

# Amplification Facilitators and Pre-Processing Methods for PCR Detection of Strictly Anaerobic Beer-Spoilage Bacteria of the Class *Clostridia* in Brewery Samples

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## ABSTRACT

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The aim of this study was to evaluate easy pre-PCR processing procedures to allow rapid and reliable detection of strictly anaerobic beer-spoilage bacteria throughout the brewing process by end-point and real-time PCR techniques. The efficiencies of the new procedures were evaluated using spiked brewery samples and specific PCRs for the target group bacteria. We found for the first time that the inclusion of 0.25% (w/v) bovine serum albumin (BSA) or 0.5% (w/v) polyvinyl pyrrolidone (PVP) in the end-point PCR mixture reduces the inhibiting effect of brewery sample extracts (3–10%, v/v) on PCR. Membrane filtration with a PVP or a sodium tri-polyphosphate-EDTA wash, and cross-flow filtration were the most promising new methods to reduce inhibitors from beer samples before cell lysis. Together with BSA, they allowed the analysis of 10% (v/v) of crude extracts instead of <3% (v/v). Moreover, we developed a one-hour procedure to prepare target DNA from process samples containing brewer's yeast. It involved removal of inhibitors by a two-step centrifugation followed by physical disruption of cells. The detection limit of the procedure was 10<sup>1</sup>–10<sup>3</sup> CFU/mL. The developed procedures help to reduce the risk of partial or complete PCR failure due to inhibition and target DNA losses, with minimal sample handling.

**Key words:** beer, brewing process, facilitator, inhibition, PCR detection, sample treatment, strictly anaerobic beer-spoilage bacteria.

## INTRODUCTION

Microbial contamination at any stage of the beer production process may lead to serious quality defects in the final product and typically high cost measures are needed to rectify the situation. The spoilage microbes encountered in the process are restricted to relatively few species due to the selectivity of the environment<sup>30</sup>. The strictly anaerobic beer-spoilage bacteria of the genera *Megasphaera*, *Pectinatus*, *Selenomonas* and *Zymophilus* spoil are able to spoil beer by producing offensive off-flavours

and turbidity<sup>6</sup>. These gram-stain-negative bacteria are phylogenetically affiliated to the *Sporomusa* sub-branch of the Gram-stain positive class *Clostridia* in the phylum *Firmicutes*<sup>6</sup>. The obligate or potential beer-spoilage species include the coccus-shaped *Megasphaera cerevisiae*, *Megasphaera sueciensis* and *Megasphaera paucivorans*, and the vibrioid *Pectinatus frisingensis*, *Pectinatus cerevisiiphilus*, *Pectinatus haikarae*, *Selenomonas lactificifex*, *Zymophilus raffinosivorans* and *Zymophilus paucivorans*. Hitherto, they have been isolated exclusively from spoiled beer or from the beer production process<sup>6,10</sup>.

The two critical control points for microbiological sampling in the beer production process are locations where microbes should be mostly absent (e.g. final beer) and where brewer's yeast is present (e.g. pitching yeast, fermenting wort)<sup>24</sup>. Detection of the beer-spoilage bacteria of the class *Clostridia* is difficult by cultivation due to the strict anaerobic conditions and high sensitivity required. In practice, the brewery samples are usually incubated without or with a reduced growth medium in filled, tightly closed bottles for 2–6 weeks or until visually turbid, followed by confirmation of the results microscopically and by smell<sup>6</sup>. This method is poorly applicable to turbid samples since haze development cannot be used to indicate microbial growth. Moreover, it does not allow for the identification of the detected bacteria.

Due to the limitations of the traditional approach, many alternative techniques, such as polymerase chain reaction (PCR), have been applied to detect spoilage microbes in brewery samples (for a review see Russell and Stewart<sup>24</sup>). PCR has allowed more specific and rapid detection of the strictly anaerobic beer spoilers than the routine enrichment methods<sup>5,7,9,11,12,18,25,27,28</sup>. However, high costs and complexity of the PCR methods compared to cultivation, the risks of false-negative and -positive results and the need for culture enrichment still limit their widespread use in brewery quality control<sup>2</sup>. The PCR analysis of spoilage bacteria in brewery samples is mainly hampered by PCR inhibitors of an unknown nature<sup>20,28,29</sup> and by the need for an easy analytical protocol that is able to detect a few viable cells in a package of beer or amongst 10<sup>6</sup>–10<sup>9</sup> brewer's yeast cells. In order to reduce the inhibiting effect of brewery samples on PCR, traditional DNA isolation and purification procedures<sup>27,34</sup> and the technique of nested PCR in combination with easy cell

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lysis methods<sup>29</sup> have been used. Although effective, these methods are complex and time-intensive for routine use. Rapid procedures for inhibitor removal have been developed but they may reduce DNA recovery from some *Pectinatus* and *Megasphaera* strains, thus decreasing the assay sensitivity<sup>28</sup>. In order to determine the presence of the strict anaerobes in packaged beer by PCR, a 1–4-day enrichment is needed due to sample size restrictions of the current membrane filtration technique<sup>5,7,9,11</sup>. Furthermore, there is no method available to prepare PCR-ready DNA from the strict anaerobes in brewery samples containing high numbers of brewer's yeast.

The aim of this study was to compare the sensitivities of conventional end-point and real-time PCR (LightCycler™) reactions to inhibition by brewery process and product samples and to evaluate easy procedures to reduce the inhibition before, during and after DNA extraction. Moreover, we developed a rapid method to prepare DNA from the anaerobic beer spoilers in process samples containing high amounts of brewer's yeast.

## MATERIALS AND METHODS

### Pure cultures

*Megasphaera cerevisiae* VTT E-981087, *Selenomonas lacticifex* VTT E-90407<sup>T</sup>, *Pectinatus frisingensis* VTT E-011871 and VTT E-91471 and lager yeast strain *Saccharomyces pastorianus* VTT A-630015 from the VTT Culture Collection<sup>31</sup> (Espoo, Finland) were used as model organisms throughout the study. The bacteria were cultivated overnight in PYF (peptone-yeast extract-fructose) broth under anaerobic conditions at 30°C<sup>6</sup>. The anaerobic conditions were created using a Whitley Anaerobic Cabinet MK III (Don Whitley Scientific Ltd., Shipley, UK) with an atmosphere of N<sub>2</sub> (80%), CO<sub>2</sub> (10%) and H<sub>2</sub> (10%) or using the Anoxomat WS8000 system (Mart Microbiology BV, the Netherlands). DNA was isolated from the bacterial pure cultures using a traditional DNA extraction method<sup>27</sup>. The yeast was grown in YM broth (Difco, Detroit, MI, USA) with 100–125 rpm shaking at 25°C for 2 days.

