNA probes immobilized in discrete locations on a few square centimeter solid supports or on identifiable beads (Rasooly and Herold, 2008). DNA microarrays can be applied to any analysis with which information can be extracted through specific hybridisation, including detection, identification and typing of microbes, discovery of new molecular markers and gene expression.

In a typical microarray experiment, the target nucleic acid stretch is amplified, labelled and hybridized with probes on the microarray. The signal from the label

in a known probe location allows identification of the target. Weber *et al.* (2008) recently showed the feasibility of an oligonucleotide microarray for the detection and identification of ten beer-spoilage *Lactobacillus*, *Megasphaera*, *Pectinatus* and *Pediococcus* species in pure culture. Probes targeted to 16S-23S spacer region differentiated inactivated and active cells. Although the expectations for the microarray technology are high, there are many challenges to be solved relating to cross-reactions, probe accessibility, sample treatment, sensitivity and quantitation. Moreover, the development and execution costs are still high (Ludwig, 2007; Rasooly and Herold, 2008).

1.7 DNA-based techniques for identification, typing and phylogenetic studies of beer-spoilage bacteria

The diagnostic probe systems described above are best used for specific detection and identification within defined target groups. They are not suitable for general purpose identification, for discovering new species or for phylogenetic studies for which other methods are needed. Non-clonal bacterial populations exhibit intraspecific genetic diversity. Typing methods aim at discriminating bacteria to the lowest subspecific level, so that clones from different sources in time and space could be discriminated (Coenye *et al.*, 2005; Medini *et al.*, 2008). Strain typing is helpful, e.g. for understanding mechanisms and sources of contamination, and potentially for identifying strains with particular traits. Certain DNA techniques also allow the inferring of evolutionary relationships for systematics and taxonomic purposes (Ludwig, 2007).

1.7.1 Whole genome similarity and composition

Two classical approaches, DDH and determination of genomic DNA guanine and cytosine content (GC mol%), are still commonly used among taxonomists for indirect whole genome characterisation.

DDH provides an estimate of the average nucleotide similarity between the whole genomic content of a pair of strains. It is based on the rationale that the more closely related the strains are, the higher the level of hybridisation between their genomes will be. The degree of DNA similarity is estimated by measuring the thermal stability or the amount of heterologous hybrids in relation to homologous DNA from the reference strain. Rossello-Mora (2006) recently reviewed various DDH procedures applied in taxonomy.

DDH is still the gold standard for species delineation. Prokaryotic species cannot be defined based on sexual reproduction boundaries. For lack of a better alternative, a pragmatic genospecies definition based on DNA similarity as measured by DDH has been defined (Wayne *et al.*, 1987). The recommended criterion states that a species generally includes strains with *ca*. 70% or greater DNA-DNA relatedness or with 5°C or less ΔT_m. These values mainly derive from correlation studies with phenotypic data. Despite this, DDH has agreed well with whole genome sequence parameters (Goris *et al.*, 2007). It has been estimated that strains with higher than 70% DDH similarity values share at least 96% DNA sequence identity (Johnson, 1973). The power of DDH lies in its ability to resolve closely related species. Hybridisation will occur to a measurable extent only if the genomes share 80–85% sequence complementarity (Ludwig, 2007). DDH has been applied to delineate and identify *Megasphaera*, *Pectinatus*, *Selenomonas* and *Zymophilus* species as well as beer-spoilage LAB (Engelmann and Weiss, 1985; Schleifer *et al.*, 1990; Funahashi *et al.*, 1998; Nakakita *et al.*, 1998).

DDH is nowadays mainly used as a taxonomic tool due to its impracticality compared to the sequence analysis. The data is not cumulative (databases cannot be built), the results from different procedures and experiments cannot be compared, and differences in the genome size and the choice of reference strain influence the outcome. Moreover, it does not provide any information concerning the involved genes and is restricted to the characterisation of culturable organisms (Ludwig, 2007; Achtman and Wagner, 2008).

The calculation of DNA GC mol% is usually based on the measurement of DNA melting temperature or buoyant density (Ludwig, 2007). It allows discrimination between organisms with different GC mol%, but does not provide infromation about phylogenetic relatedness. The GC mol% of prokaryotic genomes varies between 24 and 76. The GC mol% should be included in a new genus description, but is not required for a new species description (Stackebrandt *et al.*, 2002).

1.7.2 Pattern techniques

Numerous pattern techniques exist for the indirect comparison of DNA for species identification and strain typing (Ludwig, 2007). They allow visualisation of anonymous genetic differences by generating a banding pattern. The basic assumption is that different patterns indicate dissimilarity, whereas identical pat-

terns *per se* do not mean identity. The differences at the whole genome level or within a selected locus or loci are revealed using PCR, hybridisation, site-specific restriction enzymes, electrophoresis or their combinations.

Ribotyping involves restriction enzyme cleavage of total genomic DNA, followed by hybridisation of the electrophoretically size-separated fragments to a labelled probe homologous to a complete or a partial *rrn* operon on Southern blots (Bouchet *et al.*, 2008). Differences were shown to arise from RFLPs in the housekeeping genes immediately (~50 kb) flanking the *rrn* operon. The discrimination power of the technique depends on the restriction enzyme, the probe and the operon copy number. When using conserved sequence stretches as probes, most bacteria are typeable. Ribotyping schemes could also be designed *in silico* for fully sequenced species (Bouchet *et al.*, 2008).

When performed manually, ribotyping is a laborious and time-consuming technique that has found only limited application in brewing microbiology (Prest *et al.*, 1994; Yansanjav *et al.*, 2003). A commercial ribotyping system (Ribo-Printer™, Qualicon) for bacteria has been available on the market for more than 10 years (Bruce, 1996). It allows faster and more standardised analysis than the manual approach. It also facilitates the construction, management and exchange of identification libraries. However, an optimised manual procedure can provide better band resolution for strain typing (Bouchet *et al.*, 2008). The automated system uses *Eco*RI as a restriction enzyme and a highly conserved *rrn* region as a probe. Other enzymes can also be used. It has been applied to characterise the *Sporomusa* sub-branch beer-spoilage species, and *Hafnia alvei* and *O. proteus* strains, brewery-related pediococci and lactobacilli, and Gram-stain-positive cocci, enterobacteria and spore-forming bacteria from the brewery environment (Motoyama *et al.*, 1998 and 2000; Storgårds *et al.*, 1998; Satokari *et al.*, 2000; Barney *et al.*, 2001; Suihko and Haikara, 2001; Takeuchi *et al.*, 2005; Koivula *et al.*, 2006). In most of these studies, the technique allowed strain discrimination at the species and sub-species levels.

Automated ribotyping of *M. cerevisiae*, *P. cerevisiiphilus*, *P. frisingensis*, *S. lacticifex*, *Z. paucivorans* and *Z. raffinosivorans* strains with *Eco*RI produced patterns with conserved species-specific fragments (Motoyama *et al.*, 1998; Suihko and Haikara, 2001). The patterns from the *M. cerevisiae*, *Pectinatus* and *S. lacticifex* strains also contained polymorphic fragments for discrimination at the sub-species level. The combination of the patterns produced using two enzymes gave greater discrimination among the *M. cerevisiae* and *P. frisingensis* strains than when using a single enzyme (Motoyama *et al.*, 1998; Suihko and

Haikara, 2001). Despite this, not all the strains from different geographic locations were resolved. Ribotyping also implied that one *M. cerevisiae* and three *P. cerevisiiphilus* isolates from the beer production chain earlier identified phenotypically could represent new species (Suihko and Haikara, 2001).

Whole-genome PCR fingerprinting techniques use a single primer or primer set that is arbitrary in sequence or complementary to some repetitive DNA element (Gürtler and Mayall, 2001). When two primers anneal at a proper orientation within a few kilobases from each other, the intervening sequence can be amplifed. Variation in the number and position of the binding sites between organisms results in different gel-banding patterns. These techniques are easy, fast (8–10 h) and inexpensive, allowing high sample throughput. In relation to taxonomy, random whole genome methods are comparable to phenotypic methods due to the unknown location of the primer sites. With sequencing, it is possible to link the observed differences to actual differences in the genome.

Random amplified polymorphic DNA (RAPD) -PCR, also known as arbitrarily primed PCR, uses short (8–12 bases) arbitrary primers in low stringency conditions. It has been applied to identify brewery-related bacteria (Thompkins *et al.*, 1996), to distinguish *O. proteus* strains at biotype and isolate levels (Savard *et al.*, 1994) and to identify DNA fragments specific to beer-spoilage LAB (Fujii *et al.*, 2005). Among geographically distinct *M. elsdenii* isolates, RAPD showed little variation (Piknova *et al.*, 2006). RAPD often suffers from poor reproducibility, since many of the priming events in the low stringency conditions are sensitive to minor changes in the assay variables.

Repetitive element PCR (rep-PCR) targets repetitive DNA elements randomly distributed in the genome, such as invertedly repeated elements (BOX), polytrinucleotides (GTG_5) or repetitive extragenic palindromic sequences (REP). The primers may also bind to non-target regions (Gürtler and Mayall, 2001). Rep-PCR is generally more reproducible than RAPD owing to the higher annealing temperatures. Suiker *et al.* (2007) used BOX, GTG_5 and REP primers to characterise 72 *Pectinatus* strains from culture collections and various breweries. The discrimination power of rep-PCR varied depending on the primer and the species. GTG_5 gave distinct patterns for *P. cerevisiiphilus* and *P. frisingensis* and for a putative new species. *P. frisingensis* strains were more heterogenous than *P. cerevisiiphilus* strains. Rep-PCR has also been applied to beer-spoilage LAB (Zhu *et al.*, 2005).

In amplified ribosomal DNA restriction analysis (ARDRA) or PCR-ribotyping, differentiation is based on polymorphism in the location of restric-

tion enzyme recognition sites within the *rrn* operon. The target area is PCR-amplified with specific primers, digested with four-base pair recognizing restriction enzymes, and the fragments are electrophoretically size-separated. Due to the restricted size and conservation of the *rrn* operon, ARDRA has low discrimination power and it only provides differentiating information (Ludwig, 2007). It has been found to be suited for the species identification of lactobacilli (Singh, 2009) and non-spore-forming bacteria, Gram-stain-positive cocci and enterobacteria isolated from the brewery environment (Takeuchi *et al.*, 2005). ARDRA has not been applied to the *Sporomusa* sub-branch beer-spoilage bacteria.

1.7.3 Sequence analysis of phylogenetic markers

Sequencing involves direct determination of the primary nucleotide order of DNA. The genetic locus of interest is PCR-amplified from a pure culture or isolated by cloning from a PCR-product mixture. Sequencing is still mainly carried out using Sanger's chain termination method. In the most common modification, chain-terminating dideoxybases, each labelled with a different fluorescent label, are randomly incorporated (together with unmodified bases) into a target sequence in a linear cycle sequencing PCR, resulting in DNA fragments of varying length. The terminal base of each fragment can be determined by a fluorimeter (Clarridge, 2004). The cycle sequencing reaction is able to produce a sequence read-out of up to *ca.* 850 bp. Several new technologies allowing higher sample throughput by being faster and cheaper exist. However, they still produce rather short read-outs (50–250 bp) (Medini *et al.*, 2008).

Comparative 16S rRNA gene sequence analysis is now a basic phylogenetic tool (Ludwig and Klenk, 2005). Inference of evolutionary relationships is based on the number and character of positional differences between aligned sequences. In order to obtain the maximum amount of unskewed information, complete 16S rRNA gene sequences should be used. The gene history can be inferred from aligned sequence data using treeing methods based on models of evolution. Statistical confidence of tree branching order can be tested using re-sampling methods, such as bootstrapping. Phylogenetic trees are dynamic constructs that change according to the included data and applied analysis methods. The 16S rRNA gene sequences generally represent conserved core genes rather well, although constituting less than 0.01% of the total DNA residues (Ludwig, 2007). For example, different core markers were in good congruence concerning the assignment of *Pectinatus* spp. in the *Sporomusa* sub-branch (Haikara and

Helander, 2006). However, the 16S rRNA gene can have limited resolution at and especially below the species level (Stackebrandt and Goebel, 1994; Palys *et al.*, 2000). Another problem relating to the use of this molecule in phylogeny is that a significant degree of sequence divergence may exist among its multiple intragenomic copies. Other markers found to be useful in phylogenetic studies of brewery-related bacteria include 16S-23S spacer region, elongation factors, DNA repair and heat-shock proteins (Dobson *et al.*, 2002; Coenye and Vandamme, 2003; Ventura *et al.*, 2003; Haikara and Helander, 2006). Protein-encoding housekeeping genes may evolve even an order of magnitude faster than the 16S rRNA gene sequences (Palys *et al.*, 2000).

The 16S rRNA gene sequence data forms the backbone of the current taxonomic framework (Ludwig, 2007). It serves to delineate moderately related species and higher ranks and is a compulsory element of a new species description (Stackebrandt *et al.*, 2002). A recent correlation study with DDH showed that strains that have lower than *ca*. 99% sequence similarity usually share lower than 70% DDH similarity (Stackebrandt and Ebers, 2006). Hence, they are distinct genospecies according to the definition. Strains showing higher than 98.7–99% sequence similarity may or may not be different at the whole genome level. Therefore, their genomic uniqueness should be verified using DDH (Stackebrandt and Ebers, 2006). Adékampi *et al*. (2008) recently proposed comparative analysis of RNA polymerase β-subunit encoding gene sequences to supplement DDH for species and genus delineation. The lack of universal primers and the small size of the database (25,000 entries) still limit universal use of this marker gene. The analysis of 16S rRNA gene sequences has tentatively suggested that the beer production process harbours several undescribed species (Sakamoto *et al.*, 1997; Nakakita *et al.*, 1998; Suihko and Haikara, 2001). As emphasized by Wayne *et al*. (1987), any phylogeny-based taxonomic classification should also show phenotypic consistency.

Identification by sequencing usually involves determination of the nucleotide order of a diagnostic stretch on a conserved marker (Clarridge, 2004). The 16S rRNA gene is the best choice for the identification of an unknown isolate, since there is no other universal marker with such a vast public database. The 16S rRNA gene sequences of the *Sporomusa* sub-branch beer spoilage species, with the exception of *Z. raffinosivorans* have been deposited and can be used for their differentiation from each other (≤ 95% similarity). For most bacteria, the initial 500 bases provide sufficient information for speciation. The most informative area is usually located between the positions 60 and 100 (*E. coli*) (Ludwig and

Klenk, 2005). The partial 16S rRNA gene sequences also correctly identified *Pediococcus* brewery isolates to the species level (Satokari *et al.*, 2000; Barney *et al.*, 2001; Dobson *et al.*, 2002). The 16S rRNA gene sequence generally provides a good genus assignment, but may not allow for differentiating closely related but ecologically distinct species (Palys *et al.*, 2000). The combined use of the phenylalanyl-tRNA synthase α-subunit and the RNA polymerase α-subunit partial gene sequences had a higher discriminating power for identification of *Lactobacillus* species than the 16S rRNA gene (Naser *et al.*, 2007). The 16S-23S spacer region and *tuf* gene sequences also allowed better distinction among lactobacilli (Garcia-Martinez *et al.*, 1999; Ventura *et al.*, 2003; Singh, 2009). The 16S-23S spacer regions of the *Pectinatus*, *S. lacticifex* and *Zymophilus* were found to more divergent than their 16S rRNA genes (Motoyama and Ogata, 2000a). The flagellin genes showed even intraspecific variation between the *P. cerevisiiphilus* and *P. frisingensis* strains (Chaban *et al.*, 2005).

Accurate identification by sequencing requires a reliable database and a good-quality sequence. The best-known public databases include Genbank and Ribosomal Database Project II (RDP II). A curated public 16S rRNA sequence database was also recently established (Yarza *et al.*, 2008). A commercial MicroSeq system with a validated 16S rRNA gene sequence database and standardised reagents is also available. Hitherto, it has not been tested for the identification of brewery contaminants. As a routine tool for brewery QC, sequencing is still rather expensive due to high instrument costs (unless sharing) and being labour intensive (40 h hands-on-time for 60 samples). Clarridge (2004) estimated the running costs per sample (partial sequence) to be 50–84$. Whole sequencing services can now be outsourced, with a relatively low price. Interpreting the results can be complex. Different databases may give different similarity scores. In addition, generally accepted identification criteria do not exist. Janda and Abbot (2007) suggested 99–99.5% 16S rRNA sequence similarity (min 500–525 bp) for the species identification in clinical laboratories. When multiple species differ from each other < 0.5%, phenotypic properties should also be examined. The use of strict boundaries based on pure number differences can be misleading, since species have different genetic depths and the site of mutation might also be important. Moreover, the percent difference varies with sequence length. Poor sequence quality often results from intragenomic heterogeneity (Strömpl *et al.*, 1999).

With the decreasing cost and effort of sequencing, multilocus sequence analysis is increasingly being used to study phylogenetic relationships at the species

and genus levels in order to overcome the weaknesses of the single-gene approaches (Ludwig, 2007). The ad-hoc committee for re-evaluation of the species definition proposed sequencing of a minimum of five housekeeping genes to achieve adequate phylogenetic data (Stackebrandt *et al.*, 2002). The set of the most informative markers often need to be determined for each bacterial group (Ludwig, 2007). Multilocus sequence typing is a specific tool for grouping isolates within a species to major genetic lineages (Maiden, 2006). It is based on indexing of the number of different alleles in internal fragments (500–600 bp) of multiple housekeeping genes. The available typing schemes are maintained on a public database (http://pubmlst.org/databases.shtml), that currently includes only a few food-related bacteria.