### Brewery samples

Bottled lager style beers (alc. 0.3–4.6%, v/v) from Finland, Germany, the Netherlands and the Czech Republic were purchased from local retail sources. All-malt wort (16–17°P) was obtained from a local brewery. It was autoclaved (121°C 15 min) and clarified by decanting after settling of trub at 7°C. For the preparation of yeast-containing process samples, the clarified wort was pitched (1:100) with fresh yeast culture and incubated with 125 rpm shaking at 25°C for 1–2 days. Thereafter, the total yeast cell concentration was estimated using a Thoma counting chamber (Weber Scientific International, Middlesex, UK) and adjusted from 10<sup>7</sup> to 10<sup>8</sup> cells/mL with fermented wort from which the yeast had been removed by centrifugation. Aliquots (1 mL) of the ready-made samples were stored at –20°C.

To compare the PCR detection limits of different pre-PCR processing methods, spiked brewery samples were prepared as follows. The refreshed bacteria were serially

diluted in peptone-saline solution (Maximal Recovery Diluent, International Diagnostics Group plc, Lancashire, UK) containing 0.5 g/L cysteine-HCl (Merck, Darmstadt, Germany) and inoculated into 100 mL of beer (alc. 4.6%, v/v, 35 mg/mL iso- $\alpha$  acids) or into 1 mL of yeast-containing process samples. The same dilutions were cultivated on triplicate PYF plates and incubated anaerobically at 30°C for 3–5 days. The viable yeast cell counts of the yeast-containing samples were determined on triplicate YM plates (Difco) after a 3 day incubation at 25°C.

### End-point and real-time PCR reactions

A fragment (342 bp) of the 16S rRNA gene was amplified by group-specific end-point and real-time PCR reactions for the beer-spoilage *Megasphaera*, *Pectinatus*, *Selenomonas* and *Zymophilus* species<sup>11</sup> to evaluate the efficiency of PCR facilitators, inhibitor removal procedures, and different DNA extraction methods. The end-point PCR mixtures contained 1 × buffer (10 mM Tris-HCl, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.1% Triton X-100, pH 8.8), 200  $\mu$ M deoxyribonucleotides, 0.5  $\mu$ M An-0279f (5'-ACGATCAGTAGC-CGGT-3') and An-0603r (5'-AGCCCCGCACTTTTAAG-3') primers (Sigma-Genosys, Cambridge, UK), 1.25 U DyNAzyme II DNA polymerase and 1, 2.5 or 5  $\mu$ L template in a total volume of 50  $\mu$ L. The reagents were purchased from Finnzymes Oy (Espoo, Finland). All the reactions were performed with two replicates. The presence of a 342-bp band was interpreted as a positive result. For the real-time PCR, a capillary-based LightCycler™ instrument and a LightCycler–FastStart DNA Master SYBR Green I kit from Roche Diagnostics (Penzberg, Germany) were used. The PCR mixtures (20  $\mu$ L) contained 1 × FastStart reaction mix, 3 mM MgCl<sub>2</sub>, 0.5  $\mu$ M primers and 1 or 2  $\mu$ L template. The reactions were performed in triplicate. PCR results were expressed as crossing point (C<sub>p</sub>) values determined by the LightCycler™ software (version 3.5) based on the 2<sup>nd</sup> derivative maximum algorithm. Samples having a C<sub>p</sub> value of  $\leq$ 41 and a characteristic melting point temperature were considered positive. The efficiencies of some pre-PCR processing methods were compared by dividing their mean C<sub>p</sub> values by the mean C<sub>p</sub> value of the control treatment.

To determine whether residual inhibitors were present in the sample extracts, a known amount of *P. frisingensis* DNA was added to the PCR mixtures. The effect of anti-inhibitory treatments (Table I) was assessed by comparing the amount of DNA amplified (band intensity, or C<sub>p</sub> value) in the absence (water added instead) and in the presence of the extract. Positive and negative control reactions containing 10 ng target DNA and ultra-pure water (HPLC grade, Rathburn Chemicals Ltd., Walkerburn, UK) or 0.01 M Tris buffer (Trizma base, pH 8.0, Sigma-Aldrich Co., Dorset, UK) instead of the sample extracts were included in each run.

### PCR inhibition study with crude extracts from beer and yeast-containing brewery samples

The sensitivity of the PCR reactions to beer-derived material was studied using crude extracts from non-spiked beer samples that were prepared according to Koivula et al.<sup>15</sup> except that inhibitor removal and cell lysis steps were omitted. Briefly, a beer sample was filtered through a 0.4

**Table I.** Overview of the procedures evaluated at different stages of PCR analysis of brewery samples.

| Stage           | Bright beer (100 or 330 mL)  | Yeast-containing wort samples (1 mL)  |
|-----------------|--|---|
| Cell collection | 1. CellTrap™ filtration followed by a 100 mL water wash<br>2. Membrane filtration followed by a wash with one of the following procedures <sup>a</sup> :<br>a) 100 mL water<br>b) 5 mL 0.1 M NaOH<br>c) 5 mL 0.1 M NaOH+10 mL 0.5% SDS <sup>b</sup><br>d) 15 mL 0.01M NaOH+15 mL 0.05% SDS<br>e) 15 mL 1% (w/v) PVP-40<br>f) 15 mL 1% PVP-40+15 mL 0.01 M NaOH<br>g) 15 mL Ringer's solution containing 0.01 M EDTA and 0.2% (w/v) STPP <sup>c</sup><br>h) 30 mL 2% (v/v) Tween 20 | 1. A single-step centrifugation at high speed<br>2. A two-step centrifugation with initial low-speed centrifugation of cells in 1 mL of one of the following solutions:<br>a) Tris buffer (pH 8.0)<br>b) 0.01 M EDTA (pH 7.0)<br>c) 0.01 M EDTA+0.2 M maltose (pH 7.0)<br>d) 0.05 M EDTA (pH 7.0)<br>e) 0.5% Tween 20 |
| DNA extraction  | 1. Heating   | 1. Heating<br>2. Bead-beating<br>3. InstaGene Matrix<br>4. UltraClean™ Soil kit<br>5. Bugs'n Beads™ kit   |
| End-point PCR   | 1. 0.1, 0.2, 0.25, 0.4 or 0.6% (w/v) BSA <sup>d</sup><br>2. 0.5 or 1% (w/v) PVP-10<br>3. 0.5 or 1% (w/v) PVP-40  | 1. 0.1, 0.2, 0.25, 0.4 or 0.6% BSA <sup>d</sup><br>2. 0.5 or 1% PVP-10<br>3. 0.5 or 1% PVP-40   |
| Real-time PCR   | 1. 0.2, 0.4 or 0.6% BSA<br>2. 0.5 or 1% PVP-40   | 1. 0.2, 0.4 or 0.6% BSA<br>2. 0.5 or 1% PVP-40  |

<sup>a</sup> Tris (Trizma base, pH 8.0), EDTA, 85% sodium tri-polyphosphate solution (STPP) and polyvinyl pyrrolidone (PVP) were from Sigma Aldrich Co. (Dorset, UK), Tween 20 was from Fluka Chemie (Buchs, Switzerland), and Ringer's solution from Merck (Darmstadt, Germany).