More than 1100 prokaryotic genomes have been fully sequenced and the number is rapidly increasing. Hence, whole genome sequence analysis is becoming a feasible tool to describe the complexity and relations of bacteria. Several approaches are available to extract phylogenetic data from the whole genome sequences (Coenye *et al.*, 2005). Recently, the average nucleotide identity of the conserved core genes of a pair of strains was proposed as a DDH replacement for species delineation (Goris *et al.*, 2007). In the future, whole genome studies will probably drastically change the way we define and classify prokaryotic species. Whole genome sequences of the *Sporomusa* sub-branch beer-spoilage bacteria have not hitherto been published.

1.7.4 Comparison of the techniques

The DNA-based techniques differ from each other in their discrimination power, speed, costs and ease of use (Ludwig, 2007). Based on a few comparison studies, their discrimination power for brewery-related bacteria was in decreasing order: PCR-fingerprinting > ribotyping > 16S rRNA gene sequencing and ARDRA (Satokari *et al.*, 2000; Barney *et al.*, 2001; Takeuchi *et al.*, 2005).

The PCR-based pattern techniques (ARDRA, rep-PCR, RAPD) are generally faster, less expensive and easier to execute than ribotyping or sequence analysis. ARDRA gives reproducible, low complexity patterns and is best applied for the building of identification libraries and for interlaboratory studies (Takeuchi *et al.*, 2005). RAPD and rep-PCR generate in a shorter time more complex, but less reproducible patterns. Therefore, they are most useable for the rapid grouping of a large number of isolates prior to their identification by some database-supported technique, and for tracking specific strains. Whole genome PCR fin-

gerprinting and multilocus approaches, such as MLST, offer higher resolution for strain typing than the methods studying a single locus. For systematics and population genetic studies, techniques with a proven phylogenetic basis, such as sequence analysis of phylogenetic markers, should be used (Ludwig, 2007).

Despite the undeniable power of DNA-based techniques, a polyphasic approach incorporating genotypic, phenotypic and ecological data is needed for thorough understanding prokaryotic diversity in terms of function and species richness. The description of a new species should also be based on a polyphasic characterisation (Stackebrandt *et al*., 2002).

2. Hypotheses, rationale and specific aims of the study

The study was based on three hypotheses. The first hypothesis was that PCR enables taxon-specific, rapid and easy detection of low levels of *Sporomusa* sub-branch beer spoilers in brewery samples. In theory, PCR is capable of rapidly amplifying even a single target molecule. The design of taxon-specific PCR tests should be possible by targeting the 16S rRNA gene, which contains variable and conserved regions and for which a vast public database is available. The fact that 16S rRNA gene is present in many bacteria in multiple copies increases the likelihood of detecting low levels of cells. PCR is also easier, faster and cheaper to execute than most other specific detection techniques. The second hypothesis was that genetically atypical *Megasphaera*- and *Pectinatus*-like isolates from the beer production chain represent new species. These isolates have hitherto not been characterised extensively enough to decide whether they should be described as new species. The third hypothesis was that phylogenetic analysis of the *Sporomusa* sub-branch beer-spoilage species yields new insight into their natural relationships and habitats in view of the current 16S rRNA gene sequence database. The number of available 16S rRNA gene sequences has increased exponentially since the last phylogenetic analysis of the *Sporomusa* sub-branch. The 16S rRNA gene sequences from almost all valid species and from more than 500,000 uncultured bacteria from diverse ecosystems are now available.

The main aim of this study was to utilise DNA-based techniques in order to improve detection and identification of *Sporomusa* sub-branch beer-spoilage bacteria and to increase understanding of their biodiversity, evolutionary history and distribution in nature.

The specific aims were:

- to develop practical PCR-based tools for detection and identification of the *Sporomusa* sub-branch beer-spoilage bacteria,

- to assign the phylogenetic and taxonomic position of the atypical *Megasphaera*- and *Pectinatus*-like isolates, and

- to improve understanding of the phylogenetic relationships and natural sources of the *Sporomusa* sub-branch beer-spoilage bacteria.

3. Materials and methods

3.1 Microbial strains and their cultivation

Strains were obtained from the VTT Culture Collection or isolated from brewery samples during this study by using plate cultivation on selective or non-selective media (Papers III–V). The type strains of the proposed new species were deposited to the VTT Culture Collection and to Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). The strains were cultivated in the recommended media (http://culturecollection.vtt.fi). Unless otherwise stated, exponential phase cultures and sterile media and reagents were used throughout the study.

3.2 Brewery samples

Brewery wort (16°P) and environmental and product samples were obtained from breweries or from local supermarkets. For the determination of PCR detection limits, the beer samples (25, 100 or 330 ml) were inoculated with 10-fold dilution series of test cultures to obtain decreasing levels of contamination. Brewery process samples were prepared by inoculating fermented brewery wort samples with different yeast cell concentrations, followed by artificial contamination with bacteria as above. Non-inoculated process and product samples were used as negative control samples and to study PCR inhibition by the brewery samples.

3.3 PCR inhibitory material

Catechin (a monomeric phenol found in beer) was obtained from Sigma-Aldrich Finland Oy. PCR inhibitory material from brewery process and product samples was prepared as described in Papers I and IV. Amido black staining was used to

visualise proteinaceous material in the non-filterable beer fraction. The material retained on 0.45 µm cellulose acetate filters was stained with a 1% amido black solution for 10 min while continuously shaking, after which the filters were rinsed with a 7% acetate solution.

3.4 Enumeration of microbes from brewery samples

The viable cell counts of the target group bacteria were determined on PYF medium (Haikara and Helander, 2006). The brewer's yeast was surface-plated on yeast and malt extract medium (Difco Laboratories, USA). The total yeast cell concentration in brewery process samples was determined using haemocytometry (Paper IV). The number of bacterial cells in beer samples after various pre-PCR treatments was enumerated after membrane filtration using 4', 6-diamidino-2-phenylindole (Sigma) staining and microscopy (Paper I).

3.5 Design and evaluation of specific PCRs

Primer and probe design were carried out with the help of PC/GENE and PCRPLAN programs (Paper I) or Clustal W (Paper III). The primers targeted the 16S rRNA gene (Table 6). The specificities of candidate primers were evaluated *in silico* against GenBank and RDP II databases using basic local alignment search tool (BLAST) and Probe Check, respectively. The primers were synthesized using a PCR Mate 391 DNA Synthesizer (Papers I, II) or purchased from commercial sources (Papers III–V).

The PCR tests developed in this study are summarised in Table 6. The reactions were optimised by varying PCR mixture composition and thermal cycling conditions. Their specificity and sensitivity were determined using pure DNA from relevant target and non-target strains (Papers I, III, IV). The sensitivity of the post-PCR detection methods was studied using purified PCR products (Paper I). QIAquick PCR Purification kit (Qiagen GmbH, Germany) was used for the purification. DNA concentrations were calculated from optical density (OD) values ($OD_{260} = 1 = 50$ µg ml^{-1}).

In order to study the effect of sample-derived extracts on PCR, the reaction mixtures received a known amount of purified target DNA (Papers I, IV). The effect was assessed by comparing the real-time PCR C_p values or the final amount of the PCR product obtained in the presence and in the absence of the extracts.

Table 6. 16S rRNA gene PCR for detection and identification of the target group bacteria.

Target	Primer sequence (5' to 3')	Direction	Binding site[1]	Product (bp)	PCR format	Product detection and characterisation	Paper
M. cerevisiae	CACTGAATAGTCTATCGC	forward	204–221	403	End-point	Agarose gel (2%) electrophoresis	I–III
	AAGACCGACTTACCGAAC[2]	reverse	587–605		End-point	Colorimetric microplate hybridisation	I
Pectinatus	GCTTTTAGCTGTGTCGCTTGGA	forward	212–231	816	End-point	Agarose gel (1.2%) electrophoresis	I–III
	TGCATCTCTGCATACGTCAA[2]	reverse	1008–1027		End-point	Colorimetric microplate hybridisation	I
Sporomusa sub-branch beer-spoilage species	ACGATCAGTAGCCGGT	forward	279–294	342	End-point	Agarose gel electrophoresis (1.5%), RFLP with *Bss*HII, *Kpn*I and *Xmn*I, or *Sca*I	III, IV
	AGCCCCGCACTTTTAAG	reverse	603–620		Real-time (LightCycler®)	SYBR Green I and melting curve analysis	III, IV
Eubacteria	AGAGTTTGATCCTGGCTCAGG	forward	8–29	~1500	End-point	Confirmation of template quality	I–IV
	ACGGCAACCTTGTTACGAGTT	reverse	1492–1506				

[1] *Escherichia coli* numbering, [2] Biotinylated at the 5' end for microplate hybridisation.

3.6 Processing of brewery samples for PCR analysis

3.6.1 Sample treatment

The methods evaluated for pre-PCR processing of beer samples are summarised in Table 7. In addition, an enzymatic treatment in which membrane filters were incubated with a 200 μl aliquot of 260 U ml^{-1} xylanase (Novozymes, Denmark) at 50°C for 1 h after beer filtration was applied. The experimental scheme for the development of a pre-PCR processing method for the process samples containing high numbers of brewer's yeast cells is shown in Fig. 5.

Process sample
1 ml

16,060x*g*, 3 min

Pellet

Bacteria-binding beads
(Bugs'n Beads™ kit,
Genpoint)

Centrifugation (209x*g*,
1–5 min) in Tris buffer,
in EDTA, in EDTA-
maltose or in Tween 20

Bugs'n
Beads™ kit

UltraClean™
Soil kit (MoBio
Laboratories)

Boiling

Bead-
beating

InstaGene
Matrix
(BioRad)

Separation of bacteria

DNA extraction
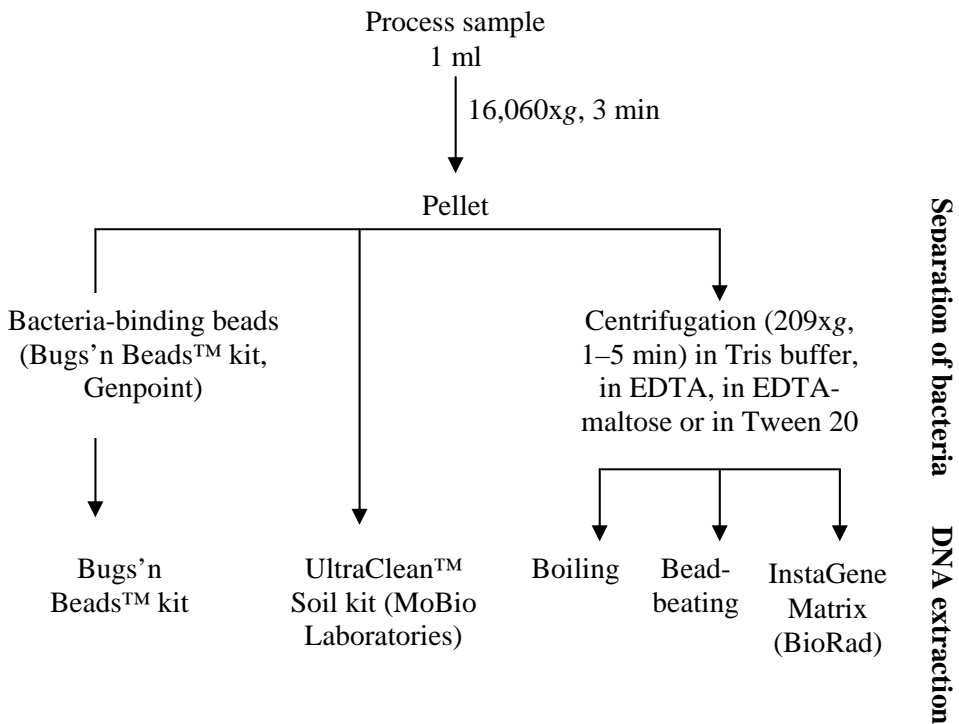
Figure 5. Experimental scheme for pre-PCR processing of yeast-containing brewery process samples (Paper IV).

Table 7. Experimental procedures for pre-PCR processing of beer samples.

Stage	Procedure	Paper
Cell collection and inhibitor removal	I) Polycarbonate membrane filtration followed by wash with one of the following procedures:	
	▪ 0.1M NaOH + 0.5% SDS	I–IV
	▪ 0.01M NaOH + 0.05% SDS	IV
	▪ 0.1M NaOH	IV
	▪ 1% PVP [1]	IV
	▪ 1% PVP+0.01M NaOH	IV
	▪ 0.01M EDTA [2] + 0.2% STPP [3]	IV
	▪ 2% Tween 20	IV
	▪ Water	I, IV
	II) CellTrap™ (Mem-teq Ventures, UK) filtration followed by water wash	IV
Cell lysis	Heating (95°C, 10 min) followed by membrane solubilisation in chloroform	III, IV
	InstaGene Matrix kit (BioRad, USA)	I, II
PCR facilitators	BSA [4] (0.1–0.6%) PVP (0.5, 1%)	IV

[1] Polyvinyl pyrrolidene, [2] Ethylene diamine tetra-acetic acid, [3] Sodium tripolyphosphate, [4] Bovine serum albumin.

3.6.2 Culture enrichment of beer samples

Several selective and non-selective media were compared for culture enrichment of the target group bacteria as described in Paper II. Two enrichment procedures were established. In the first, one part of beer sample was mixed with four parts of extra-concentrated MRS (EC-MRS: 5-fold MRS broth with 5% fructose and 0.05% cycloheximide), and incubated anaerobically at 30°C (Paper II). In the second, beer samples were mixed with an equal volume of double-concentrated MRS-fructose medium (2-MRSF: 2-fold MRS broth with 2% fructose, 0.02% cycloheximide and 0.1% cysteine), and incubated anaerobically at 27°C (Paper III). To determine minimum enrichment times for PCR detection, beer samples with various levels of active cells were analysed daily by PCR and cultivation for up to one week. In order to study the impact of stress on the growth rate, the cells (10^6–10^7 cfu ml^{-1}) were incubated anaerobically in a standard lager beer or in sterile tap water for 0, 3 or 7 d at 7°C or at 13°C. Active, non-stressed cells suspended in Ringer's solution were used as a control. The influence of the stress

treatments on the growth rate during enrichment in beer mixed with the 2-MRSF medium was evaluated by daily turbidity measurement.

3.6.3 DNase I treatment

The ability of an externally added DNAase I to reduce PCR signals from dead cells was studied using active and inactivated *M. cerevisiae* E-981087 and *S. lacticifex* E-90407 cells. The cells (10^5 cfu ml^{-1}) were inactivated by heating in sterile tap water for 20 min at 62°C, or by bubbling with sterile-filtered oxygen for 14 h at room temperature. Three 0.5 ml aliquots were drawn for the plate cultivation and for the DNase I treatment before and 0, 24, 48 and 72 h after post-inactivation storage at 0±1°C. In the DNase I treatment the harvested cells were incubated with 10 U DNase I (RNase-free, Roche Oy, Espoo) in 100 µl of 1 x DNase buffer (10 mM Tris, 250 mM $MgCl_2$, and 0.5 mM $CaCl_2$, pH 7.5) at 37°C for 1 h. After reharvesting, the cells were washed once with 1 ml of 10 mM Tris (pH 8.0) and boiled in 0.1 ml of 10 mM Tris for 15 min to lyse the cells and to inactivate possible traces of the enzyme. Control samples did not receive the enzyme. The DNA extracts were analysed in duplicate by real-time PCR. In preliminary experiments, it was determined that the DNase I is able to hydrolyse 10 ng of DNA in 0.5 h; that DNA does not spontaneously degrade during the 1 h incubation at 37°C; and the enzyme buffer does not interfere with PCR. More-over, it was shown that washing in combination with boiling inactivates DNase I.

3.7 Characterisation of pure culture isolates

The procedures used for characterising the pure culture isolates are summarised in Table 8.

3.8 Sequences

The 16S rRNA gene sequences determined in this study were deposited to Gen-Bank with the following accession numbers: DQ217599, DQ223729, DQ223730, DQ223731, EU589443, EU589444, EU589445, EU589446, EU589447, EU589448 and EU589449.

Table 8. Experimental procedures for genotypic and phenotypic characterisation of pure culture isolates.

Analysis	Paper
DNA extraction:	
▪ traditional method	I–IV
▪ InstaGene Matrix	I, III
▪ bead-beating	V
16S rRNA gene sequencing:	
▪ partial gene (*ca.* 500 bp)	III
▪ complete gene (*ca.* 1400–1500 bp)	V
Comparative sequence analysis:	
▪ sequence compilation using DNAMAN (Lynnon Biosoft, Canada)	V
▪ sequence comparisons to GenBank/EMBL/DDBJ and RDP II databases	III, V
Phylogenetic analysis using ARB (Technische Universität Münich, Germany):	V
▪ neighbour joining	
▪ maximum parsimony	
▪ maximum likelihood	
DNA-DNA hybridisation	V
Genomic DNA guanine and cytosine content	V
Automated ribotyping with *Eco*RI (Riboprinter® Microbial Characterization System, DuPont Qualicon™, USA)	V
Ability to grow in beer	V
Cellular morphology	III, V
Gas chromatography of volatile fatty acids	V
High performance liquid chromatography of non-volatile fatty acids	V
Other biochemical and physiological characteristics:	
▪ acetoin production, arginine hydrolysis, bile resistance, nitrate reduction and urease activity (Rosco A/S, Denmark)	V
▪ acid production from carbohydrates	V
▪ aerotolerance	III, V
▪ antibiotic susceptibility (An-ident discs, Oxoid, UK)	V
▪ catalase and oxidase activities	III, V
▪ growth in selective and non-selective media	V
▪ temperature limits of growth	V
▪ utilisation of organic acids	V

3.9 Statistical analyses

Statistical significance of the difference between various enrichment media or between various pre-PCR treatments was tested using analysis of variance ($\alpha = 0.05$) or Student's t-test ($\alpha = 0.05$) or both (Papers I, IV). Regression analysis was used for determining the relationship between initial DNA concentrations and real-time PCR C_p values (Paper III). The analyses were performed using the Statistical Toolpak of Microsoft Excel. Dunnett's test was used to compare the influence of PCR facilitators and inhibitors with a common control sample (Paper IV).