<sup>b</sup> Ref. 28

<sup>c</sup> Ref. 20

<sup>d</sup> The molecular biology grade BSA from Roche (Espoo, Finland), standard grade BSA from Sigma-Aldrich Co. (>98% purity) and New England Bio Labs Inc. (Finnzymes Oy, Finland) were tested.

µm polycarbonate membrane (Ø 47 mm, Millipore, Bedford, USA) followed by wash with 100 mL purified water (Milli-Q UF water purification system, Millipore Oy, Espoo, Finland). The membrane was transferred into a 1.5 mL micro-centrifuge tube and the non-filterable fraction was released in 200 µL 0.01 M Tris buffer by vortexing for 10–15 sec (Vortex-Genie 2™, Scientific Industries Si, Bohemia, NY, USA). Finally, the membrane was dissolved in an 800 µL chloroform: isoamyl alcohol (24:1) mixture. The water phase was recovered for PCR by centrifugation (16,060 × *g*, 2 min) in 2 mL phase-lock-gel tubes (Phase Lock Gel Light, Eppendorf AG, Germany).

The main components of yeast-containing brewery samples, i.e. yeast cells and fermented wort, were investigated for their inhibitory effect as follows. The effect of yeast cells was studied by harvesting known numbers of cells from YM broth and wort cultures by centrifugation (16,060 × *g*, 3 min). The cells were washed with 1 mL ultra-pure water and suspended in 0.01 M Tris buffer (pH 8.0) to obtain different cell densities. The cells were subjected to a 10 min lysis by heating at 95°C or by bead-beating (Vortex-Genie 2™) with 0.1 g glass beads (150–212 µm, acid-washed, G1145, Sigma-Aldrich Co.) at maximum speed, after which the debris was centrifuged away (209 × *g*, 5 min). The supernatant was used for PCR.

### Effect of PCR facilitators on PCR inhibition by brewery samples

For relief of PCR inhibition, the supplementation of the PCR mixtures with non-acetylated BSA from three different suppliers or with PVP-40 or PVP-10 was tested (Table I). The stock solutions of the facilitators were prepared in sterile ultra-pure water except for the BSA solution from Roche, which was used as supplied. The final concentrations of BSA in the PCR mixtures varied from

0.1 to 0.6% (w/v). PVPs were added at 0.5 and 1% (w/v) concentrations without and with 0.25% (w/v) BSA (Sigma-Aldrich).

### Removal of PCR inhibitors from beer during the filtration step

To reduce the amount of inhibitors remaining on the membranes after beer filtration, different washing procedures were applied. Aliquots of 100 mL or 330 mL beer (alc. 4.6%, v/v, 35 mg/mL iso- $\alpha$  acids) were vacuum-filtered and subjected to the washing procedures described in Table I. Before and after applying the washing chemicals, 50 mL purified water was passed through. The washed membranes were immersed in 200 µL 0.01 M Tris buffer (pH 8.0), and the non-filterable material was released as in the inhibition study and used for PCR. Alternatively, disposable CellTrap™ devices (Mem-teq Ventures Ltd., UK) composed of 0.2 µm nominal size polypropylene hollow-fibres were used for beer filtration according to the manufacturer's instructions. After filtration, 100 mL purified water was passed through. The trapped material was eluted for PCR in 200 µL 0.01 M Tris buffer (pH 8.0).

### PCR detection limits of pre-processing methods for beer samples

PCR detection limits when applying selected amplification facilitators and pre-processing methods (see results) were determined using 100 mL aliquots of spiked beer samples. Membrane filtration followed by a 100 mL water rinse alone and with 5 mL 0.1 M NaOH and 10 mL 0.5% SDS was used as a negative and a positive control treatment. The cells were disrupted by heating and the DNA was recovered for PCR as in the inhibition study above.

## Comparison of different pre-processing methods for yeast-containing brewery samples

The efficiencies of different pre-processing methods (Table I) were compared by analysing 1 mL aliquots of yeast-containing ( $3 \times 10^7$  or  $1 \times 10^8$  cells/mL) wort samples inoculated with serial dilutions of *S. lactificex* or *M. cerevisiae*. The cells were collected for the processing by centrifugation ( $16,060 \times g$ , 3 min). An UltraClean™ Soil kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) was used according to the instruction for maximum yields. DNA was finally eluted in 50  $\mu$ L. A Bugs'n Beads™ kit (Genpoint, Oslo, Norway) was used with a few modifications. The cell pellets were suspended in 800  $\mu$ L of B&W buffer and 20  $\mu$ L of the bead solution (7.5 mg/mL) provided with the kit. Thereafter, the cell suspension was shaken at room temperature for 1 h instead of 5 min, since this was shown to improve the recovery of *S. lactificex* cells from 1% to 49% (data not shown). The beads were collected using a Dynal magnet (Oslo, Norway), incubated in 50  $\mu$ L lysis buffer at 80°C for 5 min and then treated following the kit's instructions. The DNA was eluted for PCR with 30  $\mu$ L ultra-pure water at 90°C for 5 min.

A single-step and a two-step centrifugation were compared to concentrate the bacteria before cell lysis by heating, bead-beating or InstaGene Matrix (BioRad, Hercules, CA, USA). In the single-step method, the pellets were washed ( $16,060 \times g$ , 3 min) with 1 mL ultra-pure water and suspended in 100  $\mu$ L 0.01 M Tris buffer (pH 8.0) or