4. Results and discussion

4.1 Development and evaluation of PCR tests for *Sporomusa* sub-branch beer-spoilage bacteria (Papers I, III)

Diagnostic PCR encompasses three basic steps: pre-PCR processing, amplification and detection of PCR products. All these steps influence the assay performance (Malorny *et al*., 2003). One aim of this study was to design qualitative PCR tests with narrow- and broad-range specificities for the *Sporomusa* sub-branch beer-spoilage bacteria. We used the 16S rRNA gene as the primer target, because the spoilage potential of these bacteria varies between the species. Moreover, this gene exhibits sequence variation at various taxonomic ranks and a vast public database is available (Ludwig, 2007). Most of the target-group bacteria were also known to possess multiple 16S rRNA gene copies (Motoyama and Ogata, 2000a), allowing for higher sensitivity than using a single copy gene. Specificities and sensitivities of the PCR tests were assessed using purified DNA from the target and non-target strains (Papers I, III). Artificially contaminated and real brewery samples were also analysed (Paper III). Three different PCR formats were evaluated for their feasibility for routine QC (Papers I, III).

4.1.1 Detection of *Megasphaera cerevisiae* and *Pectinatus* by end-point PCR with colorimetric microplate hybridisation or gel electrophoresis (Paper I)

Post-PCR analysis by colorimetric microplate hybridisation has many potential benefits over the electrophoretic techniques (Soumet *et al*., 1995; Koskiniemi *et al*., 1997; Laitinen *et al*., 2002). It has earlier been used to detect and identify various pathogenic and intestinal bacteria and viruses. In this study, microplate hybridisation was first applied to the beer-spoilage bacteria. Our aim was to

improve the post-PCR analysis in comparison to agarose gel electrophoresis. Specific end-point PCRs and a colorimetric microplate hybridisation assay were established for *M. cerevisiae* and *Pectinatus* bacteria (*P. cerevisiiphilus*, *P. frisingensis*). These organisms were at the time the only absolute beer spoilage organisms in the *Sporomusa* sub-branch.

Comparative 16S rRNA gene sequence analysis revealed potential signature sequences for an *M. cerevisiae*-specific forward and a reverse primer in the V2 and V4 regions. For the genus *Pectinatus*, our earlier designed primer set was applied in the same reaction conditions as the *M. cerevisiae*-specific primers in order to allow their parallel use. It amplifies a 815-bp fragment between the V2 and V6 regions (Satokari *et al*., 1997). In the microplate assay, the biotinylated PCR products were captured on streptavidin-coated microtitre wells and hybridised with a semiconserved digoxigenin-labelled detection probe. The hybridisation was visualised using ELISA (Koskiniemi *et al*., 1997). A cut-off value based on the optical densities of negative control and blank samples was defined to distinguish between negative and positive PCR results.

Specificity testing showed that both PCRs combined with either the gel electrophoresis or with the microplate assay, differentiated their target species from other bacteria likely to occur in the brewery samples (Table 9). The anomalous products amplified from a few non-target strains in the *M. cerevisiae*-specific PCR did not give a positive signal in the microplate assay. In the gel electrophoresis, they were separated from the specific products by size. The analysis of the real brewery samples confirmed the specificities of the reactions (Paper III: Table 5). We later showed that the *Pectinatus*-specific PCR also yields a correct-sized product from *Pectinatus haikarae*, a new beer spoiler described in Paper V. The reaction was rather weak due to three and two mismatches in the forward and in the reverse primer sequence, respectively (Table 9). The *M. cerevisiae*-specific PCR did not cross-react with the new, closely related *Megasphaera* species (Table 9, Paper V). The forward primer had five mismatches and two gaps, and the reverse primer six mismatches compared to the 16S rRNA gene sequences of these species.

The microplate assay was ten times more sensitive than the gel electrophoresis, detecting 5×10^8 molecules. These sensitivities are in the range reported in previous comparisons of the two methods (Koskiniemi *et al*., 1997; Laitinen *et al*., 2002). The interpretation of weak positive results was more objective in the microplate assay than in the gel electrophoresis, since the cut-off value could be used. However, the microplate assay was more laborious and time-consuming when performed manually.

Table 9. Specificities of the PCR primer sets and post-PCR detection methods developed in this study.

Strains (VTT)	PCR product with universal primers	End-point PCR			End-point and real-time PCRs		
		816-bp product with *Pectinatus* primers	403-bp product with *M. cerevisiae* primers	342-bp product with group-specific primers	Restriction pattern (bp) with *BssHII, KpnI, XmnI*	Restriction pattern (bp) with *ScaI*	T_m of real-time PCR products (°C)
M. cerevisiae E-79111[T], E-981087, E-79110, E-84195 [1]	+[2]	–[3]	+	+	39 + 89 + 214	342	89.0±0.07
M. elsdenii E-84221[T]	+	–	–	+	39 + 89 + 214	342	88.8
M.microniciformis E-032113[T]	+	–	–	+	39 + 89 + 214	342	88.5
Megasphaera paucivorans E-032341[T], E-042576	+	–	–	+	39 + 89 + 214	342	88.8±0.0
Megasphaera sueciensis E-97791[T]	+	–	–	+	39 + 89 + 214	342	89.1
P. cerevisiiphilus E-79103[T], E-81132	+	+	–	+	75 + *ca.* 130	342	89.0±0.1
P. frisingensis E-79100[T], E-011871, E-91471, E-981088	+	+	–	+	75 + *ca.* 130	342	89.3±0.0
P. haikarae E-88329[T], E-89371, E-88330, E-97914	+	±[4]	–	+	75 + *ca.* 130	342	89.4±0.08
S. lacticifex E-90407[T], E-86273	+	–	–	+	75 + *ca.* 130	144, 198	88.0±0.14

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Species	PCR product with universal primers	End-point PCR			End-point and real-time PCRs		
		816-bp product with *Pectinatus* primers	403-bp product with *M. cerevisiae* primers	342-bp product with group-specific primers	Restriction pattern (bp) with *BssH*II, *Kpn*I, *Xmn*I	Restriction pattern (bp) with *Sca*I	T_m of real-time PCR products (°C)
Z. paucivorans E-90405[T]	+	–	–	+	139 + 203	342	87.6
Z. raffinosivorans E-90406[T]	+	–	–	+	139 + 203	342	87.6

[1] No data with group-specific primers, [2] Expected size band or C_p value < 41, [3] No visible band or C_p value < 41, [4] Faint band.

No amplification with any of the specific primers from *Acetobacter pasteurianus* E-97830[T], *Brevibacillus parabrevis* E-83171, *Gluconobacter oxydans* E-97864[T], *Enterobacter aerogenes* E-88325, *Enterococcus faecium* E-90381, *Kocuria kristinae* E-82147, *Kocuria varians* E-82148, *L. brevis* E-91458[T], *Lactobacillus buchneri* E-75036, *L. casei* E-85225[T], *L. coryniformis* E-93487[T], *Lactobacillus curvattus* E-90391[T], *Lactobacillus delbrueckii* E-79097[T], *Lactobacillus fermentum* E-93489[T], *Lactobacillus fructivorans* E-91473[T], *L. lindneri* E-91460[T], *Lactobacillus paracasei* E-93490[T], *L. plantarum* E-79098[T], *Lactobacillus perolens* E-90345, *Lactococcus lactis* E-90414, *Leuconostoc citreum* E-93497, *O. proteus* E-78073[T], *Pantoea agglomerans* E-90413[T], *Pectobacterium carotovorum* E-981115[T], *P. damnosus* E-91459[T], *P. inopinatus* E-96661[T], *Pediococcus pentosaceus* E-88317[T], *Saccharomyces pastorianus* A-63015, *Staphylococcus warneri* E-82149 and *Zymomonas mobilis* E-82099[T].

4.1.2 Detection of *Sporomusa* sub-branch beer-spoilage bacteria by real-time PCR and PCR-RFLP (Paper III)

Beer contaminations due to *Sporomusa* sub-branch spoilage bacteria are typically sporadic and a large fraction of routine samples test negative (Haikara and Helander, 2006). Hence, QC of brewery samples using a specific PCR for each potential spoilage species or genus is impractical and expensive. PCR tests with narrow-range specificity may also give a false negative result in the case of a genetic variant strain or a new spoilage species. For example, the new beer-spoilage *Megasphaera* spp. were not detected using the *M. cerevisiae*-specific PCR (Table 9). Moreover, the increasing trend to produce new low-alcohol products is reducing selectivity of the beer environment and may provide more growth opportunities for new and potential spoilers.

We designed a specific primer set to detect the group of all nine potential and absolute *Sporomusa* sub-branch beer-spoilage bacteria in a single test (Table 9). The putative group-specific signature sequences bordered a polymorphic 342-bp fragment spanning the V3 and a part of the V4 region (Fig. 6). Testing specificity *in silico* predicted that neither primer binds to brewery-related non-target species. A few perfect sequence hits with phylogenetically close but ecologically distant species were inevitable, since the beer-spoilage species are not a coherent group in the *Sporomusa* sub-branch (Fig. 14, p. 106). The possible cross-reacting species have not been detected in breweries using culture-dependent or – independent techniques (Back, 2005; Timke *et al.*, 2005a, b, c; Storgårds *et al.*, 2006). They are mainly found in the gut and rumen, lake sediments or clinical samples (Schink *et al.*, 1982; Strömpl *et al.*, 1999; Jousimies-Somer *et al.*, 2002; Marchandin *et al.*, 2003; Hespell *et al.*, 2006). Hence, false positive results due to their detection in brewery samples are unlikely. By contrast, our group-specific primer set could minimise the risk of missing yet-unknown target group spoilage bacteria. Based on the large number of 16S rRNA gene sequences from unidentified bacteria in the public databases, diversity in the *Sporomusa* sub-branch is underestimated (Marchandin *et al.*, 2003; Zozaya-Hincliffe *et al.*, 2008).
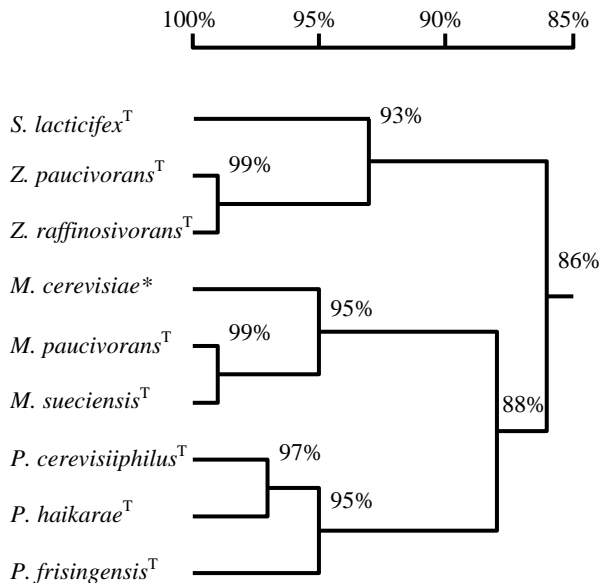
Figure 6. Dendrogram showing sequence similarity (%) of the group-specific PCR products among the target group bacteria.* Strain E-85230.

The group-specific primer set was successfully applied in the real-time PCR with SYBR Green I dye and MCA and in the end-point PCR with agarose gel electrophoresis. PCR analysis of a large number of pure culture isolates from the brewing process and from the bottling hall environment verified the specificities of the reactions for brewery application (Table 9; Paper III: Table 5). The samples from the bottling hall environment probably contain the greatest biodiversity in breweries due to low level of beer-derived growth inhibitors (Timke *et al.*, 2005a).

Sensitivity testing showed that both PCR applications could amplify trace amounts of the target DNA (Paper III: Table 2). Theoretically, even one to thirty cells were detected, assuming that they contain the same amount of DNA than *M. elsdenii* (3 femtograms) (Marchandin *et al.*, 2003). The real-time PCR was slightly more sensitive than the end-point PCR counterpart. The reason for this could be that the same template amount led to higher DNA concentration in the real-time PCR due to the 40% smaller reaction volume.

MCA is an easy, inexpensive and fast method to distinguish PCR products of identical size but different sequence (Ririe *et al.*, 1997). The potential of MCA for differentiating polymorphic fragments amplified in consensus or group-

specific PCRs rarely has been exploited (Pietilä *et al*., 2000; Nicolas *et al*., 2002; Tanriverdi *et al*., 2002; Mangold *et al*., 2005). In this study, it was shown that MCA of the group-specific PCR products can be used to classify the target bacteria in 10 min on the basis of their spoilage potential. Two sub-groups with a mean T_m difference of 1.4°C were distinguished (Table 9):

1. the absolute beer-spoilage *Megasphaera* and *Pectinatus* species (89.21°C±0.19);

2. the potential beer-spoilage *Selenomonas* and *Zymophilus* species (87.8°C±0.24).

The T_m difference between the two sub-groups was statistically significant over a wide target concentration range with pure culture strains (10 fg – 1 ng, t-test, $p < 0.001$) and with spiked beer samples (10^1–10^6 cfu; t-test, $p < 0.001$) (Paper III: Tables 2 and 3). MCA also correctly identified the products amplified from real brewery samples (Paper III: Table 5). The T_m difference between same-sized DNA fragments mainly depends on their GC content. It has been estimated that T_m will change by 1°C with each 2.4% change in GC% (Wittwer *et al*., 2001). In this study, the mean GC% difference between the sub-groups was 2.9% which translates to 1.2°C. This is very close to the measured difference (1.4°C). In previous studies, 1–2°C difference was also needed to reliably distinguish the PCR products by MCA (Wittwer *et al.*, 1997; Mangold *et al*., 2005).

In routine application of MCA, reference DNA samples representing both sub-groups should be included in each run for the product identification. The use of absolute values is unreliable, since DNA melting is sensitive to minor variations in reaction variables, such as dye or salt concentration, that may vary due to e.g. a reagent batch change (Ririe *et al*., 1997; Rantakokko-Jalava and Jalava, 2001; Mangold *et al*., 2005). In the future, new dyes that are less sensitive to variations could be used instead of SYBR Green I (Gudnason *et al*., 2007). The differentiation within the target group could also be based on sequence-specific, fluorogenic probes (Mackay *et al*., 2007). However, this approach is more expensive.

In this study, we designed a RFLP procedure to discriminate the target group bacteria at the genus level after the end-point PCR amplification. RFLP reveals DNA polymorphism within a specific genetic locus on the basis of the variation in the restriction enzyme recognizition sites. The final procedure involved a triple digestion with *Bss*HII, *Kpn*I and *Xmn*I and a separate incubation with *Sca*I. It was shown to produce the predicted genus-specific restriction profiles, except that fragments with < 10 bp difference could not be resolved in the agarose gel

(Table 9). RFLP analysis could also identify mixed contaminations consisting of two to three genera (Paper III: Table 5). It is a flexible tool for post-PCR analysis. Table 10 shows some other potentially useful enzymes. Although RFLP analysis is not able to provide as high discrimination power as the sequencing of the group-specific PCR products (Fig. 6), it is a faster and less laborious technique as it does not require prior pure culturing or cloning in the case of a mixed contamination. The whole analysis can be completed in 22 h. The digestion time might be shortened to 1–3 h to obtain the results in one working day.

Table 10. Examples of potentially useful restriction enzymes for identification of group-specific PCR products (*ca.* 342 bp).

Specificity	Restriction enzyme	Predicted RFLP profile [1]
M. sueciensis	*Xma*III	30, 312
Megasphaera	*Mae*III	138, 204
Megasphaera, Pectinatus	*Bsc*91I	139, 203 132, 210
Pectinatus, S. lacticifex, Zymophilus	*Xmn*I	139,203
Megasphaera, Pectinatus, S. lacticifex	*Kpn*I	130, 212
M. sueciensis, M. paucivorans	*Msc*I	157, 185
Zymophilus	*Mae*I	123, 219

[1] Predictions are based on the sequences of the strains shown in Fig. 6.

4.2 Application of PCR to finished beer (Papers I-IV)

PCR analysis of the spoilage bacteria in beer is faced by many challenges. The sample matrix contains unknown inhibitors (DiMichele and Lewis, 1993; Satokari *et al.*, 1997; Yasui *et al.*, 1997) and there is a need to detect only a few viable cells in a large sample volume (Jespersen and Jakobsen, 1996). For routine QC, the assay procedure also needs to be fast and easy to use in order to minimise contamination risk and to allow sufficient sample throughput (Brandl and Geiger, 2003; Haikara *et al.*, 2003).

4.2.1 PCR inhibition and evaluation of pre-PCR processing methods (Papers I, II, IV)

We evaluated different methods for pre-PCR processing of beer samples with the aim of developing an easy, rapid, inexpensive and sensitive procedure. In brewery trials, complexity and high costs compared to cultivation have been identified among the major factors limiting PCR implementation in the brewing industry (Brandl and Geiger, 2003).