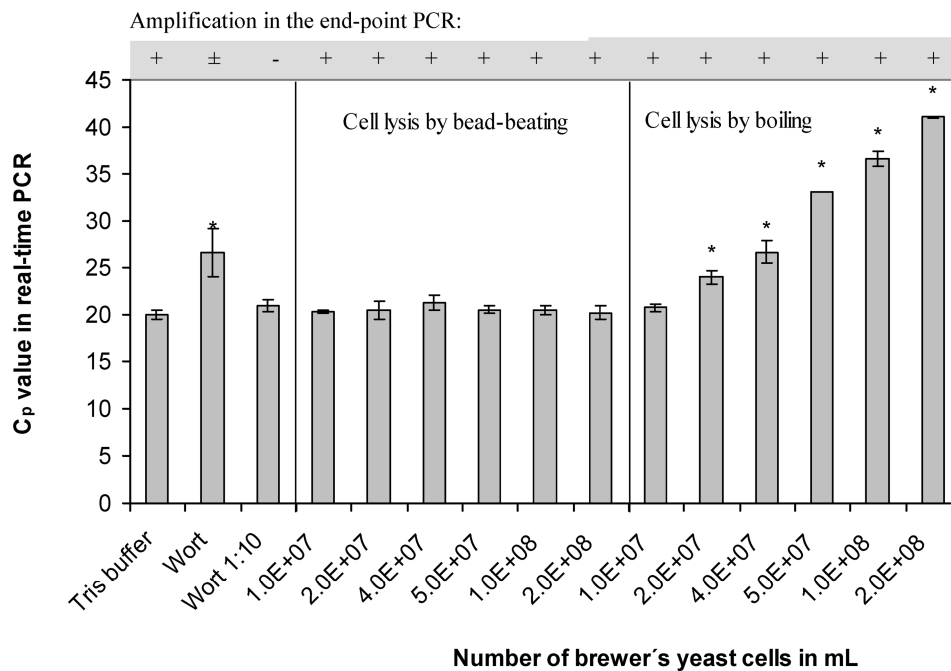
the matrix. In the two-step method, the pellets were suspended in 1 mL ultra-pure water, vigorously vortexed for 10–15 sec and centrifuged at  $209 \times g$  for 5 min to selectively remove the yeast cells. The bacteria were harvested from the supernatants ( $16,060 \times g$ , 3 min) in new microtubes. The cell lysis by bead-beating and heating was performed as in the inhibition study. After the cell lysis, debris was removed by centrifugation. The InstaGene Matrix was used as described by Juvonen et al.<sup>9</sup>

## Effects of cell dispersants on PCR detection of bacteria in yeast-containing brewery samples

The effects of different potential cell dispersants (Table I) on the PCR detection of the model bacteria were tested with 1 mL brewery wort samples containing  $10^8$  cells/mL of brewer's yeast and *ca.*  $5 \times 10^5$  CFU/mL *S. lactificex* or *M. cerevisiae*. All dispersant solutions were sterile-filtered. The cells in 1 mL aliquots were pelleted and then suspended in 1 mL of the various dispersants followed by centrifugation at  $209 \times g$  for 5 min. The cells were harvested ( $16,060 \times g$  3 min) from the supernatant in new microtubes, washed once with 1 mL ultra-pure water and finally suspended in 100  $\mu$ L 0.01 M Tris (pH 8.0). The DNA was liberated from the cells by heating. Three replicate samples were processed for each treatment.

## Statistical analyses

The influence of brewery sample extracts and PCR facilitators on the real-time amplification was assessed using Dunnett's test ( $\alpha = 0.01$ ) in comparison to the control reactions. The significance of differences between the mean  $C_p$



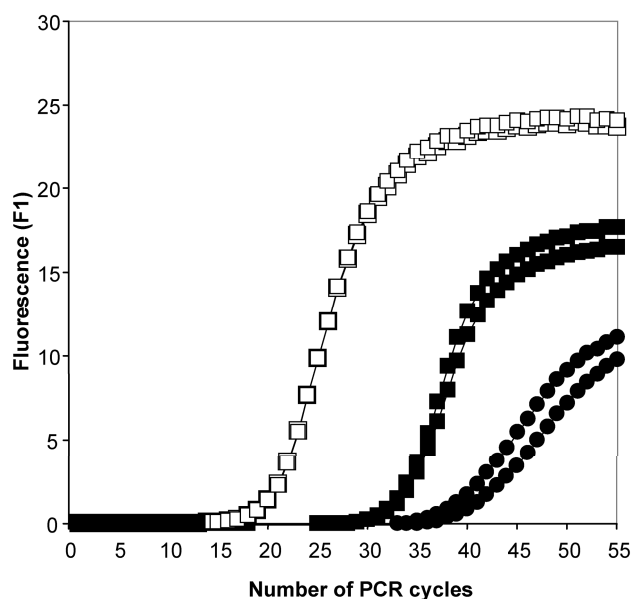
**Fig. 1.** Effects of constituents of yeast-containing brewery process samples on the end-point and real-time PCR amplifications. Washed, YM-grown brewer's yeast cells were prepared for the analysis by bead-beating or heating. Wort, crude cell extracts or 0.01 M Tris buffer (positive control) was added (5%, v/v) to the PCR mixtures with 10 ng *P. frisingensis* DNA. Symbols: +, amplification; ±, weak amplification;  $C_p$ , crossing point cycle number; \*, significant effect on the real-time amplification compared to the control reaction (Dunnett's test,  $\alpha = 0.01$ ).

values of different pre-PCR treatments were determined by the Student's two-tailed t-test ( $\alpha = 0.05$ ) assuming equal variances. The F-test ( $\alpha = 0.05$ ) was used to test the equality of the variances.

## RESULTS

### Inhibition of end-point and real-time PCR reactions by brewery samples

Sensitivities of the model end-point and real-time PCR reactions to substances present in crude extracts from beer and brewery process samples were compared. Bacteria are normally concentrated from beer samples for PCR by membrane filtration. Therefore, we studied the inhibiting effect of the sample components concentrating on the membranes with the cells. To evaluate the inhibiting effect, the non-filterable material collected from different volumes of beer was added to the PCR mixtures in a water suspension (3, 5 or 10%, v/v) with purified target



**Fig. 2.** Effect of crude extracts from wort-grown brewer's yeast cells on the real-time PCR amplification of *P. frisingensis* DNA (10 ng). The extracts were prepared by bead-beating of the washed cells ( $1 \times 10^8$  cells/mL) in 0.01 M Tris buffer (pH 8.0) for 10 min, and thereafter analysed in duplicate PCR reactions. Symbols: (□) no yeast, (■), 1  $\mu$ L of crude extract, (●) 2  $\mu$ L of crude extract.

DNA. The two PCR reactions differed in their sensitivity to the beer-derived material. The end-point PCR failed in the presence of 3% (v/v) of the extract from 100 mL beer, while the real-time PCR was only inhibited in the presence of  $\geq 5\%$  (v/v) of the extract from 330 mL beer. Thus, the real-time PCR tolerated four to five times higher amounts of the inhibitory material than the end-point PCR. Neither of the reactions was affected by 10% (v/v) of the extract from 25 mL of beer.

In order to study the contributions of different constituents of yeast-containing process samples to PCR inhibition, fermented wort and crude cell extracts from YM-grown and wort-grown yeast cells were separately analysed by PCR. Heating and bead-beating were compared for preparation of the crude cell extracts. Wort inhibited the real-time PCR only slightly, whereas the end-point PCR was more strongly affected (Fig. 1). Wort dilution by a factor of 10 eliminated the inhibition (Fig. 1). The inhibiting effect of the crude extracts depended on the cell lysis method, on the concentration and the growth medium of the cells and on the PCR format (Figs. 1 and 2). The extracts prepared from the YM-grown cells ( $\leq 2 \times 10^8$  cells  $\text{mL}^{-1}$ ) by bead-beating were not inhibitory. The extracts prepared from the same culture ( $\geq 2 \times 10^7$  cells  $\text{mL}^{-1}$ ) by boiling inhibited the real-time PCR but the end-point PCR was not affected. The wort-grown yeast cells inhibited both PCR reactions irrespective of the cell lysis method employed (Fig. 2, Table II), indicating that the cells were binding inhibitors from the wort.

### Facilitation of PCR amplification

**End-point PCR.** Three BSA products and PVP-10 and PVP-40 were evaluated to facilitate the end-point amplification in the presence of inhibitors from various types of brewery samples. PVP-10 and one of the BSA products (New England Biolabs) were themselves inhibitory at all tested concentrations. The maximum concentrations of PVP-40 and the other BSAs that had no influence on PCR sensitivity in the absence of inhibitors were 0.5% (w/v) and 0.25% (w/v), respectively. The inclusion of the maximum tolerated amount of BSA or PVP-40 in the PCR mixture overcame the inhibition caused by wort and crude extracts from beer and brewer's yeast (Table II). The reaction sensitivity was the same with the sample extracts as with ultra-pure water. BSA and PVP-40 were further challenged with extracts from five commercial lager beers (Table III). BSA allowed PCR amplification in the presence of  $\geq 5\%$  (v/v) instead of  $\leq 2\%$  (v/v) of the extracts. PVP-40 was less efficient. Synergistic effects were not

**Table II.** Influence of facilitators on the end-point amplification of serial dilutions of purified *P. frisingensis* DNA in the absence and presence of wort or crude extracts from beer or yeast samples.

| Sample (5%, v/v, per reaction)                            | Detection limit in the end-point PCR (pg DNA) <sup>a</sup> |                                |                    |
|---|--|--------------------------------|--------------------|
|   | No facilitator   | BSA <sup>b</sup> , 0.25% (w/v) | PVP-40, 0.5% (w/v) |
| Ultra-pure water  | 0.2  | 0.2                            | 0.2                |
| Extract from beer (330 mL) <sup>c</sup>                   | >2000  | 0.2                            | 0.2                |
| Fermented wort  | 2000   | 0.2                            | 0.2                |
| Extract from YM-grown yeast ( $1 \times 10^8$ cells/mL)   | 0.2  | 0.2                            | 0.2                |
| Extract from wort-grown yeast ( $1 \times 10^8$ cells/mL) | >2000  | 0.2                            | 0.2                |

<sup>a</sup> The lowest amount of target DNA in 50  $\mu$ L PCR reaction resulting in a visible band.

<sup>b</sup> Sigma-Aldrich.

<sup>c</sup> 3% (v/v) of the extract per reaction was used.

observed when both facilitators were simultaneously used (data not shown).

To determine the influence of the BSA addition on the end-point PCR detection limits, DNA extracts (10%, v/v) from 100 mL beer samples containing different amounts of *M. cerevisiae* cells were analysed without and with 0.25% (w/v) BSA (Sigma-Aldrich). The detection limit with BSA was  $2 \times 10^2$  CFU/100 mL, whereas in the absence of BSA not even the highest level,  $2 \times 10^5$  CFU/100 mL, was detected (data not shown).

**Real-time PCR.** The abilities of BSA and PVP-40 to relieve the inhibition of the real-time PCR by brewery samples were also evaluated. In the absence of inhibitors, the reaction tolerated 0.2% (w/v) BSA or 1% (w/v) PVP-40 ( $\alpha = 0.01$ , Dunnett's test). However, use of either of the facilitators did not reproducibly reduce the inhibiting effect of wort or crude yeast or beer extracts (5%, v/v) on

the amplification compared to the non-facilitated reactions ( $\alpha = 0.01$ , Dunnett's test).

### Removal of PCR inhibitors from beer samples during the filtration step

In order to reduce the amount of PCR inhibitors during the filtration step, the washing of polycarbonate membranes with chemical dispersants, chelators or surfactants, and cross-flow filtration using CellTrap™ devices were investigated. The results showed that only those washes which involved 0.1 M NaOH allowed PCR amplification in the presence of the extracts (3–10%, v/v) from 330 mL beer (data not shown). When 0.25% (w/v) BSA (Sigma-Aldrich) was added to the end-point PCR mixture, 10% (v/v) of any extract was tolerated except for the extracts from Tween 20 (5%, v/v) and the mere water wash (3%, v/v) (data not shown). Hence, in most cases higher volume of the extract could be analysed by combining an inhibitor removal with BSA.

The eluates (5–10%, v/v) recovered from the Cell-Trap™ devices after filtration of 330 mL beer did not influence the real-time PCR, but the end-point PCR was inhibited unless BSA was used (data not shown). When 100 mL beer was passed through, the end-point PCR tolerated 5% (v/v), while 10% (v/v) could be analysed in combination with BSA. The detection limits of the Cell-Trap™ devices and the membrane filtration were compared by analysing 100 mL beer samples inoculated with a *P. frisingensis* VTT E-011871 dilution series. The Cell-Trap™ filtration followed by a mere water wash allowed as sensitive a detection ( $7 \times 10^2 - 7 \times 10^3$  CFU/100 mL) as the membrane filtration followed by 0.1 M NaOH, 0.5% SDS and water wash by both PCR formats. Our results indicated that the CellTrap™ filtration was more effective in removing inhibitors from the beer samples.

**Table III.** The percentage (% v/v) of beer extracts tolerated in 50- $\mu$ L end-point PCR reactions.

| Beer <sup>a</sup> | Volume <sup>b</sup> | End-point PCR <sup>c</sup> |                  |                    |
|-------------------|---------------------|----------------------------|------------------|--------------------|
|                   |                     | No facilitator             | BSA, 0.25% (w/v) | PVP-40, 0.5% (w/v) |
| 1                 | 330                 | 2 <sup>d</sup>             | ≥10              | 10 <sup>d</sup>    |
| 2                 | 230                 | 2 <sup>d</sup>             | ≥10              | 5                  |
| 3                 | 230                 | <2                         | 5                | <2                 |
| 4                 | 330                 | 2 <sup>d</sup>             | ≥10              | 10 <sup>e</sup>    |
| 5                 | 330                 | 2 <sup>d</sup>             | ≥10              | 10 <sup>d</sup>    |

<sup>a</sup> Commercial lager beers containing 0.3–4.6% (v/v) of alcohol.