The first step for pre-PCR processing of beer samples usually involves collecting cells by membrane filtration. Our PCR inhibition studies showed that the non-filterable material retained on the membrane filters contains potent PCR inhibitors and cannot directly be applied to PCR (Papers I, IV). The inhibiting effect of different lager beer brands varied considerably (Paper IV: Table III). Moreover, we found that real-time PCR tolerated at least a 5-fold higher amount of the inhibiting material than the end-point counterpart (Paper IV). The differences between the PCR formats in their reagent mixture composition, reaction kinetics and detection principle could explain this result. The commercial real-time PCR master mixture contains BSA, which possibly relieved the inhibition (Kreader, 1996; Teo *et al*., 2002). DNA polymerases are also known to differ widely in their sensitivity to inhibitors (Rådström *et al*., 2004).

Two easy strategies were evaluated to minimise the influence of the beer-derived material on PCR, i.e. removal of inhibitors during the membrane filtration step and suppression of inhibitors during PCR amplifications.

Washing membrane filters with 0.1M NaOH and 0.5% SDS (mod. from Yasui *et al*., 1997) was shown to be an efficient method for removing PCR inhibitors from a wide range of commercial beers (100–330 ml) (Papers I–IV). It allowed at least a 20-fold increase in the beer volume tolerated by the PCR reactions. The PCR detection limits in beer and in water were identical, confirming the absence of residual inhibitors in the extracts (data not shown). Hence, further DNA purification was not needed. Labelling DNA of the cells on non-washed and NaOH-SDS washed membrane filters with a fluorochrome suggested that the wash causes DNA losses from some *Pectinatus* and *Megasphaera* strains (Paper I). This phenomenon was not observed in an earlier study with Gram-stain-positive beer-spoilage LAB (Yasui *et al*., 1997). It is known that NaOH and SDS lyse Gram-stain-negative cells, but normally 2–10 times stronger solutions were used than in our study (Ciccolini *et al*., 1998). Helander *et al*. (2004) showed that cell walls of the beer-spoilage *Megasphaera* and *Pectinatus* are unique in many re-

spects and may not be as effective barriers as those of classical Gram-stain-negative bacteria. SDS and NaOH also need to be carefully rinsed away from samples to avoid PCR inhibition (Rossen *et al.*, 1992). For the above reasons, we also evaluated potentially less destructive washes. They included various modifications of the original protocol, or the use of chelators, dispersants or non-ionic detergents. Of these, only 0.1M NaOH was effective alone. This implies that SDS could be omitted from the procedure to avoid its possible adverse effects. NaOH solubilises a wide range of organic deposits, such as proteins, and was also used to remove hop-derived polyphenolics associated with brewer's yeast cells (Tsang *et al.*, 1979; Nand, 1987).

The filtration capacity of the membrane filters was limited to 230–330 ml of beer (Paper IV: Table III). Cross-flow filtration through hollow-fibre membranes allows substantial increase in the filtration volume compared to the standard membrane filtration (Polaczyk *et al.*, 2008). In our study, small disposable devices based on this principle (CellTrap™) and capable of filtering up to 2.5 l of beer were evaluated. They were shown to retain fewer inhibitors than the polycarbonate membranes. As a result, two- to three-fold higher beer volumes were tolerated in the PCR reactions. They also facilitated the DNA extraction, as the cells were simply back-flushed in the eluate. In the future, such devices may prove to be a viable alternative to membrane filtration for high-volume cell concentration for PCR. Further studies are needed to optimise the analytical procedure.

This study showed for the first time that the inclusion of BSA (0.25%, w/v) or PVP (0.5–1%, w/v) into the end-point PCR mixture markedly reduces the inhibiting effect of the substances present in beer. BSA was generally a more effective PCR facilitator than PVP (Paper IV: Tables II and III). In the presence of BSA, a 5- to 30-fold higher amount of the inhibiting material was tolerated. In practice, 5-10 vol-% of the crude beer extract (200 µl) from a packaged beer could be analysed without inhibitor removal. Hence, the efficacy of BSA in the inhibition relief was comparable to that of the NaOH-SDS wash. BSA at a similar level (0.1–0.6%, w/v) also facilitated PCR amplification in the presence of plant and soil extracts, and lake water, food, blood or faeces (Kreader, 1996; Rådström *et al.*, 2004). PVP (1–2%, w/v) has been documented to be able to neutralize the effects of phenolic contamination on PCR (Koonjul *et al.*, 1999; Teo *et al.*, 2002).

The combination of a gentle inhibitor removal method, such as PVP-40 or STPP-EDTA wash or cross-flow filtration, with the use of BSA was more effective than either strategy alone, allowing for more than three-fold increase in the

tolerated beer volume (Paper IV). The possible detrimental effect of strong NaOH and SDS on PCR could be overcome by using these alternative washes in combination with a PCR facilitator.

Based on this study, some conclusions about the identity of PCR inhibitors present in beer could be drawn. The PCR-inhibiting material retaining on the membrane filters had the following properties.

- It may contain arabinoxylans, since xylanase treatment occasionally reduced its inhibiting effect.

- It was stainable with amido black that preferably reacts with proteins. The inhibition was not detected when this material was removed using the NaOH-SDS wash.

- Reagents able to react with phenolic compounds or metal ions reduced its inhibitory effect.

- It was mostly water-soluble, since organic extractions did not relieve the inhibition.

This data suggests that phenolic compounds associated with beer macromolecules are one class of PCR inhibitors in beer. We also showed that catechin, a monomer constituent of procyanidin polyphenols, inhibits PCR at the concentrations (5–10 mg l^{-1}) found in beer (Table 11) (Madigan *et al*., 1994; De Pascual-Teresa *et al*., 2000). Phenolic compounds are often associated with organic macromolecules. In cereals, the polyphenols are partly linked with cell-wall arabinoxylans and proteins (Faulds and Williamson, 1999). Certain polyphenols, especially procyanidins and prodelphinidins, are also known to form insoluble complexes with beer proteins. A polymeric PVP that contains the same – NH functional group as some beer proteins can specifically adsorb these complexes (Madigan *et al*., 1994; Freeman, 2006). Most plant-derived polyphenols also coisolate with DNA in the organic extractions (Koonjul *et al*., 1999). Phenolic compounds are known for their PCR-inhibiting properties. They are thought to inhibit the PCR by binding to DNA and proteins, or by interfering with the interaction between DNA polymerase and its template (Wilson, 1997).

Table 11. Effect of catechin on end-point PCR amplification with DynaZyme and *Taq* DNA polymerases.

Catechin (mg l^{-1})	DynaZyme polymerase	*Taq* polymerase
0.1	+[1]	+
0.5	+	+
5	+	–
10	–[2]	–
50	–	–

[1] No inhibition, [2] PCR totally inhibited.

Our study also suggests the presence of other PCR inhibitors in beer. BSA with a broader range of anti-inhibitory activity was a more effective PCR facilitator than PVP, which is mainly phenol-specific (Wilson, 1997; Teo *et al*., 2002).

   Two easy and rapid cell lysis methods, i.e. boiling and InstaGene Matrix, were applied in combination with the anti-inhibitory treatments for detecting the target bacteria in artificially contaminated beers (Papers I–IV). They produced comparable detection limits. Both methods have their benefits and drawbacks. The InstaGene Matrix could also be used for DNA extraction from beer-spoilage LAB (Stewart and Dowhanick, 1996; Haakensen *et al*., 2007) that were not efficiently lysed by boiling (data not shown). Boiling was a cheaper and quicker method that could be combined with the membrane solubilisation procedure. However, this procedure involves the use of organic solvents.

4.2.2 PCR detection limits (Papers I, III, IV)

The detection limits of the developed PCR tests were studied in combination with different easy pre-PCR processing methods by assaying artificially contaminated beers. Although different sample volumes were used in Papers I–IV for practical reasons, all the procedures allowed for analysing at least 100 ml of standard lager beer. The detection limit of the *M. cerevisiae-* and *Pectinatus*-specific end-point PCR assay was ≥ 5 x 10^3 cfu and ≥ 5 x 10^5 cfu, respectively, in 100 ml of beer (Paper I). The poor assay sensitivity with some strains could be partly explained by premature lysis of a part of the cells during the NaOH-SDS wash which was used for inhibitor removal. The agarose gel electrophoresis and the microplate assay were equally sensitive post-PCR detection methods (Paper I). This contrasts with the results obtained with purified PCR products. A reason for this could be that the unincorporated biotinylated primers competed

with the PCR products for binding sites on the microplate wells, leading to the signal reduction. The group-specific PCR assays detected from less than 10^1 to 10^3 cfu in 25 ml of beer (Paper III: Table 3, Paper IV). These detection limits closely corresponded to the theoretical minimum of the assays which was 4×10^1 cfu 25 ml^{-1} for end-point PCR and 1×10^2 cfu 25 ml^{-1} for real-time PCR.

The sensitivity of a PCR assay is influenced by pre-PCR processing, PCR amplification and detection steps (Malorny *et al.*, 2003). Hence, several possibilities exist to explain the variation in the detection limits of the developed PCR assays (Papers I, III). Differential lysis of the strains during pre-PCR processing was considered likely to contribute to the observed variation (Paper I). The variation could also be due to a difference in *rrn* copy number between the strains (Marchandin *et al.*, 2003; Bouchet *et al.*, 2008), to point mutations in the primer sites or to differences in the PCR amplification efficiencies (Paper III). Furthermore, the cell counts estimated by the plate count method may have been inaccurate due to possible cell clumping and inability of the cultivation method to detect dead and unculturable cells (Amann *et al.*, 1995). PCR inhibition as the cause of variable results is unlikely, since no inhibition was detected when the sample extracts were amplified with a known amount of target DNA.

The detection of even a single viable beer-spoilage microbe in a package of beer is a general requirement in brewery QC (Jespersen and Jakobsen, 1996; Back, 2005). In practice, this high sensitivity is not possible due to inevitable target losses during pre-PCR processing and the small final sample size, and due to statistical uncertainty of single-cell detection. Although traditional DNA extraction procedures can provide better sensitivity than rapid pre-PCR processing, they are too laborious and time-consuming for routine QC (Tsuchiya *et al.*, 1992 and 1993; Yasui *et al.*, 1997; Satokari *et al.*, 1997). Moreover, an extremely sensitive PCR assay would be prone to incorrect interpretations concerning the spoilage risk, since even a few dead cells in a sample or a trace contamination from the work environment could lead to a positive result. For these reasons, a growth-based enrichment step was incorporated into the PCR assays instead of further improving the assay sensitivities.

4.2.3 Improvement of PCR assay sensitivities by culture enrichment (Papers II, III)

This study was the first to establish pre-PCR enrichment procedures for beer-spoilage bacteria. We set the following minimum criteria for the enrichment procedure:

- easy application,
- rapid growth of a wide range of anaerobic beer-spoilage bacteria,
- no interference with PCR, and
- reasonable costs.

Direct enrichment in a concentrated growth medium, also known as the forcing test, was the technique of choice, since membrane filtration of beer samples before the enrichment may inactivate strictly anaerobic beer spoilers (Haikara, 1985a; Brandl and Geiger, 2005). Moreover, this technique was easy and could be used for any beer-spoilage microbe. Since PCR is a specific method and the number of competing microbes in beer is generally low, strictly selective enrichment conditions were not considered necessary in order to improve the growth and recovery of the target bacteria.

In Paper II, we established a new procedure for the pre-PCR enrichment of the absolute beer-spoilage bacteria. It used a five-fold concentrated MRS broth (EC-MRS) for maximising the sample volume, while providing the same level of nutrients as in a single-strength medium. The medium was fortified with fructose to enhance the growth of *M. cerevisiae* and with cycloheximide to suppress the yeast growth. At the same addition level, EC-MRS broth supported faster growth of the target bacteria than NBB-C and C-MRS media routinely used in the forcing tests (Paper II: Fig. 1). It also outperformed these media when they were used according to EBC *Analytica Microbiologica* (Hage *et al.*, 2005). For the detection of low levels of contamination by the *M. cerevisiae*- and *Pectinatus*-specific PCR assays, an enrichment time of 2–4 d was needed (Table 12).

Table 12. Enrichment times for the detection of the target bacteria (≤ 10 cfu 100 ml^{-1}) by *M. cerevisiae*- and *Pectinatus*-specific PCR assays in beer forced with EC-MRS medium.

Species	No. of strains	Enrichment time (d) in beer with	
		< 2.8 vol-% alc.	3.7–4.7 vol-% alc.
M. cerevisiae	4	2–3	nd
P. cerevisiiphilus	1	nd [1]	4
P. frisingensis	2–3	2	2–3
P. haikarae	1	nd	3

[1] Not done.

In Paper III, a double-concentrated MRS-fructose broth (2-MRSF) applied with an equal volume of the beer sample was used to enrich potential and absolute beer-spoilage bacteria for their detection by the group-specific PCRs. The medium was developed and validated in a collaborated EU-project for rapid enrichment of beer-spoilage LAB and the *Sporomusa* sub-branch bacteria (Brandl and Geiger, 2005). In this medium, fewer than 10 cfu were detected after 1–3 d enrichment, usually already after 1 d (Table 13).

Table 13. Enrichment times for the detection of the target bacteria ($\leq$ 10 cfu 25 ml^{-1}) by the group-specific PCR assays in beer forced with 2-MRSF medium.

Species	No. of strains	Enrichment time (d) in beer with		Time saving (d) [1]
		< 2.8 vol-% alc.	3.7–4.7 vol-% alc.	
M. cerevisiae	1	1	1	4–6
P. frisingensis	2	1	1–2	3–4
S. lacticifex	1	1	1	1–2
Z. raffinosivorans	1	2–3	2–3	1–2

[1] Compared to visual turbidity development in the same medium.

In beverages and food, bacteria are exposed to various stresses, and the enrichment times predicted using active laboratory cultures may not be sufficient in practice (Uyttendale *et al*., 1998). Therefore, we looked at the impact of possible brewery-related stress conditions on the growth rate of *M. cerevisiae* E-981087. The log-phase cells were cold-stored in beer or in water for 0, 3 or 7 d before the enrichment. Long-term exposure of the cells to beer or to starvation did not influence their growth (turbidity development) compared to active cells (Fig. 7). However, a transient passage in beer or water caused slight growth retardation (Fig. 7). A 90% decrease in the number of culturable cells was detected after 7 d storage (data not shown). Taken together, our results suggest that *M. cerevisiae* is capable of long-term survival and maintenance of high cell division capacity in beer and in water at low temperatures. In the study of Chihib and Tholozan (1999), *P. frisingensis* also recovered rapidly from a cooling treatment in growth-permissive conditions. Our results further indicate that part of the population enters into a viable but non-culturable state that cannot be detected on PYF agar, but readily grows in the beer-based enrichment medium. During beer adaptation, *L. lindneri* cells also gradually lost their culturability on standard media, although they were able to grow in a beer-based medium (Suzuki *et al*., 2006a). It appears that pro-

longed exposure of *M. cerevisiae* to sub-optimal conditions triggers an adaptive stress-tolerance response in the cells – a common survival strategy in bacteria (Aertsen and Michiels, 2004).
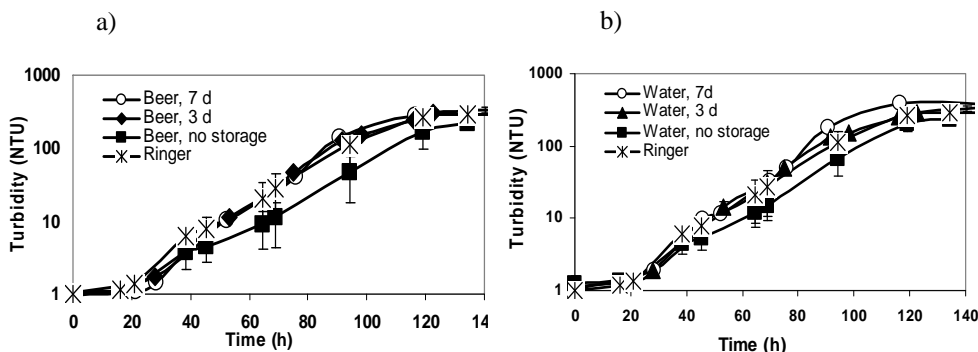
Figure 7. Growth of stressed and active (Ringer) *M. cerevisiae* E-981087 cells in beer mixed with 2-MRSF in anaerobic conditions at 27°C after a) 0-, 3- and 7-day storage in beer at 7°C, or b) 0-, 3- and 7-day storage in water at 13°C. Growth is expressed as the mean nephelometric turbidity unit (NTU) of three replicates (± SD). The experiment was performed twice.

Even in combination with culture enrichment, PCR offered a considerable time saving compared to the forcing test. In the case of the group-specific PCR, the time saving ranged from one to more than six days (Table 13). The preliminary industrial trials indicated that 3–4 d of enrichment is sufficient for presence-absence testing by PCR (data not shown). In practice, the detection of *Megasphaera* and *Pectinatus* contamination in beer by the forcing test takes 3–4 and 2–3 weeks (Haikara and Helander, 2006). The enrichment also ensured that the detected cells were viable. Moreover, the MRS-based enrichment media established in this study were PCR-compatible and could be produced from a commercial base. The selective SMMP medium strongly inhibited PCR and could not be applied in combination with the rapid cell lysis methods.

A potential drawback of culture enrichment is that the growth of non-target organisms might lead to the suppression of the target bacteria, decreasing the assay sensitivity. This problem was not encountered in an industrial trial in which 2-MRSF medium was used in parallel with the more selective NBB-C medium (data not shown). In the future, the assay sensitivity could be improved by collecting cells from several packages of beer using powerful concentration techniques rather than by culture enrichment.

Based on our results, a flexible PCR-based procedure for the presence-absence testing of the *Sporomusa* sub-branch spoilage bacteria in beer is proposed. It can be adjusted based on individual brewery needs (Fig. 8). After the enrichment step, 20 samples could be processed in *ca.* 2 h 20 min. The consumable costs per sample were estimated to be 3–4 € The enrichment medium costs for 25–100 ml beer volumes were comparable to costs for the NBB-C method (EC-MRS: 2 €, NBB-C: 0.2–2 €).