<sup>b</sup> A bottle of 330 mL or maximum filterable amount of beer was analysed as described in Materials and Methods.

<sup>c</sup> The reactions contained 2, 5 or 10% (v/v) of the extract with 10 ng *P. frisingensis* DNA. BSA was from Sigma-Aldrich.

<sup>d</sup> Very faint band.

<sup>e</sup> Faint band.

**Table IV.** PCR detection limits of different sample treatment methods for *S. lactificifex* VTT E-90407<sup>T</sup> and *M. cerevisiae* VTT E-981087 in 1 mL of fermented brewery wort containing  $3 \times 10^7$  yeast cells.

| Species                | DNA extraction   | Centrifugation   | End-point PCR <sup>a</sup>                   |                     | Real-time PCR                                |                     |                                   |
|------------------------|------------------|------------------|--|---------------------|--|---------------------|-----------------------------------|
|                        |                  |                  | Optimum extract volume, % (v/v) <sup>b</sup> | CFU/mL <sup>c</sup> | Optimum extract volume, % (v/v) <sup>d</sup> | CFU/mL <sup>e</sup> | C <sub>p</sub> ratio <sup>f</sup> |
| <i>S. lactificifex</i> | Heating          | 1-step           | 2–10   | $3 \times 10^1$     | 5  | $3 \times 10^3$     | 1.08                              |
|                        |                  | 2-step           | 5–10   | $3 \times 10^1$     | 10   | $3 \times 10^2$     | 1.00                              |
|                        | Bead-beating     | 1-step           | 2–10   | $3 \times 10^1$     | 5  | $3 \times 10^2$     | 1.03                              |
|                        |                  | 2-step           | 2–10   | $3 \times 10^1$     | 10   | $3 \times 10^2$     | 1.02                              |
|                        | InstaGene Matrix | 1-step           | 2–10   | $3 \times 10^1$     | 5  | $3 \times 10^2$     | 1.05                              |
|                        |                  | 2-step           | 2–10   | $3 \times 10^1$     | 10   | $3 \times 10^2$     | 0.99                              |
| <i>M. cerevisiae</i>   | Bugs'n Beads     | 1-step           | 5–10   | $3 \times 10^2$     | 10   | $3 \times 10^2$     | 1.19                              |
|                        |                  | UltraClean™ Soil | 1-step                                       | 5–10                | $3 \times 10^1$                              | 10                  | $3 \times 10^2$                   |
|                        | Heating          | 1-step           | 2  | $2 \times 10^3$     | 5  | $2 \times 10^4$     | 1.02                              |
|                        |                  | 2-step           | 5–10   | $2 \times 10^2$     | 10   | $2 \times 10^4$     | 1.00                              |
|                        | Bead-beating     | 1-step           | 5–10   | $2 \times 10^3$     | 5  | $2 \times 10^3$     | 0.91                              |
|                        |                  | 2-step           | 5–10   | $2 \times 10^2$     | 10   | $2 \times 10^3$     | 0.91                              |
| InstaGene Matrix       | 1-step           | 2                | $2 \times 10^3$                              | 5                   | $2 \times 10^3$                              | 0.98                |                                   |
|                        |                  | 5–10             | $2 \times 10^2$                              | 10                  | $2 \times 10^3$                              | 0.94                |                                   |
|                        | 2-step           | 10               | $2 \times 10^2$                              | 10                  | $2 \times 10^3$                              | 1.01                |                                   |
|                        |                  | 5–10             | $2 \times 10^3$                              | 10                  | $2 \times 10^3$                              | 0.99                |                                   |

<sup>a</sup> The PCR mixture contained 0.25% (w/v) BSA.

<sup>b</sup> The reactions contained 2, 5 or 10% (v/v) of the extract.

<sup>c</sup> The lowest contamination level giving a visible band.

<sup>d</sup> The reactions contained 5 or 10% (v/v) of the extract.

<sup>e</sup> The lowest contamination level with C<sub>p</sub> value of ca. 41 cycles.

<sup>f</sup> Sum of C<sub>p</sub> values in relation to the heating method with 2-step centrifugation.

## Pre-processing of yeast-containing brewery samples

**Comparison of pre-processing methods.** Five easy methods were compared to prepare DNA from *S. lactificifex* (rod) and *M. cerevisiae* (coccus) in 1-mL samples of yeast-containing brewery wort. The direct cell lysis methods, i.e. heating, bead-beating and InstaGene Matrix, were evaluated with a single-step centrifugation to concentrate all cells and with a two-step process involving an initial low-speed centrifugation for yeast cell removal. BSA (0.25%, w/v) was included in the end-point PCRs as an amplification facilitator.

In general, all the methods allowed direct detection of low levels (*ca.*  $10^1$ – $10^3$  CFU/mL) of the strict anaerobes in the brewery process samples (Table IV). The handling time of 10 samples was 1 h for the heating and bead-beating methods, 1.5 h for the InstaGene Matrix and 2–3 h for the two kits. The end-point PCR was generally ten times more sensitive than the real-time PCR counterpart. The direct cell lysis methods required the use of BSA in the end-point PCR. Its omission increased the detection limits by 10–1,000-fold, indicating that the extracts contained residual inhibitors (data not shown). The use of two-step centrifugation tended to improve the detection limits of the direct lysis methods as it allowed the analysis of a higher extract volume or reduced the real-time PCR  $C_p$  values or both (Table IV). Its positive influence was most obvious in the real-time PCR analysis of the crude extracts prepared using the heating or the InstaGene Matrix

method. Overall, the bead-beating and the InstaGene Matrix methods preceded by the two-step centrifugation enabled the most sensitive PCR detection of the strict anaerobes in the yeast-containing brewery samples.

**Impact of low-speed centrifugation with cell dispersants on PCR detection.** Low-speed centrifugation of thick yeast-containing wort samples ( $10^8$  cell mL<sup>-1</sup>) with potential cell dispersants was evaluated because flocculation of the bacteria—with each other, with yeast cells or with other sample components—could impair their separation. Based on the  $C_p$  values, the use of EDTA, EDTA–maltose or Tween 20 solution instead of Tris buffer improved the detection of both strains in the samples (Table V). This was particularly evident in the case of the flocculating *M. cerevisiae* strain. Moreover, the improvement in the detectability was more pronounced with 2  $\mu$ L than with 1  $\mu$ L crude extract, suggesting that the dispersants also removed sample-derived inhibitors. The PCR detection limits with the four most promising dispersants were determined for the two model strains. The detection limits for *M. cerevisiae* were 10 times lower when EDTA, EDTA-maltose or Tween 20 was used instead of Tris buffer (Table VI).

## DISCUSSION

Brewery samples are complex media that are known to contain substances that may be carried along with DNA through sample processing and inhibit the end-point PCR<sup>3,20,27–29</sup>. However, data concerning their influence on the real-time PCR was not available. As a basis for this study, we compared the inhibition of the conventional end-point PCR and the SYBR Green I based real-time PCR (LightCycler™) by brewery samples. The real-time PCR was found to be more sensitive to brewer's yeast constituents than to wort or beer-derived substances. The reverse was true for the end-point PCR. This was probably due to differences between the two systems in their PCR mixture composition (e.g. enzyme, buffer), amplification kinetics (stepwise vs. kinetic) and principle of measurement (real-time vs. end-point). Based on Teo et al.<sup>32</sup>, the real-time PCR master mixture contained BSA, which was likely to facilitate the amplification in the presence of inhibitors. By contrast, the end-point PCR generally resists higher amounts of non-target DNA than LightCycler™ PCR<sup>22,32,37</sup>. This study revealed that both PCR systems were influenced by the crude extracts from wort-

**Table V.** Influence of the use of potential cell dispersants during low-speed centrifugation on the detection of *S. lactificifex* VTT E-90407<sup>T</sup> and *M. cerevisiae* VTT E-981087 in yeast-containing wort samples as assessed by real-time PCR.

| Treatment                 | Relative $C_p$ value in real-time PCR <sup>a</sup> |           |                      |           |
|---------------------------|--|-----------|----------------------|-----------|
|                           | <i>S. lactificifex</i>                             |           | <i>M. cerevisiae</i> |           |
|                           | 1 $\mu$ L  | 2 $\mu$ L | 1 $\mu$ L            | 2 $\mu$ L |
| Tris buffer (control)     | 1.00   | 1.00      | 1.00                 | 1.00      |
| 0.5% (w/v) Tween 20       | 0.93*  | 0.90*     | 1.00                 | 0.95*     |
| 0.01 M EDTA               | 0.99   | 0.97*     | nd <sup>b</sup>      | nd        |
| 0.05 M EDTA               | 0.96*  | 0.91*     | 0.92*                | 0.93*     |
| 0.01 M EDTA–0.2 M maltose | 0.95*  | 0.91*     | 0.92*                | 0.88*     |

<sup>a</sup> The mean  $C_p$  values of the treatments are expressed relative to the mean  $C_p$  value of the control treatment. A significant effect in the Dunnett's test ( $\alpha = 0.01$ ) is indicated by an asterisk.

<sup>b</sup> Not determined.

**Table VI.** Influence of potential cell dispersants on PCR detection limits for *S. lactificifex* VTT E-90407<sup>T</sup> and *M. cerevisiae* VTT E-981087 in 1 mL of fermented brewery wort containing  $1 \times 10^8$  brewer's yeast cells.

| Species                | Treatment                 | End-point PCR                                |                 | Real-time PCR                                |                 |
|------------------------|---------------------------|--|-----------------|--|-----------------|
|                        |                           | Optimum extract volume, % (v/v) <sup>a</sup> | CFU/mL          | Optimum extract volume, % (v/v) <sup>b</sup> | CFU/mL          |
| <i>S. lactificifex</i> | Tris buffer (control)     | 5  | $6 \times 10^1$ | 10   | $6 \times 10^2$ |
|                        | 0.5% (w/v) Tween 20       | 5  | $6 \times 10^1$ | 10   | $6 \times 10^2$ |
|                        | 0.01 M EDTA–0.2 M maltose | 5  | $6 \times 10^1$ | 10   | $6 \times 10^2$ |
|                        | 0.05 M EDTA               | 2  | $6 \times 10^1$ | 5  | $6 \times 10^2$ |
| <i>M. cerevisiae</i>   | Tris buffer (control)     | 5  | $5 \times 10^3$ | 5 or 10                                      | $5 \times 10^4$ |
|                        | 0.5% (w/v) Tween 20       | 5  | $5 \times 10^2$ | 5 or 10                                      | $5 \times 10^3$ |
|                        | 0.01 M EDTA–0.2 M maltose | 5  | $5 \times 10^2$ | 5 or 10                                      | $5 \times 10^3$ |
|                        | 0.05 M EDTA               | 5  | $5 \times 10^2$ | 5 or 10                                      | $5 \times 10^3$ |

<sup>a</sup> The reactions contained 2, 5 or 10% (v/v) of the extract.

<sup>b</sup> The reactions contained 5 or 10% (v/v) of the extract.

grown brewer's yeast. Brewer's yeast is known to adsorb PCR-inhibiting polyphenols from wort<sup>20,37</sup>, which may explain the result. In conclusion, the inhibition study implied that the end-point PCR requires more extensive relief of inhibition from beer-derived material than the real-time PCR and that both systems benefit from the removal of brewer's yeast cells from the process samples.

We evaluated two different approaches to minimise the inhibition of PCR by brewery samples, namely the inclusion of amplification facilitators into the PCR mixtures and the removal of inhibitors before or during the DNA extraction. For the first time it was shown that the use of BSA or PVP-40 as a facilitator efficiently relieves the inhibiting effect of brewery samples on the end-point PCR. The effective concentration of BSA was 0.25% (w/v), which agrees well with the values reported for other sample matrices<sup>14,23</sup>. Standard and molecular biology grade BSA performed equally well, but the former is more economic to use. PVP-40 was effective at a 0.5–1% (w/v) concentration. In the study of Koonjul et al.<sup>13</sup>, 0.5–2% (w/v) PVP with  $M_w$  of 25,000–30,000 reversed the inhibition of the end-point PCR by plant-derived polyphenols. In line with previous studies<sup>1,14</sup>, neither of the facilitators interfered with the amplification in the absence of inhibitors and no synergy was found between them. The real-time PCR did not benefit from the facilitators probably because the master mixture already contained BSA<sup>32</sup>. Many inhibiting substances, which are attenuated by PVP and BSA, carry phenolic groups<sup>13,14,37</sup>. PVP is known to selectively adsorb polyphenols from beer and is used in some breweries for this purpose in order to prevent non-biological haze formation<sup>33</sup>. Thus, BSA and PVP-40 may have protected the polymerase from inactivation by scavenging phenolic compounds in the extracts. Taking into consideration the complexity of brewery samples, it is likely that many substances take part in the inhibition. This is supported by the fact that BSA, which is able to sequester a broader range of compounds than PVP-40<sup>37</sup>, was more effective.