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┌─────────────────────────────────────┐
│           Finished beer             │
│         ▪ 100–330 ml                │
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┌─────────────────────────────────────┐
│     Forcing of beer samples with    │
│     2-MRSF or EC-MRS medium         │
│         ▪ 3–4 d 27–30°C             │
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│   Collection of cells by filtration on │
│       polycarbonate membrane        │
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┌─────────────────────────────────────┐
│      Removal of PCR inhibitors      │
│   ▪ membrane wash with 1%  PVP,     │
│     0.01M EDTA-0.2% STPP or         │
│        0.1M NaOH solution           │
└─────────────────────────────────────┘
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│           DNA isolation             │
│       ▪ InstaGene Matrix            │
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┌─────────────────────────────────────┐
│   PCR in the presence of BSA (0.25%,│
│         w/v) for inactivation of    │
│         residual inhibitors         │
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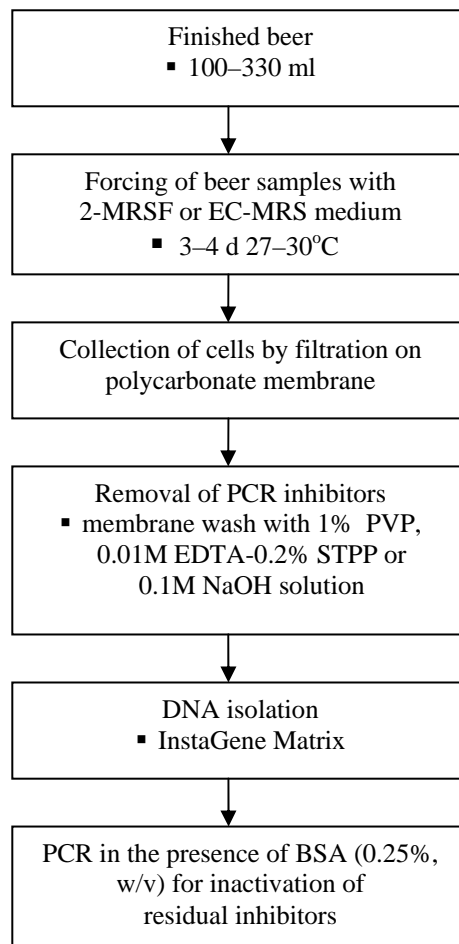
Figure 8. Proposed procedures for pre-PCR processing of beer samples.

4.3 Application of PCR to yeast-containing brewery process samples (Paper IV)

In addition to finished beer, the *Sporomusa* sub-branch beer-spoilage bacteria have been isolated from pitching yeast and from unfiltered beer (Haikara, 1989; Schleifer *et al.*, 1990; Seidel-Rüfer, 1990). The relevance of the primary process contamination to beer quality is still poorly known. A culture-independent detection method could provide new information to link the presence of these bacteria with possible quality defects. In this study, PCR was applied to the monitoring of the *Sporomusa* sub-branch beer-spoilage bacteria in unfiltered brewery process samples. These samples contain high numbers of brewer's yeast cells and cannot be concentrated by membrane filtration. Therefore, another method was needed for their pre-PCR processing.

4.3.1 Evaluation of pre-PCR processing methods

Wort and brewer's yeast cells, the constituents of yeast-containing brewery samples, were shown to have a synergistic inhibitory effect on the end-point and real-time PCR amplifications (Paper IV: Figs. 1 and 2, Table 2). Brewer's yeast cells could have bound inhibitors from wort, since the wort-grown cells were more inhibitory than cells grown in wort-free medium (Paper IV: Figs. 1 and 2, Table 2). It is probable that yeast-bound phenolic substances were involved in the inhibition (Pecar *et al.*, 1999). The real-time PCR was mainly sensitive to pure yeast constituents, whereas the end-point counterpart was mainly influenced by the wort constituents (Paper IV: Fig. 1, Table 2). In the real-time PCR, a high concentration of non-target DNA may have masked target amplification by causing high background fluorescence due to incomplete denaturation during the rapid cycling (Teo *et al.*, 2002). BSA inherently present only in the real-time PCR mixture probably relieved the inhibition of PCR by the wort.

   To overcome PCR inhibition caused by yeast-containing brewery samples, different PCR facilitators, and cell separation and DNA extraction methods were evaluated. *M. cerevisiae* E-981087 (floc-forming coccus) and *S. lacticifex* E-90407^{T} (rod) were selected as the model bacteria. Two PCR facilitators, i.e. BSA and PVP-40, were found to reduce the inhibition of the end-point PCR by the process samples (Paper IV: Table 2). PCR sensitivity was the same as in the reactions with water instead of the sample extracts. BSA was subsequently included in the end-point PCR mixtures.

Spoilage bacteria can easily be collected from the non-filterable brewery samples by centrifugation. In this study, we found that separation of the bacteria from heavier sample constituents by low-speed centrifugation was beneficial for their PCR detection (Fig. 9). The result implied that the inhibitory components were preferentially removed in the applied conditions. This approach has earlier been succesfully used to improve the rapid detection of beer-spoilage pediococci and *O. proteus* (Whiting *et al*., 1992; Koivula *et al*., 2006).
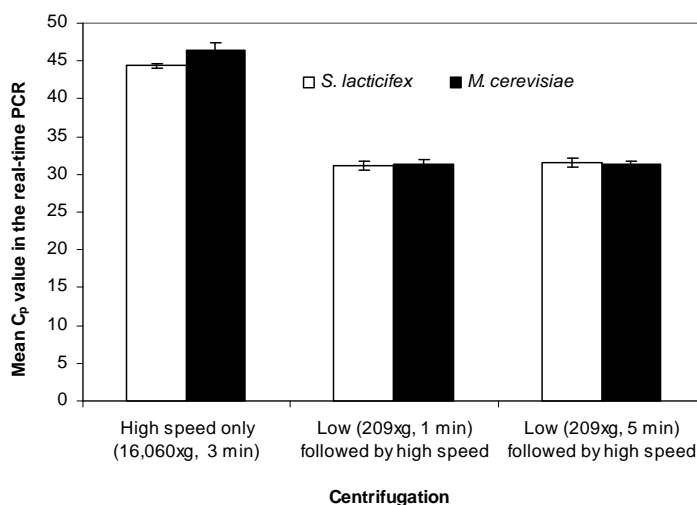
Figure 9. Effect of centrifugation on the real-time PCR detection of *S. lacticifex* and *M. cerevisiae* (*ca*.10^4–10^5 cfu ml^{-1}) in 1 ml wort samples containing brewer's yeast (10^8 cells ml^{-1}). Both yeast and bacteria were collected for PCR by high-speed centrifugation, or the cell pellet was also further subjected to low speed-centrifugation and the cells in the supernatant were recovered for use. Heating was used for cell lysis for all samples.

Comparison of different cell lysis methods in combination with low-speed centrifugation, and two commercial DNA extraction and purification kits, showed no major difference in sensitivity (Table 14). Obviously, the improved template quality brought about by the purification step did not compensate for the extra target DNA losses. Hence, using the more expensive and laborious procedures with DNA purification was not necessary provided the impact of residual inhibitors was relieved with BSA. Based on its ease of use, low costs, speed, good sensitivity, amenability to automation and compatibility with both PCR formats, bead-beating is proposed for DNA release from the target group bacteria in brewery process samples.

Table 14. Comparison of DNA extraction methods for process samples containing high numbers of brewer's yeast cells.

Method	Needed equipment	Time, h	Reagent costs per sample, €	Theoretical minimum, cells ml^{-1} [2]	PCR detection limit, cfu ml^{-1}	
					end-point	real-time
Bead-beating [1]	Mixer, centrifuge	1	< 0.5	20–100	30–200	300–2,000
Heating [1]	Thermal incubator, mixer, centrifuge	1	< 0.5	20–100	30–200	300–20,000
InstaGene Matrix [1]	Thermal incubator, mixer, centrifuge	1.5	*ca.* 1	20–100	30–200	300–2,000
Bugs'n Beads™ kit	Thermal incubator, magnet, mixer (centrifuge)	3	*ca.* 4	6–30	200–300	300–2,000
Ultra Clean™ Soil kit	Thermal incubator, mixer, centrifuge	2	*ca.* 4	10–50	30–2,000	300–2,000

[1] The brewer's yeast cells were removed by low-speed centrifugation before recovery and lysis of bacterial cells, [2] 1–5 µl used for PCR.

Brewer's yeast cells and many brewery bacteria (co)flocculate (Peng *et al.,* 2001). In theory, this phenomenon could reduce the efficacy of the cell mass-based separation by centrifugation. Flocculation of many brewery-related microbes is a calcium-dependent process that involves binding of lectin-like cell-wall proteins to specific sugar residues on adjacent cells. It can be specifically inhibited by certain sugars (e.g., mannose, maltose) and chelators (e.g., EDTA) (Verstrepen *et al.,* 2003). We showed in this study that EDTA, without or with maltose, and Tween 20 significantly enhance PCR detection of the target bacteria (Paper IV: Table V). The positive effects were thought to be due to the removal of sample-derived inhibitors as well as specific blocking of the flocculation.

Based on our results, a rapid procedure is proposed for the pre-PCR processing of yeast-containing process samples (Fig. 10). Using this procedure, 20 samples were processed in 1 h – 1 h 30 min.

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┌─────────────────────────────────────┐
│      1 ml of yeast-containing        │
│          process samples             │
│    ▪ ≤10⁸ cells ml⁻¹                 │
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                  │
                  ▼
┌─────────────────────────────────────┐
│  Collection of cells by centrifugation│
│    ▪ 16,060x g, 3 min                │
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                  ▼
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│ Separation of bacteria from brewer's yeast│
│       by low-speed centrifugation    │
│  ▪ 1 ml 0.01 M EDTA and 0.2 M maltose│
│           ▪ 209x g, 5 min            │
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│ ▪ Collection of cells from the supernatant│
│          by centrifugation           │
│        ▪ 16,060x g, 3 min            │
└─────────────────────────────────────┘
                  │
                  ▼
┌─────────────────────────────────────┐
│           Washing of cells           │
│            ▪ 1 ml water              │
│        ▪ 16,060x g, 1 min            │
└─────────────────────────────────────┘
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│      Cell lysis by bead-beating      │
│        ▪ 0.2 ml bead solution        │
│           ▪ 10 min vortex            │
└─────────────────────────────────────┘
                  │
                  ▼
┌─────────────────────────────────────┐
│            Centrifugation            │
│        ▪ 16,060x g, 1 min            │
└─────────────────────────────────────┘
                  │
                  ▼
┌─────────────────────────────────────┐
│         Supernatant for PCR          │
└─────────────────────────────────────┘
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│ PCR in the presence of BSA (0.25%, w/v)│
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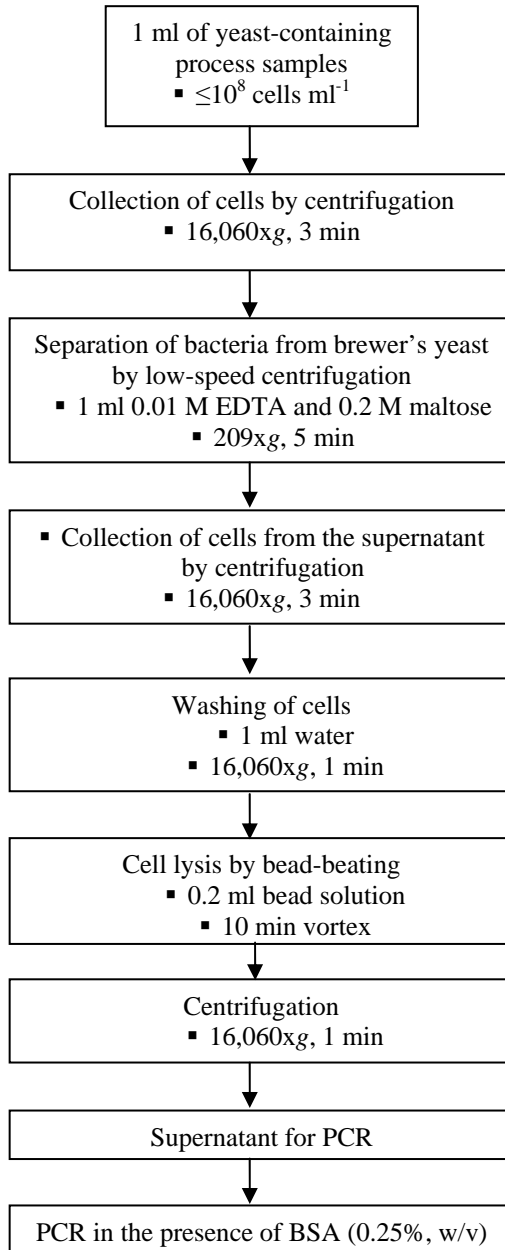
Figure 10. Pre-PCR processing of yeast-containing brewery process samples.

4.3.2 PCR detection limits

The group-specific PCR assays for the yeast-containing process samples allowed the detection of 10^1–10^3 cfu against 10^7–10^8 yeast cells ml^{-1}. This detection limit nearly meets the guideline, 1 cfu ml^{-1}, suggested for process samples (Jespersen and Jakobsen, 1996). In order to detect 1 cfu ml^{-1}, the cells could be collected from 10–100 ml instead of from 1 ml, or a 1–2 d enrichment step could be incorporated into the PCR assay. The detection limits for process samples were of the same order of magnitude as in the finished beer for the corresponding strains (Paper III: Table 3), indicating effectiveness of the pre-PCR processing step. In the study of Koivula *et al.* (2006), the PCR detection limit for *O. proteus* was *ca.* 20-fold higher in yeast slurry than in beer.

4.3.3 PCR detection of inactivated cells without and with DNase I treatment

DNA in cells is usually detectable with PCR for long periods after their inactivation. DNA stability varies depending on the organism, inactivation treatment, sample matrix and storage conditions (Nogva *et al*., 2000; Keer and Birch, 2003; Wolffs *et al*., 2005). We monitored the DNA stability in heat-inactivated *M. cerevisiae* and *S. lacticifex* cells stored in water at 0ºC for 3 d. These conditions mimicked the storage of recycled brewer's yeast. Our results showed slow DNA degradation in the inactivated cells (Fig. 11). DNA appeared to be better protected in *M. cerevisiae* than in *S. lacticifex* cells (Fig. 11). In heat-inactivated *C. jejuni*, the initial loss of DNA was rapid, followed by a slower decay (Nogva *et al*., 2000).

An approach involving externally added DNA-degrading enzyme, DNase I, was evaluated to selectively reduce the PCR signals from inactivated cells. The DNase I treatment did not influence the level of DNA in the active cultures, but it led to 10–40-fold signal reduction in the inactivated cultures compared to the control samples without DNase I (Fig. 11). Despite this, DNA was still PCR-detectable after the 3 d storage. Hence, the mild heat treatment appeared to be insufficient for fully exposing DNA for hydrolysis. This was also supported by the fact that the DNA decay kinetics with DNase I followed its natural decay (Fig. 11). Our study suggests that false positive PCR results due to the detection of DNA in the heat-inactivated cells are possible despite the DNase I treatment. It is likely that the DNA degradation would be faster at higher inactivation and

storage temperatures (Nogva *et al.*, 2000; Wolffs *et al.*, 2005). In the future, alternative strategies, such as blocking of DNA synthesis in membrane-compromised cells by propidium monoazide or incorpation of a short culture enrichment into the assay, could provide a more distinct live-dead discrimination (Nocker *et al.*, 2007).
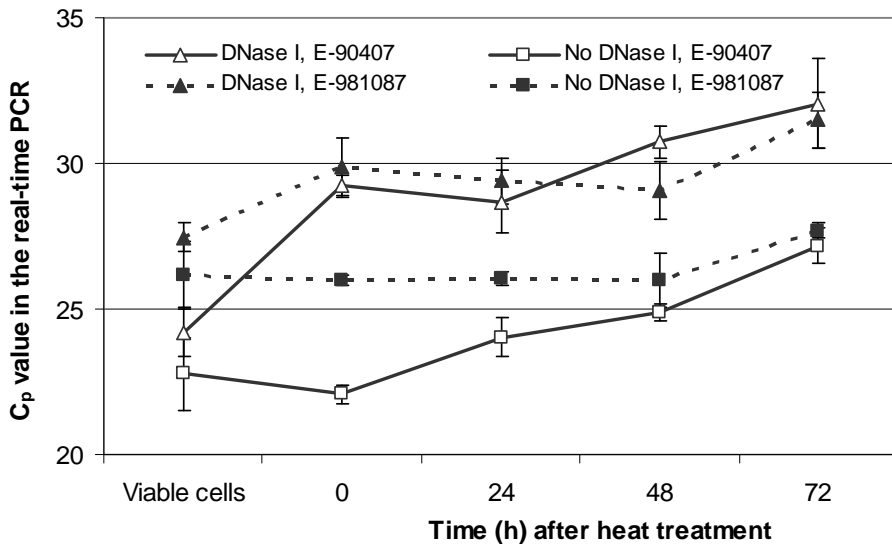

Figure 11. Influence of externally added DNase I on real-time PCR signals from active and heat-inactivated (62°C 20 min) *S. lacticifex* E-90407[T] and *M. cerevisiae* E-981087 cultures (10[5] cfu ml^{-1}). The inactivated cells were stored in water at 0°C.

We also attempted to inactivate growing cultures by bubbling with oxygen. Interestingly, *M. cerevisiae* and *S. lacticifex* survived a 22 h exposure to oxygen. Moreover, *M. cerevisiae* remained fully culturable during the 3 d post-treatment storage at 0°C, whereas the viable cell counts of *S. lacticifex* decreased below the detection limit (10 cfu ml^{-1}) in 4 h (data not shown). The absolute beer-spoilage bacterium *P. frisingensis* is also known to be rather oxygen-tolerant, showing a D_{oxy}-value of 55 h in wort at saturated oxygen content at 30°C (Chowdhury *et al.*, 1995). Low temperatures might have further increased the resistance of *M. cerevisiae* to oxygen, as reported for *P. cerevisiiphilus* (Chowdhury *et al.*, 1995). Our results preliminarily suggest that good tolerance of *M. cerevisiae* towards oxygen and low temperature stresses may partly explain its establishment in breweries.

4.4 Comparison of the PCR tests to a culture-dependent identification approach (Paper III)

The developed PCR tests were compared to a culture-dependent approach for identification of their target bacteria in spoiled beers and in brewery environmental samples (Table 15). The different methods were in good agreement. In a few samples, PCR detected the target bacteria when they could not be found by sequencing of the various types of isolated colonies. This was most probably due to the PCR detection of unculturable or dead cells or due to overgrowth of the target bacteria by other microbes in the enriched samples. It was noted that the SMMP medium designed for the detection of *Megasphaera* and *Pectinatus* bacteria in beer was not selective when applied for the environmental samples, possibly due to the greater load and diversity of competing microbes.

Table 15. Comparison of the developed PCR tests with a culture-dependent approach for the identification of *Megasphaera* and *Pectinatus* in naturally spoiled beers and in positive SMMP enrichment cultures from brewery environmental samples.

Sample		Group-specific PCR with RFLP analysis	*Pectinatus*- and *M. cerevisiae*-specific PCR	Cultivation followed by 16S rRNA gene sequence analysis of typical isolates
1	Bottling hall floor	*Pectinatus*	*M. cerevisiae* *Pectinatus*	*P. frisingensis*
2	"	*Pectinatus*	not detected	not detected
3	"	*Megasphaera* *Pectinatus*	*M. cerevisiae* *Pectinatus*	*P. frisingensis*
4	"	*Megasphaera* *Pectinatus*	*M. cerevisiae* *Pectinatus*	*M. cerevisiae* *P. frisingensis*
5	Corking machine	*Pectinatus*	*Pectinatus*	not detected
6	"	not detected	not detected	not detected
7	Spoiled lager beer	*Megasphaera* *Pectinatus*	*M. cerevisiae* *Pectinatus*	*M. cerevisiae*
8	Spoiled diet beer	*Pectinatus*	*Pectinatus*	*P. haikarae*

4.5 Application of the developed PCR assays in breweries (Papers I–IV)

The developed PCR assays provide a toolbox for rapid detection and identification of the strictly anaerobic beer spoilers. Fig. 12 shows a possible strategy for their application in breweries. It is obvious that no single PCR method is ideal for every situation, as there is no single cultivation method for all beer-spoilage bacteria. The group-specific PCR reactions provide a cost-effective approach with a high-throughput capacity for initial screening of brewery samples for any known beer spoiler in the *Sporomusa* sub-branch. RFLP analysis or the PCR tests with narrow-range specificity or both could then be used to further identify the detected bacteria. The individual PCR tests could also be used stand-alone for troubleshooting, hygiene monitoring and random checks, and for complementing the routine cultivation methods for contaminant identification. PCR is a particularly useful identification tool for the strictly anaerobic bacteria that can rarely be cultivated in industry settings. As a culture-independent technique, PCR could also shed new light on distribution of the anaerobic beer spoilers within and outside breweries.

There are many criteria to consider when selecting a PCR format for a QC laboratory, including primary application (troubleshooting/routine analysis), necessary sample capacity, work safety, contamination risks, user-friendliness, automation possibilities and operative (reagent, labour, space) and instrument costs. In the course of this study, three generations of PCR formats were applied: 1) agarose gel electrophoresis, 2) colorimetric microplate hybridisation and 3) real-time PCR with SYBR Green I. Each was found to have its own benefits and drawbacks (Table 16). Real-time PCR was undoubtly the most practical technique for routine QC. It offered many benefits compared to end-point PCR, including speed, ease of use and low risk of carry-over contaminations. Labour savings also compensated for the higher reagent costs. However, rather high capital investment for the instrumentation may still be prohibitive to its use in smaller breweries. The end-point PCR could be a useful platform for breweries that occasionally want to use PCR for troubleshooting and contaminant identification but are not ready to invest in a real-time instrument. However, the prices of the real-time instruments are continuously decreasing. Although the microplate assay facilitated the interpretation of weak positive results and reduced the need for harmful reagents, it was more laborious and expensive to use and not more sensitive than agarose gel electrophoresis as the end-point PCR readout. Therefore, the latter may be better suited for the end-point detection than the

non-automated microplate assay. However, the microplate hybridisation might prove a convenient format for high-throughput identification in combination with multiple taxon-specific and functional probes ("DNA macrochip") instead of the semiconserved detection probe used in this study.
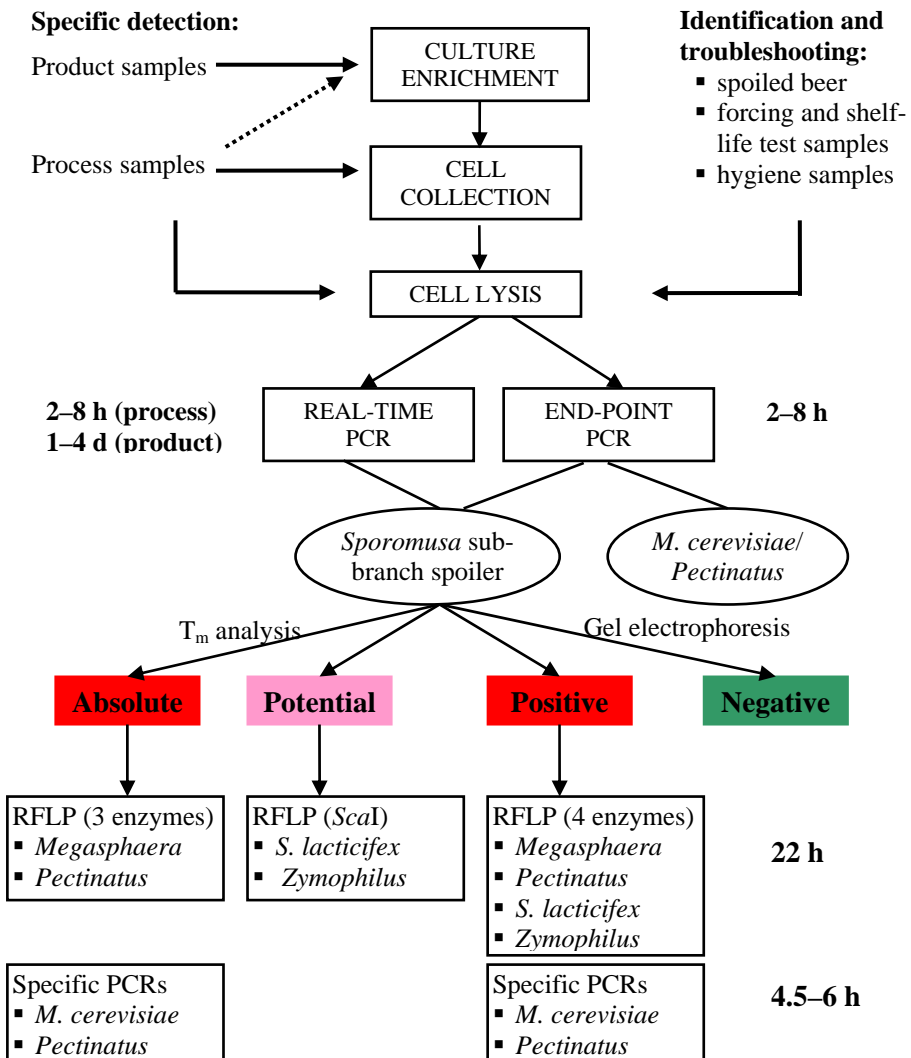

Figure 12. Overview of possible application of the developed PCR assays in brewery QC.

Table 16. Comparison of various PCR formats for detection of the *Sporomusa* sub-branch beer-spoilage bacteria in brewery samples.

Property	End-point PCR and gel electrophoresis	End-point PCR and microplate hybridisation	Real-time PCR with SYBR Green I
PCRs in a run [1]	96	96	32
Duration of PCR analysis	4.5–5 h	5.5–6 h	0.75 h
Throughput in a working day:			
▪ PCRs	96	96	200
▪ Brewery samples [2]			
* non-filterable	40–60	40–60	75–105
* filterable	15–25	15–25	30–40
Post-PCR handling	Yes	Yes	No
Risk of PCR carry-over contamination	High [3]	High [3]	Low
Automation level	Low	Low (can be automated)	High
Harmful reagents	Yes	No	Few
Interpretation of results	Subjective (visual)	Objective (cut-off value)	Objective (cut-off value)
PCR reagent costs	Low (≤ 3 €)	Low to medium (< 5 €)	Medium (5 €)
Labour costs	Medium	Medium to high	Low
Instrumentation costs	Low (≥ 4 000 €)	Low (≥ 4 000 €)	Medium to high (25 000–130 000 €) [4]
PCR applications	*Sporomusa* sub-branch spoilers, *M. cerevisiae*, *Pectinatus*	*M. cerevisiae*, *Pectinatus*	*Sporomusa* sub-branch spoilers

[1] Capacity depends on the instrument. Real-time PCR instruments for 96–384 samples are also available, [2] Entire end-point PCR procedure was estimated to take 4.5 h with agarose gel electrophoresis and 5.5 h with microplate hybridisation, [3] Contamination control system may be added to prevent PCR carryover, [4] Mothershed and Whitney (2006).

4.6 New *Megasphaera* and *Pectinatus* species in the beer production chain (Paper V)

Genetic characterisation of 13 *M. cerevisiae* isolates from the beer production chain by Suihko and Haikara (2001) suggested that one of them is potentially a new species. Moreover, we isolated from a spoiled beer exceptionally slow-growing Gram-stain-negative anaerobic cocci that gave a negative result in the *M. cerevisiae*-specific PCR (Table 9, p. 73). A *Pectinatus*-like strain (DSM 20764, E-97914) originating from German beer was deposited to DSMZ in the 1990s. Based on the strain information, it shared less than 20% DDH value with *P. cerevisiiphilus* and *P. frisingensis* type strains. Further genetic characterisation of this and similar strains from Finland also implied that they could represent a new *Pectinatus* species (Suihko and Haikara, 2001). In this study, a polyphasic characterisation of the *Megasphaera*- like and *Pectinatus*-like strains was carried out for their phylogenetic and taxonomic assignment.

4.6.1 Polyphasic characterisation of *Megasphaera*-like isolates

The three *Megasphaera*-like strains characterised were E-032341[T] and E-042576 isolated from beer produced in Italy (5 vol-% alc., pH 4.3) and E-97791[T] isolated from beer produced in Sweden (2.8 vol-% alc., pH 4.9).

The three strains shared almost identical 16S rRNA gene sequences (99.3–100%). The phylogenetic analysis suggested that they represent new species within the *Anaeroglobus-Megasphaera* group of the *Sporomusa* sub-branch (Paper V: Fig. 1). Their 16S rRNA gene sequence similarities to the nearest type species (*A. geminatus*, *M. cerevisiae, M. elsdenii, M. micronuciformis*) were far below 98.7–99%, the range below which a new isolate can be defined as a distinct genospecies (Stackebrandt and Ebers, 2006). The DNA GC content of the strains was within or slightly below the range of the genus *Megasphaera* (Engelmann and Weiss, 1985; Marchandin *et al.*, 2003) and far below that of the genus *Anaeroglobus* (Carlier *et al.*, 2002)

Genetic relatedness between E-97791[T] and E-032341[T] was further studied using DDH, since it provides better resolution than the 16S rRNA gene sequence analysis above 98.7–99% sequence similarities (Stackebrandt and Ebers, 2006). The results revealed that E-97791[T] and E-032341[T] are not related to each other or to any *Megasphaera* type strain at the species level (DDH value $< 70\%$; Wayne *et al.*, 1987) (Table 17). It also verified that E-97791[T] and E-032341[T] are

the closest relatives among these strains. Ribotyping further supported the assignment of E-97791[T] into separate genospecies from the strains E-032341[T] and E-042576 (Fig. 13, p. 102).

Table 17. DDH similarity values between the representative *Megasphaera*-like strains and the type strains of the genus *Megasphaera*.

Strain	M. paucivorans E-032341[T]	M. sueciensis E-97791[T]
M. cerevisiae E-79111[T]	3.1	22.0
M. elsdenii E-84221[T]	23.6	7.2
M. micronuciformis E-032113[T]	17.1	28.9
M. paucivorans E-032341[T]		41.0

Physiological and biochemical characterisation reinforced the phylogenetic assignment and uniqueness of both genospecies (Table 18). All strains produced C4–C6 fatty acids – a typical feature of the genus *Megasphaera* (Marchandin *et al.*, 2003; Haikara and Helander, 2006). They were distinguishable from the known *Anaeroglobus* and *Megasphaera* species based on several phenotypic criteria (Paper V: Table 1). The new genospecies differed from each other in their metabolite profiles, cell size and growth rate (Paper V: Table 1). The main metabolite of E-97791[T] was isovalerate, whereas E-032341[T] and E-042576 produced almost equal amounts of isovalerate and caproate. Pyruvate, gluconate and glucuronate were the only carbon sources supporting good growth.

In conclusion, the phylogenetic analysis supported by DNA GC content, cell morphology and metabolic end-products showed that the *Megasphaera*-like strains do indeed belong to the genus *Megasphaera*. The strains represented two new genospecies when a species is defined as a group of strains sharing at least 70% DDH value as recommended by Wayne *et al.* (1987). Their description as two new species was also justified, since they could be discriminated from each other and from the known species phenotypically (Paper V: Table 1) (Wayne *et al.*, 1987). The names *Megasphaera* paucivorans sp. nov. (pau.ci.vo'rans. L. adj. paucus, few, little, L. part. adj. vorans, devouring; N.L. part. adj. *paucivorans*, devouring a few substrates), including E-032341[T] (= DSM 16981[T]) and E-042576, and *Megasphaera sueciensis* sp. nov. (sue.ci.en' sis. N.L. fem. adj.

sueciensis pertaining to Sweden), including E-97791^T (= DSM 17042^T), were validly published for these species (Paper V).

4.6.2 Polyphasic characterisation of *Pectinatus*-like isolates

Four *Pectinatus*-like strains were characterised. Strains E-88329^T and E-88330 were isolated from the air of a brewery bottling hall and E-89371 from spoiled beer produced in Finland (2.7 vol-% alc.). Strain E-97914 originated from German beer.

The strains shared identical 16S rRNA gene sequences (1467 ntd). Based on the phylogenetic analyses their nearest relatives were *P. cerevisiiphilus*^{T} and *P. frisingensis*^{T}, with 95.6% and 93.6% similarity (Paper V: Fig. 2). The result suggested that the strains belong to a new genospecies, since sequence similarities below 98.7–99% range usually correlate with lower than 70% DDH similarity (Stackebrandt and Ebers, 2006). Ribotyping with *Eco*RI also revealed a homogenous group, distinct from the *P. cerevisiiphilus* and *P. frisingensis* strain groups (Paper V: suppl. Fig. 2). Moreover, the GC mol% of a representative strain (E-88329^T) fell within the radius of the genus *Pectinatus* and outside that of the related *Selenomonas* genus (Schleifer *et al.*, 1990; Hespell *et al.*, 2006).

The strains were shown to possess morphological features of *Pectinatus*, *viz* slightly curved to helical cell shape and formation of an "X" pattern during movement that separates them from selenomonads with tumbling motility (Schleifer *et al.*, 1990; Hespell *et al.*, 2006). The metabolites (propionate, acetate, H$_2$S and acetoin) also supported their inclusion into the genus *Pectinatus*, and distinguished them from other rod-shaped strict anaerobes found in breweries (Schleifer *et al.*, 1990; Haikara and Helander, 2006). The strains differed phenotypically from the known *Pectinatus* species (Paper V: Table 2). Most notably, they were catalase positive. Moreover, they formed acid from lactose but not from D-salicin and did not grow at 37°C in contrast to most *P. cerevisiiphilus* and *P. frisingensis* isolates.

In conclusion, comparative 16S rRNA gene sequence analysis, supported by phenotypic characterisation, proved that the *Pectinatus*-like strains belonged to the genus *Pectinatus*. Based on the lower than 98.7% sequence similarity with known species and on unique phenotypic features, *Pectinatus haikarae* (N.L. gen. n. *haikarae*, of Haikara) was described. The original genus description by Lee *et al.* (1978) was amended to include the phenotypic characteristics of *P. haikarae* (Paper V).

Hitherto, *P. haikarae* has only been associated with low-alcohol beer. In addition to German and Finnish isolates characterised here, we have isolated this species from low-alcohol beers produced in Sweden (Paper III) and in Norway (R. Juvonen, unpubl. data). Based on the cross-reactivities of flagellar antibodies, Chaban *et al.* (2005) hypothesised that *P. cerevisiiphilus* descends from *P. frisingensis* and not *vice versa*. *P. haikarae* may have further diverged from *P. cerevisiiphilus* as a result of better adaptation to the brewery environment. *P. haikarae* has a preference for lower temperature that may be related to the fact that brewing is carried out at low temperatures. Moreover, unlike *P. cerevisiiphilus* it produces catalase, which may enhance its survival in oxygenic niches in the brewery (Rocha *et al.*, 1996).