Due to the generally low concentration of microbes per volume of beer, the cells are concentrated for PCR by membrane filtration. As shown in this and earlier studies this step also concentrates PCR inhibitors<sup>28</sup>. Washing of the membrane filters with strong alkali (0.1 M) and SDS (0.5%) is an established method for rapid removal of PCR inhibitors from beer samples<sup>9,11,28,38</sup>. Although usually effective, NaOH and SDS are in their use concentrations strong permeabilising agents that may lead to DNA losses from *Pectinatus* and *Megasphaera* bacteria before DNA extraction<sup>26,28</sup>. They are also potent PCR inhibitors<sup>22</sup>. We evaluated the use of potentially less destructive chemicals, many of which had earlier been applied to scavenge inhibitors from brewer's yeast slurries, wine, plant materials and food samples prior to PCR<sup>4,16,20,37</sup>. The washes with PVP-40, with STPP and EDTA and with the dilute SDS and NaOH solutions were as effective as the established method when BSA was present in the reactions. Hence, the possible adverse effects of strong alkali and SDS on the PCR analysis may be overcome by using the alternative treatments presented here. This study also indicated that NaOH is the key compound for removing inhibitors, suggesting that SDS may be omitted from the established procedure.

The cross-flow filtration with the CellTrap™ devices was shown to be a potential alternative to the routinely used membrane filtration to concentrate the strictly anaerobic bacteria from beer samples for PCR. The devices may also reduce or exclude the need for anti-inhibitory treatments as they retain fewer inhibitors than the membrane filters. Furthermore, large volume samples (up to several litres) could be analysed to increase the assay sensitivity. DNA extraction is also facilitated since the cells are eluted from the devices. Further studies are warranted to determine the suitability of the system for diverse microbes and to optimise the entire analytical procedure.

For the first time, different procedures were compared to prepare DNA from the strict anaerobes in brewery process samples. When the impact of residual inhibitors was relieved with BSA, the direct cell lysis methods had sensitivities that were equal to or greater than those of the commercial kits involving multiple purification steps. As the commercial kits had the highest theoretical sensitivity, it is likely that some target DNA was lost during purifications. Simple heating appeared to release or to generate more PCR-inhibiting substances from the process samples than bead-beating. Chelex 100, a chelator used in the InstaGene Matrix method, probably scavenged some inhibitors as the extracts were less inhibitory despite the heating. This is in line with the results of Panicker et al.<sup>19</sup> for PCR detection of *Vibrio vulnificus* in water. Due to its ease of use, rapidity, low costs and compatibility with both PCR systems, the bead-beating is regarded overall as the best method to disrupt cells of the strict anaerobes in process samples containing brewer's yeast.

Low-speed centrifugation prior to cell lysis was shown to enhance PCR detection of the strict anaerobes in the yeast-containing process samples. The result implies that the interfering sample components were preferentially removed in the applied conditions. Low-speed centrifugation also improved the detectability of *Pediococcus damnosus* cells in pitching yeast in an immunoassay<sup>36</sup> and was successfully used to recover *Obesumbacterium proteus* cells in non-filtered beer<sup>15</sup>. Flocculation, in which microbial cells spontaneously and reversibly aggregate together, is a common phenomenon in microbiology. It has also been reported to occur between brewery contaminating bacteria and brewer's yeast<sup>21</sup>. In this study, we applied potential cell dispersants to promote the separation of the strict anaerobes from brewer's yeast during the centrifugation step. Our results suggest that Tween 20 and especially EDTA, without or with maltose, enhance this process particularly when bacteria have a tendency to flocculate. Flocculation of brewer's yeast is usually mediated by binding of lectin-type receptors to cell-wall mannose residues on adjacent cells. Hence, it is probable that EDTA chelated  $Ca^{2+}$  ions and maltose blocked the cell-surface receptors, both needed in flocculation<sup>21,35</sup>. As a non-ionic surfactant, Tween 20 probably functioned as a non-specific dispersant. EDTA and Tween 20 probably also removed inhibitors from the samples<sup>20,37</sup>.

Based on the obtained results, an easy 1-hour protocol is proposed to prepare PCR-ready DNA from the strict anaerobes in the yeast-containing brewery samples. It involves the removal of yeast cells by low-speed centrifugation in a dispersant, preferably maltose and EDTA, and

physical disruption of the pelleted bacteria, preferably by bead-beating. The procedure is fast (approx. 20 samples/h), and does not require expensive equipment or reagents or involve toxic chemicals. The detection level of the procedure, *ca.*  $10^1$ – $10^3$  CFU/mL ( $<10^0$ – $10^2$  CFU per PCR), agrees well with the detection limit reported for the same group-specific PCRs for beer samples<sup>11</sup>. Using LightCycler™ PCR coupled with a rapid sample treatment, Koivula et al.<sup>15</sup> detected  $2 \times 10^2$  –  $2 \times 10^3$  CFU of *O. proteus* against  $2 \times 10^8$  yeast cells. However, their end-point PCR reaction, which did not contain BSA, was 10–100 times less sensitive. Maugueret and Walker<sup>17</sup> only detected  $10^7$  CFU/mL of *O. proteus* when they reduced the number of yeast cells in wort by centrifugation before DNA extraction. It is probable that the rather high centrifugal speed ( $2500 \times g$ ) used in their study also pelleted bacteria. Stewart and Dowhanick<sup>29</sup> detected low levels of lactic acid bacteria in pitching yeast ( $1:10^8$ ) by nested PCR, which is a laborious technique prone to carry-over contamination. According to Jespersen and Jakobsen<sup>8</sup>, the sensitivity required for detection of spoilage bacteria in process samples is 1 CFU/mL. This could be achieved by collecting bacteria from a 10–100 mL sample volume or by combining a short enrichment step with the presented PCR assay.

The methods presented here can be exploited in the PCR analysis of brewery samples and may also be applied to other similar matrices. BSA or PVP-40 could be routinely added to the end-point PCR mixtures to reduce the risk of inhibition and to allow the analysis of crude extracts. Further, the combination of a gentle inhibitor removal and rapid cell lysis methods with the use of PCR facilitators reduces the risk of partial or complete PCR failure, while allowing minimal sample processing, thus potentially improving the assay sensitivity, reliability and speed. This study also implies that the various PCR techniques may need specific analytical procedures due to their different sensitivities to inhibitors. In the future, the combination of large volume cell concentration and effective DNA extraction methods with automation may allow direct single-cell detection with minimal cross-contamination risks. Meanwhile, simple and rapid pre-PCR processing methods, as presented in this study, combined with culture enrichment, remain the most practical and reliable procedures to detect trace contamination of beer-spoilage bacteria for routine quality control.

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