4.6.3 Detection, isolation and identification of the new species in breweries

M. paucivorans, *M. sueciensis* and *P. haikarae* could be detected and isolated from brewery samples using the same cultivation media and techniques as for the other brewery-related *Megasphaera* and *Pectinatus* species (Paper V). Non-selective media found to support their growth included PYF, PYG and MRS with 1% fructose. The colonies of the new *Megasphaera* species and *P. haikarae* appeared after 3–4 d and 1–2 d at 30°C, respectively. PY medium with 1% (w/v) pyruvate or gluconate allowed maximal growth of *M. paucivorans* and *M. sueciensis* and is recommended for their cultivation. The SMMP medium developed for the selective detection of *Megasphaera* and *Pectinatus* in beer also supported the growth of the new species. *M. paucivorans* and *M. sueciensis* changed the medium colour from dark violet to yellow/light violet like *M. cerevisiae* (Anon., 1998). *P. haikarae* did not produce colour change, in common with the other *Pectinatus* species.

M. paucivorans, *M. sueciensis* and *P. haikarae* could be specifically detected by PCR. The *Pectinatus*-specific primers of Satokari *et al.* (1997) were shown also to recognize *P. haikarae* (Table 9, p. 73). The developed group-specific PCR tests (Paper III) allowed the detection of all three species in a single reaction. Moreover, specific primer sets have been published in the literature for their individual identification (Sakamoto *et al.*, 1997; Iijima *et al.*, 2008).

Ribotyping with *Eco*RI appeared be a suitable DNA fingerprinting method for the identification of the *Megasphaera* (Fig. 13) and *Pectinatus* species (Paper V: suppl. Fig. 2). The distinction between *M. cerevisiae* and *M. sueciensis* can be

verified using *Pvu*II, as shown by Suihko and Haikara (2001). The generated patterns have been deposited in the VTT database for future use. *P. haikarae* could also be differentiated from the other *Pectinatus* spp. by 16S rRNA gene sequence analysis (< 95.6% similarity to the nearest species). The 16S rRNA gene sequences of *M. paucivorans* and *M. sueciensis* differed by 11 bases and 2 gaps. Most of the differences (9/13) were scattered around the V1–V4 region. In order to identify possible signature codons for the species identification, more strains (when available) will need to be characterised in the future.
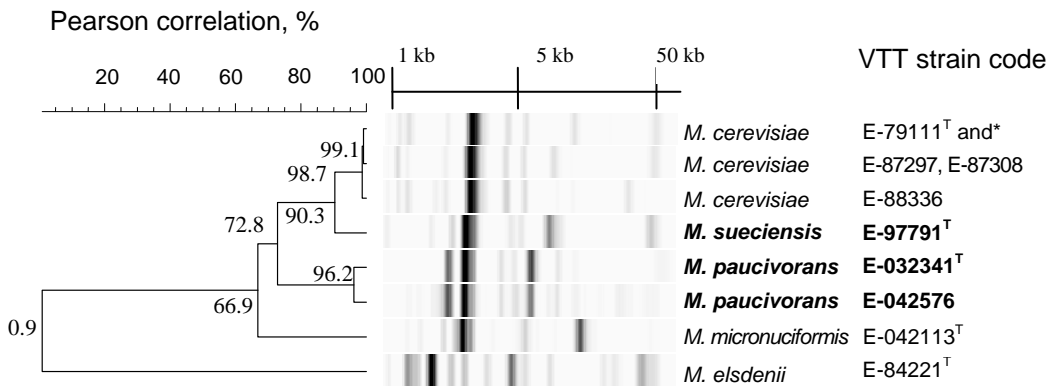

Figure 13. Ribotyping patterns of the known and new *Megasphaera* species generated using *EcoR*I and RiboPrinter® Microbial Characterization System. The dendrogram was constructed using Pearson correlation coefficient and UPGMA clustering. *E-79110, E-84195, E-85230, E-86267, E-86272, E-89375, E-90412 and E-981087.

Phenotypic characteristics useful for differentiating the brewery-related *Megasphaera* and *Pectinatus* species are shown in Tables 18 and 19. *M. paucivorans* and *M. sueciensis* were best separated from each other by analysing volatile fatty acids produced in PYF medium. They were easy to distinguish from *M. cerevisiae* owing to their smaller cell size, poorer growth on the standard media and the inability to use DL-lactate or fructose.

Table 18. Differential characteristics of brewery-related *Megasphaera* species.

Characteristic	*M. cerevisiae* [1]	*M. paucivorans* [2]	*M. sueciensis* [3]
Cell size (µm)	1.5–2.1	1.2–1.9 x 1.0–1.4	1.0–1.4 x 0.8–1.2
Colonies appear on PYF at 30°C	1–2 d	3 d	4 d
Acid from fructose	+	–	–
Utilisation of DL-lactate	+	–	–
Major volatile fatty acids in PYF medium [4]	**C**, iV, B	**iV, C**	**iV**, B, C, V
GC mol%	42.4–44.8	40.5	43.1

[1] Type strain and 11 other strains tested by Engelmann and Weiss (1985), [2] E-032341[T] and E-042576, [3] E-97791[T], [4] Relative amount is ≥ 10%. The products in bold-face constitute 40–60% of the total amount. B, butyric acid; iV, isovaleric acid; V, valeric acid; C, caproic acid.
Symbols: +, positive; –, negative.

Strictly anaerobic, Gram-stain-negative, catalase-positive, motile rods found in brewery samples can be tentatively identified as *P. haikarae*. The carbohydrate utilisation tests do not provide conclusive identification alone, since some *P. cerevisiiphilus* and *P. frisingensis* strains are able to grow on D-salicin and *P. cerevisiiphilus* may utilise lactose (Schleifer *et al*., 1990).

This study also indicated that gas chromatographic analysis of volatile fatty acids may be a useful tool to detect beer contamination by *M. paucivorans*. In beer, this species produced butyrate (> 99%) with traces of isovalerate and caproate, in common with *M. cerevisiae* (Haikara and Lounatmaa, 1987). Butyrate is the main product of pyruvate metabolism in *M. cerevisiae* (Engelmann and Weiss, 1985), suggesting that *Megasphaera* species could derive their energy for growth in beer from pyruvate.

Table 19. Differential characteristics of brewery-related *Pectinatus* species.

Characteristic	*P. cerevisiiphilus* [1]	*P. frisingensis* [2]	*P. haikarae* [3]
Catalase activity	–	–	+
Growth at 37°C	+	+	–
Acid production from:			
D-cellobiose	–	+	–
i-inositol	–	+	+
Lactose	–	–	+
α-D-melibiose	+	–	+
N-acetyl-glucosamine	–	+	–
D-salicin	+	+	–
D-xylose	+	–	+

[1] Type strain and 10 other strains (Paper V; Haikara *et al*., 1981; Schleifer *et al*., 1990), [2] Type strain and 13 other strains (Paper V; Haikara *et al.*, 1981; Schleifer *et al*., 1990), [3] Strains E-88329[T], E-88330, E-97914 and E-89371.
Symbols: +, 75% or more of the strains are positive; –, 75% or more of the strains are negative.

4.6.4 Phylogenetic analysis of the *Sporomusa* sub-branch of the class "*Clostridia*"

Marchandin *et al*. (2003) performed a phylogenetic analysis of the *Sporomusa* sub-branch members with a special focus on clinical species. Since then many species have been reclassified and described with concomitant deposition of their 16S rRNA gene sequence in public databases. Moreover, sequences from hundreds of uncultured *Megasphaera*-affiliated bacteria have become available. We carried out a phylogenetic analysis based on the current 16S rRNA gene database including all beer-spoilage species, the type species of the *Sporomusa* sub-branch and closely related phylotypes selected from sequence databases. For this purpose, the 16S rRNA gene sequence of the potential beer-spoilage bacterium *Z. raffinosivorans* was also determined.

Overall topology of the phylogenetic trees inferred using the neighbour joining method and maximum likelihood and parsimony analyses were in good congruence (data not shown). The consensus neighbour-joining tree is shown in Fig. 14.

In the inferred tree, the *Anaeroglobus* and *Megasphaera* species and phylotypes were separated into a well-supported cluster. A statistically significant relative branching order within the cluster was not resolved due to low bootstrap support on some nodes, in agreement with a previous study (Marchandin *et al*., 2003). In terms of the 16S rRNA gene sequence similarity, the genus *Megas-*

phaera was very broad. *M. elsdenii* (the type species) shared 92–93% sequence similarity with the four other species. A recent calculation of taxa boundaries based on the 16S rRNA gene sequences from 451 genera showed a minimum similarity of 94.9% ± 0.4 to the type species within a genus (Yarza *et al*., 2008). Although this value cannot be taken as an absolute cut-off value, it clearly indicates an unusually high diversity in the genus *Megasphaera*. Its heterogeneity is also supported by the high GC mol% range among the species (40.5–52.6%) (Paper V). Furthermore, the *Megasphaera* spp. are physiologically and ecologically diverse (Paper V; Marchandin *et al*., 2003; Haikara and Helander, 2006). Their further characterisation by using additional phylogenetic (e.g., heat-shock proteins) and chemotaxonomic markers (e.g., cellular fatty acids, lipopolysaccharides) could provide data needed for their reorganization into more homogenic and ecologically more meaningful genera.

Six major groups (sequence similarity ≥ 92.5%) were distinguished within the *Anaeroglobus-Megasphaera* cluster. These groups mostly correlated with the source of the bacteria. The brewery-related species formed a moderately supported sub-group that did not include any sequences from bacteria found in other sources. This suggests that *M. cerevisiae*, *M. paucivorans* and *M. sueciensis* may be uniquely adapted to the brewery environment. In evolutionary genetics, each ecologically distinct group is predicted to eventually diverge into its own sequence cluster for any gene in the genome (Palys *et al*., 2000). The phylogenetic analysis also suggested that at least ten new, mainly yet uncultured species exist in the *Anaeroglobus-Megasphaera* group (< 98.7% similarity to the known species).

The nearest phylogenetic relative to the *Pectinatus* spp. (89.9% to the type species *P. cerevisiiphilus*) was *Megamonas hypermegale* isolated from chicken gut. It was only recently suggested that this species should be transferred from the family *Bacteroidetes* to the lineage of *Firmicutes* (Morotomi *et al*., 2007).
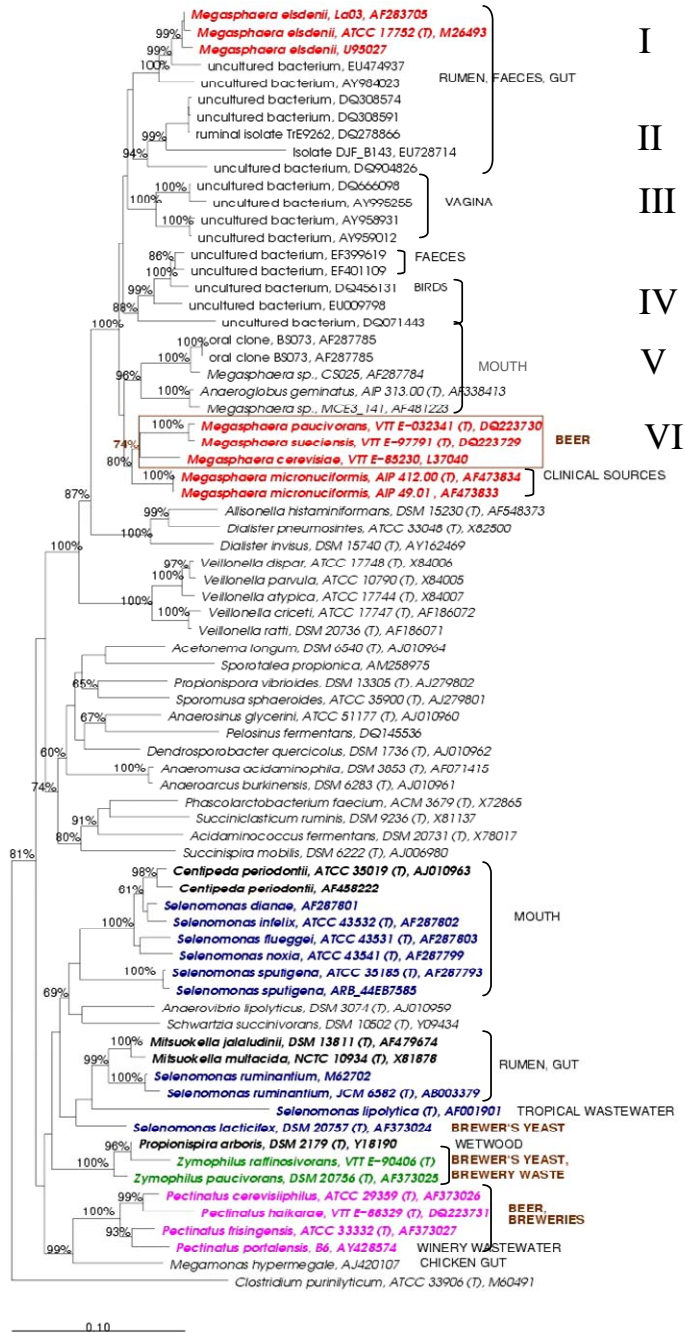
Figure 14. Consensus neighbour-joining tree of 16S rRNA gene sequences (1309 nt) of *Sporomusa* sub-branch type species and representative phylotypes. Bootstrap values (percentages of 1000 replications) above 60% are shown. Sequence names are followed by their GenBank accession numbers. (T) indicates type strain. Bar = 10 % sequence divergence.

The genus *Selenomonas* currently includes one brewery-related species, *S. lacticifex*; six oral species *S. artemidis*, *S. dianae*, *S. flueggei*, *S. infelix*, *S. noxia* and *S. sputigena*; *S. lipolytica* found in wastewater and a ruminal species *S. ruminantium* (ssp. *lactilytica*, ssp. *ruminantium*). Our phylogenetic analysis included all the species except *S. artemidis* (no sequence available). It clearly showed that the genus *Selenomonas* is polyphyletic i.e. originating from several ancestors. The species were intermixed with *Anaerovibrio lipolyticus*, *Centipeda periodontii*, *Mitsuokella* spp. and *Schwarzia succinivorans*, although not all the nodes were statistically supported (bootstrap value < 60%). Sequence similarities of *Selenomonas* spp. to the type species (*S. sputigena*) varied from 82% to 90%. Based on the analysis of the 16S rRNA gene sequences from genera with three or more species, Yarza *et al*. (2008) found that species within a genus have a mean similarity of 96.4% to the type species. The 10% range in the GC mol% also pinpoints heterogeneity of the genus *Selenomonas* (Hespell *et al.*, 2006).

Our phylogenetic analysis was not able to reveal reliably the phylogenetic position of *S. lacticifex* in the *Pectinatus-Selenomonas-Sporomusa* group. This species formed a deeply-branched lineage with low bootstrap support. *S. ruminantium* subsp. and the two *Mitsuokella* spp. were sister taxons in a well-supported clade, sharing *ca*. 95% sequence similarity with each other. These species are fermentative organisms that inhabit a similar ecosystem (rumen, gut). However, *Mitsuokella* bacteria are clearly distinct from selenomonads in being non-motile rods that produce lactate, acetate and succinate (Land *et al.*, 2002). Thus, they may deserve a separate genus status. Interestingly, the oral *Selenomonas* spp. with the exception of *S. sputigena* formed a well-supported tight cluster with another oral anaerobe *C. periodontii* with *ca*. 96–98% sequence similarity. In this group, *C. periodontii* strains clustered together (98% bootstrap value), but the exact branching order of the *Selenomonas* species was not resolved. This species cluster showed only distant relationship to *S. sputigena* (≤ 89.7%) and even less to the other selenomonads. The range of GC mol% among these oral species (including *C. periodontii*) is narrow (52–58%) compared to the range among all the *Selenomonas* spp. (48–58%) (Hespell *et al.*, 2006). In addition to their genetic and ecological similarities, the oral selenomonads and *C. periodontii* produce propionate, acetate and/or lactate as the major metabolites (Lai *et al.*, 1983; Hespell *et al.*, 2006). However, the selenomonads have flagella arranged in tufts on the concave side of the cell (Hespell *et al.*, 2006), whereas the flagella on *C. periodontii* are inserted in a spiral path along the body (Lai *et al.*, 1983). Whether this merits a separate genus status is a future deci-

sion. It is tentatively suggested here to assign a new genus status to *S. lacticifex*, to *S. ruminantium* subspecies, and to *S. lipolytica*, to include the oral selenomonads (except *S. sputigena*) in the genus *Centipeda* and to retain *S. sputigena* as the only representative of the genus *Selenomonas*.

The phylogenetic analysis supported the close relationships between *Z. paucivorans* and *Z. raffinosivorans* (95.9% similarity), earlier established using DDH (Schleifer *et al*., 1990) and 16S-23S spacer analysis (Motoyama and Ogata, 2000a). Furthermore, it showed for the first time that the *Zymophilus* species are in fact affiliated to the genus *Propionispira* (Schink *et al*., 1982). The sequence similarity to *Propionispira arboris*, the only species in the genus, was 97.5–98%. *P. arboris* and *Z. raffinosivorans* formed a well-supported group that was linked to *Z. paucivorans* with a 100% bootstrap value. Based on the literature, all three species are also phenotypically similar (Schink *et al*., 1982; Schleifer *et al*., 1990). They mainly produce acetate and propionate from glucose, and have similar cell shape and substrate utilisation patterns, pH growth range and GC mol% (37–41%). No definite rules to delineate genera exist. However, it is recommended that they should be monophyletic and possess a distinct phenotypic trait not shared by neighbour genera (Wayne *et al*., 1987). In order to find a unique genus-specific phenotypic trait among the three species, further chemotaxonomic, ultrastructural and metabolic characterisation will be needed. The affiliation of the *Zymophilus* spp. to *Propionispira* also sheds light on their ecology. *P. arboris* is a N_2 fixing anaerobe that is commonly isolated from alkaline wetwood (Schink *et al*., 1982). This suggests that *Z. paucivorans* and *Z. raffinosivorans* may have been carried to breweries with plant material (hops, malt).

5. Conclusions

The practical PCR-based assays developed in this study provide a flexible tool-box that allows the brewer to obtain more rapid and specific information about the presence and identity of the *Sporomusa* sub-branch spoilage bacteria in the beer production process than is possible by using cultivation methods. Breweries can react to contamination at an earlier stage to minimise product recall and disposal and negative impact on company reputation. This should ensure financial benefits to the industry. Furthermore, integration of the information from the DNA-based analyses with phenotypic and ecological characteristics improved understanding of the biodiversity, natural relationships and habitats of the *Sporomusa* sub-branch beer-spoilage bacteria. The findings can be exploited for taxonomic classification of these bacteria and for surveillance and control of contaminations.

The specific findings of this thesis were as follows.

The 16S rRNA gene was found to contain suitable signature sequences for designing specific PCR primers for *M. cerevisiae*, the genus *Pectinatus* and the *Sporomusa* sub-branch beer-spoilage group. The PCR tests based on these primers reliably differentiated the target bacteria from other microbes likely to be found in the beer production chain. The *Pectinatus*- and *M. cerevisiae*-specific PCR tests are most useful for the identification and troubleshooting purposes. The group-specific PCR tests provide a cost-effective tool for routine screening of brewery samples for all nine spoilage species in a single reaction. Moreover, they allow tentative identification based on spoilage potential or genus identity.

This study provided new information about the identity and attenuation of the PCR inhibitors present in the brewery samples. Phenolic compounds associated with beer macromolecules and with brewer's yeast cells were recognized as the inhibitors in the sample extracts. Supplementation of PCR reactions with BSA was found to be an effective, easy and inexpensive means to alleviate PCR inhi-

bition by brewery samples. BSA could even preclude the need for inhibitor removal, and it is proposed as a standard component of PCR mixtures for the analysis of brewery samples. Rapid inhibitor removal combined with physical cell lysis methods and with the use of PCR facilitators was shown to be an easy, fast (1–2.5 h) and inexpensive (0.5–4 €) approach for the pre-PCR processing of brewery process and product samples. The optimal procedure should be selected on a case-by-case basis, because the inhibitory effect of the samples can vary from one beer type to another. This study also implied that the end-point and real-time PCR formats may need separate pre-PCR optimisation due to their different resistance to inhibitors. Culture enrichment combined with the easy pre-PCR processing methods was found to be a practical approach for the detection of a low level of viable cells (≤ 10 cfu 100 ml^{-1}) in a packaged beer. It did not require special skills and overcame the risk of false positive results from a few dead cells. The developed beer-based enrichment media enabled faster PCR detection of the target group bacteria than routine media and appeared also to be beneficial for recovering stressed cells. A minimum enrichment time of 3–4 d for bacterial presence-absence testing is proposed.

Each evaluated PCR format had specific benefits and drawbacks regarding routine application. Real-time PCR is undoubtedly the most practical choice for routine QC, but it still requires substantial capital investment. End-point PCR with gel electrophoresis represents a low cost alternative to real-time PCR. Microplate hybridisation can provide a convenient format for high-throughput identification of brewery contaminants when applied as a DNA macroarray with multiple taxon-specific and functional probes.

Based on the polyphasic characterisation, the genetically atypical *Megasphaera*- and *Pectinatus*-like strains were demonstrated to represent three new species for which the names *M. paucivorans*, *M. sueciensis* and *P. haikarae* were validly published. The description of these new species enabled establishment of phenotypic and genotypic methods for their detection and identification. The selective and non-selective media applied for cultivation of *P. cerevisiiphilus*, *P. frisingensis* and *M. cerevisiae* were found to be suitable also for *M. paucivorans*, *M. sueciensis* and *P. haikarae*. *P. haikarae* could be discriminated from the other brewery-related *Pectinatus* species based on acid formation from lactose but not from D-salicin, a positive catalase reaction and inability to grow at 37°C. It appears to be better adapted to grow in oxygenic low-temperature environments of breweries than *P. cerevisiiphilus* or *P. frisingensis*. *M. paucivorans* and *M. sueciensis* could be best discriminated from each other by metabolic end-

product analysis. In brewing microbiology, their most useful differential characteristics from *M. cerevisiae* were slower growth on PYF medium, smaller cell size and inability to use lactate and fructose. DNA-based characterisation by ribotyping with *Eco*RI enzyme discriminated between all the *Megasphaera* and *Pectinatus* species. The complete 16S rRNA gene sequence analysis also allowed unambiguous identification of *P. haikarae*. In order to identify the possible species-specific signature nucleotides for discriminating between *M. paucivorans* and *M. sueciensis* more strains need to be analysed.

Three key findings were made on the basis of the phylogenetic analysis of the *Sporomusa* sub-branch bacteria. First, the genus *Selenomonas* was shown to be polyphyletic and most species should be assigned to new genera in order to reflect their phylogenetic, ecological and phenotypic relatedness. Second, the brewery-related *Megasphaera* species were found to form a distinct sub-group in the dendrogram. No sequences in this sub-group derived from other sources, suggesting that *M. cerevisiae*, *M. paucivorans* and *M. sueciensis* may be uniquely adapted to the brewery habitat. Third, *Z. paucivorans* and *Z. raffinosivorans* isolated from breweries were in fact members of the genus *Propionispira* isolated from wetwood of trees. This suggests that *Zymophilus* species might be plant-associated in their natural habitat and carried along with plant material to breweries.

This study also revealed that *M. cerevisiae* E-981087 is capable of long-term survival in various stress conditions that it could encounter in the brewing process (starvation, exposure to beer or oxygen, and cold storage). The results imply that *M. cerevisiae* has efficient stress tolerance mechanisms. This finding could partly explain its persistence in the brewery environment and establishment as a beer-spoilage organism.

6. Future outlook

The developed PCR-based assays could further be improved and modified. In the future, validation by interlaboratory demonstration of robustness, and construction of internal controls to detect failure at any analysis stage would facilitate their implementation in the industry. Moreover, the group-specific primer set could be applied with genus- or species-specific identification probes in PCR-ELISA or in real-time PCR to confirm and further identify the PCR products. It could also be suitable for studying the diversity and population dynamics of the target group bacteria within and outside breweries using community fingerprinting techniques such as denaturing gradient electrohoresis or chromatography or sequence analysis.

All alternative detection methods for beer-spoilage bacteria still rely on culture enrichment for reaching adequate sensitivity. This essentially precludes instant detection. The enrichment step may be fully avoided by applying new powerful concentration techniques that allow tens of litres of beer to be filtered. It has been shown with water samples that the detection of < 100 cfu in 100 l is possible using ultrafiltration in combination with fast DNA extraction and real-time PCR (Polaczyk *et al.*, 2008). Cell collection from multiple packages would also increase the likelihood of detecting secondary contaminations. New microfluidic systems could prove to be useful for high-throughput separation of bacteria from yeast-containing samples. They are cost-effective, and easy to use and manufacture (Wu *et al.*, 2009). Propidium monoazide or another viability stain could be incorporated into the real-time PCR for discriminating between viable and dead cells on the basis of membrane integrity (Nocker *et al.*, 2007). By multiplexing, the information content of a single test could be increased, while minimising the assay costs and work load. Taxon-specific (at various ranks and for various organisms) and functional genes could be targeted simultaneously in order to classify the contaminants based on their taxonomic status

and spoilage potential. Several multiplex platforms such as microarrays and various bead technologies are now available (Rasooly and Herold, 2008). Finally, integration of the sample treatment, detection and data analysis steps is needed to minimise human intervention. Many sample preparation stations exist on the market, but they are mainly for low volume processing. The ultimate aim would be an instant, low-cost, non-expert, high-throughput method that enables specific detection of low levels of those organisms that will eventually cause beer spoilage.

The genera *Megasphaera* and *Selenomonas* currently include phylogenetically, physiologically and ecologically diverse species. In the future, the analysis of additional phylogenetic and taxonomic markers will be required to resolve their evolutionary relationships and classification. In addition, *P. arboris* and *Zymophilus* spp. needs further phenotypic and chemotaxonomic characterisation to establish possible discriminatory characteristics for redefining the genus *Propionispira*.

Beer-spoilage properties of *Sporomusa* sub-branch bacteria are inadequately understood. Even basic information about the growth limits of the new species in beer with regard to e.g. ethanol and oxygen content, pH, hop acids and other beer constituents is still lacking. It is currently not known whether beer-spoilage ability is a strain-specific feature among the *Megasphaera* and *Pectinatus* species, as it is among LAB (Suzuki *et al*., 2006b). If strain-specific differences exist, it would be interesting to try to determine possible genetic determinants of the spoilage ability. Moreover, beer-related factors governing growth of strict anaerobes are only partly understood. We have observed that beer products with similar alcohol content and pH value can greatly vary in their ability to support the growth of the strict anaerobes. Could the differences be related to their different organic acid and carbohydrate profiles or raw materials? The sensitivities of the new low-alcohol product types to growth of the potential and absolute spoilage bacteria of the *Sporomusa* sub-branch also remain to be elucidated.

The findings of this and earlier studies indicate that some *M. cerevisiae* and *Pectinatus* strains possess remarkable ability to survive in the sub-optimal conditions encountered in the beer production chain (Chowdhury *et al*., 1995; Haikara and Helander, 2006). Understanding of the molecular basis of stress tolerance responses, e.g., by applying proteomics, and relation of stress tolerance to beer-spoilage activity could help determine what is needed to prevent and control contamination, as well as develop tools for screening of possible beer-tolerant strains.

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Title

DNA-based detection and characterisation of strictly anaerobic beer-spoilage bacteria

Abstract

Megasphaera cerevisiae, *Pectinatus cerevisiiphilus*, *Pectinatus frisingensis*, *Selenomonas lacticifex*, *Zymophilus paucivorans* and *Zymophilus raffinosivorans* are strictly anaerobic Gram-stain-negative bacteria that are able to spoil beer by producing off-flavours and turbidity. They have only been isolated from the beer production chain. The species are phylogenetically affiliated to the *Sporomusa* sub-branch in the class "*Clostridia*". Routine cultivation methods for detection of strictly anaerobic bacteria in breweries are time-consuming and do not allow species identification. The main aim of this study was to utilise DNA-based techniques in order to improve detection and identification of the *Sporomusa* sub-branch beer-spoilage bacteria and to increase understanding of their biodiversity, evolution and natural sources. Practical PCR-based assays were developed for monitoring of *M. cerevisiae*, *Pectinatus* spp. and the group of *Sporomusa* sub-branch beer spoilers in brewery process and product samples. The developed assays reliably differentiated the target bacteria from other brewery-related microbes. The contaminant detection in process samples (10^1–10^3 cfu ml^{-1}) could be accomplished in 2–8 h. Low levels of viable cells in finished beer (≤10 cfu 100 ml^{-1}) were detected after 1–4 d culture enrichment. Time saving compared to cultivation methods was up to 6 d. Based on a polyphasic approach, this study also revealed the existence of three new anaerobic spoilage species in the beer production chain, i.e. *Megasphaera paucivorans*, *Megasphaera sueciensis* and *Pectinatus haikarae*. The description of these species enabled establishment of phenotypic and DNA-based methods for their detection and identification. The 16S rRNA gene based phylogenetic analysis of the *Sporomusa* sub-branch showed that the genus *Selenomonas* originates from several ancestors and will require reclassification. Moreover, *Z. paucivorans* and *Z. raffinosivorans* were found to be in fact members of the genus *Propionispira*. This relationship implies that they were carried to breweries along with plant material. The brewery-related *Megasphaera* species formed a distinct sub-group that did not include any sequences from other sources, suggesting that *M. cerevisiae*, *M. paucivorans* and *M. sueciensis* may be uniquely adapted to the brewery ecosystem. *M. cerevisiae* was also shown to exhibit remarkable resistance against many brewery-related stress conditions. This may partly explain why it is a brewery contaminant. This study showed that DNA-based techniques provide useful tools for obtaining more rapid and specific information about the presence and identity of the strictly anaerobic spoilage bacteria in the beer production chain than is possible using cultivation methods. This should ensure financial benefits to the industry and better product quality to customers. In addition, DNA-based analyses provided new insight into the biodiversity as well as natural sources and relations of the *Sporomusa* sub-branch bacteria. The data can be exploited for taxonomic classification of these bacteria and for surveillance and control of contaminations.

Tekijä(t)
Riikka Juvonen

Nimeke

Ehdottoman anaerobisten oluen pilaajabakteerien osoittaminen ja karakterisointi DNA-pohjaisilla menetelmillä

Tiivistelmä

Megasphaera cerevisiae, *Pectinatus cerevisiiphilus*, *Pectinatus frisingensis*, *Selenomonas lacticifex*, *Zymophilus paucivorans* ja *Zymophilus raffinosivorans* ovat ehdottoman anaerobeja (happiherkkiä) oluen pilaajabakteereita. Ne aiheuttavat oluessa samennusta sekä haju- ja makuvirheitä. Lajit kuuluvat firmikuuttien pääjaksossa ns. *Sporomusa*-alahaaraan. Niitä on toistaiseksi tavattu vain oluen tuotantoketjusta. Näiden bakteerien monitorointi panimonäytteistä perinteisin viljelymenetelmin on hidasta eikä sillä saada tarkkaa lajitunnistusta. Tämän tutkimuksen tavoitteena oli parantaa *Sporomusa*-alahaaran oluenpilaajien osoittamista ja tunnistamista sekä tuoda uutta tietoa niiden monimuotoisuudesta, sukulaisuussuhteista ja lähteistä DNA-pohjaisia tekniikoita hyödyntämällä. Tutkimuksessa kehitettiin helpot ja spesifiset polymeraasiketjureaktioon perustuvat testit *M. cerevisiae* -lajille, *Pectinatus*-suvulle sekä *Sporomusa*-alahaaran pilaajabakteeriryhmälle. Uudet testit mahdollistivat kontaminaation (10^1–10^3 pmy ml^{-1}) havaitsemisen prosessinäytteistä 2–8 tunnissa. Lisäksi osoitus oli spesifinen. Valmiista oluesta muutama elinkykyinen solu (≤10 pmy 100 ml^{-1}) voitiin todeta 1–4 vrk:n rikastuskasvatuksen jälkeen. Saavutettu ajansäästö viljelymenetelmään verrattuna oli jopa kuusi vrk. Tutkimuksessa kuvattiin lisäksi kolme uutta anaerobista oluenpilaajalajia: *Megasphaera paucivorans*, *Megasphaera sueciensis* ja *Pectinatus haikarae*. Niiden tunnistamiseksi löydettiin sekä DNA:n että fenotyyppisten tuntomerkkien analysointiin perustuvat menetelmät. *Sporomusa*-alahaaran 16S rRNA geenisekvenssien vertailuun perustunut fylogeneettinen analyysi osoitti *Selenomonas*-suvun olevan polyfyleettinen ja vaativan tulevaisuudessa uudelleenluokittelua. Lisäksi se osoitti, että *Zymophilus*-lajit kuuluvat itse asiassa *Propionispira*-sukuun. Tämä sukulaisuussuhde viittaa ensi kertaa näiden bakteerien kasvialkuperään. Olutta pilaavien *Megasphaera*-lajien ryhmittyminen erilleen muista lähteistä löydettyjen bakteerien sekvensseistä viittasi siihen, että ne ovat sopeutuneet elämään vain panimoympäristössä. Lisäksi tutkimuksessa havaittiin, että *M. cerevisiae* kykenee kestämään erilaisia panimoprosessin stressiolosuhteita erittäin hyvin. Tulos selittänee osittain, miksi laji on panimokontaminantti. Tutkimuksen tulokset osoittivat, että DNA-pohjaiset tekniikat tarjoavat hyödyllisiä työkaluja, joiden avulla on mahdollista osoittaa ja tunnistaa happiherkät pilaajabakteerit oluen tuotantoketjusta entistä nopeammin ja täsmällisemmin. Tämä auttaa vähentämään kontaminaatioista johtuvia teollisuuden tappioita sekä takaamaan kuluttajalle paremman tuotelaadun. Lisäksi *Sporomusa*-alahaaran pilaajabakteerien DNA-pohjainen karakterisointi toi uutta tietoa niiden monimuotoisuudesta, sukulaisuussuhteista sekä ekologiasta, jota voidaan hyödyntää näiden organismien luokittelussa sekä kontaminaatioiden hallinnassa ja valvonnassa.

Julkaisuaika
Marraskuu 2009

Kieli
Englanti, suom. tiiv.

Sivuja
134 s. + liitt. 50 s.

Projektin nimi
Mikrobiologiset laadunvalvontamenetelmät, PCR-pohjaisten menetelmien kehittäminen ja kokeilu panimoteollisuuden laadunvalvontaan

Toimeksiantaja(t)
AB Pripps Bryggerier, EU, Elintarvikkeiden tutkimussäätiö, Nutek, PBL, Raisio Oyj:n tutkimussäätiö, Tekes, VTT

Avainsanat
Beer, brewing, identification, PCR, *Pectinatus*, phylogeny, *Megasphaera*, rapid detection, *Selenomonas*, spoilage, taxonomy, *Zymophilus*

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