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Effect of autolytic by-products on PCR-detection of beer spoilers in yeast slurry

The detection of wild yeasts and bacteria within stores of brewing yeast is a standard activity executed at most commercial breweries, however due to the complexity of the sample matrix often contaminants remain undetected. Wild *Saccharomyces* spp., particularly the dextrin hydrolyzing variant *S. cerevisiae* var. *diastaticus*, can pose a unique problem for commercial brewers as these organisms are morphologically similar to brewer's yeast and may lead to the undesired recall of affected batches. It is for this reason that a reliable method has to be developed to detect low concentrations as quickly as possible. Here we propose a new DNA extraction procedure and real-time PCR assay that reliably detects *S. cerevisiae* var. *diastaticus* without the timely preenrichments. Furthermore the method removes the inhibitory effects traditionally caused by high concentrations of brewing yeast and autolytic degradation products.

Descriptors: beer, yeast slurry, *S. cerevisiae* var. *diastaticus*, autolytic by-products, PCR-inhibition, sample treatment, DNA-extraction, real-time PCR

1 Introduction

Maintaining high purity yeast is vital to ensure high-quality and consistent taste of commercially produced beers [1-3]. The contamination of brewing yeast by spoilage organisms has been shown to result in undesirable consequences for the finished product, including but not limited to the manifestation of unwanted flavors, turbidity, ropiness, gushing, added carbonation, elevated alcohol content, and over attenuation [1,4-5].

S. cerevisiae var. *diastaticus* has recently emerged as one of the most severe spoilage microorganisms affecting the brewing industry, particularly craft brewers [5-7]. The detection of *S. cerevisiae* var. *diastaticus* via the conventional plate culture method remains problematic, primarily due to the physiological likeness between the contaminant and the traditional brewing yeast, *S. cerevisiae*. Furthermore, *S. cerevisiae* var. *diastaticus* detection by means of conventional plate culture method, which examines yeasts ability to metabolize dissimilar carbon sources compared to brewer's yeast, requires several days prior to obtaining a conclusive result and often did not allow for the detection of low-level contaminations [5-8].

More recently, the development of readily available real-time PCR assays has allowed for the rapid detection of *S. cerevisiae* var. *diastaticus* and many other beer spoiling organisms [1,7]. However, the standard requirement for preenrichment, absence of a selective media for all organisms, and the amount of matrix inhibitors reduce the practical application of real-time PCR for analysis of pitching yeast, propagation yeast and freshly harvested yeast for contamination. Potential PCR-inhibitors are e.g. high concentrations of brewing yeast and their autolysis by-products, polyphenols, polysaccharides, alcohol and proteins [5, 9-10]. In this study, the influence of yeast slurry protein inhibitors, yeast cell concentration and pH value (data not shown) are shown to impact the sensitivity of real-time PCR detection of beer spoilers.

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2 Materials and Methods

2.1 Test strains and growth conditions

The strains used in this study were *S. cerevisiae* var. *diastaticus* and *Lactobacillus brevis* (data not shown). They were provided by a brewery (Duvel Moortgat, Belgium). *S. cerevisiae* var. *diastaticus* was grown in YM broth (3 g yeast extract, 3 g peptone, 3 g malt extract, 10 g dextrose, add water to 1 l) for 48 h at 36 °C. Dextrose was used instead of glucose as *S. cerevisiae* var. *diastaticus* showed better growth due to dextrin hydrolyzing properties. *Lactobacillus brevis* was grown anaerobically in MRS broth (Merck) for 48 h at 28 °C.

2.2 Authentic yeast slurry samples

Yeast slurry samples of unknown number of *S. cerevisiae* cells were provided by a Belgian brewery (Duvel Moortgat). The *S. cerevisiae* (culture yeast) cell concentrations were determined by a cell counting chamber (Neubauer improved). The counted *S. cerevisiae* cells varied from 10^7 to 10^{10} cfu/ml, for further experiments samples were concentrated up to 10^{12} by centrifugation.

2.3 Artificial contamination of yeast slurry samples

S. cerevisiae var. *diastaticus* (3.9 x 10^9 cfu/ml) and *Lactobacillus brevis* (5.0 x 10^9 cfu/ml) were serially diluted in sterile Ringer

solution. For the following study cell concentrations of 1x10^3 cfu/ml up to 1x10^9 cfu/ml were used for spiking authentic yeast slurry samples.

2.4 Determination of cells concentrations, pH value and proteins

The cell concentrations of *S. cerevisiae* and *S. cerevisiae* var. *diastaticus* were determined by using the Neubauer improved cell counting chamber (depth 0.100 mm) and the Thoma cell count chamber (depth 0.02 mm) for *Lactobacillus brevis*. Measuring of optical density was done with A_{600} in a spectrophotometer (Eppendorf BioSpectrometer). The pH values of the yeast slurry samples were determined by pH indicator strips (Macherey-Nagel). The proteins were measured after homogenization of the samples photometrically at 280 nm (UV range, Eppendorf BioSpectrometer, Table 1).

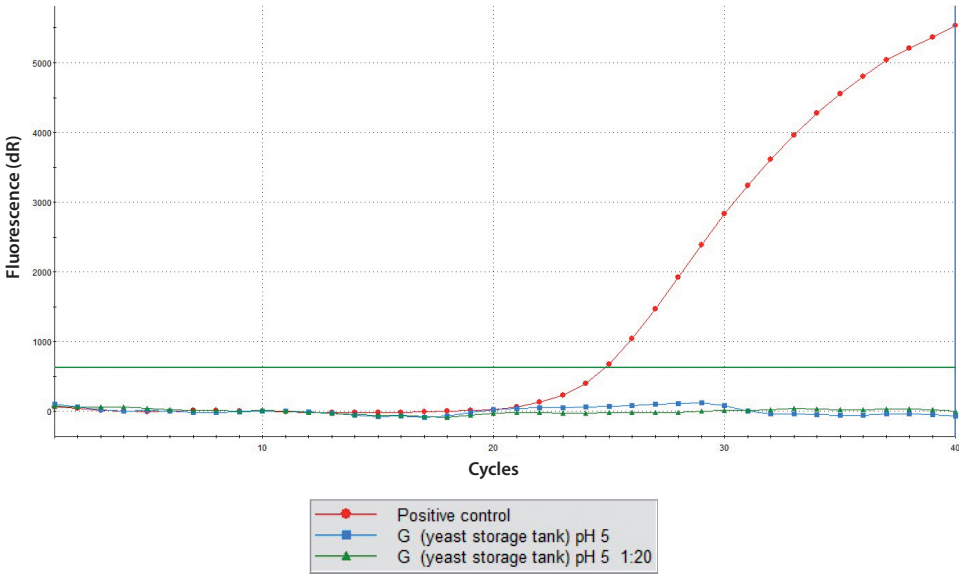

Fig. 1 **PCR-analysis of 10^9 cfu/ml *S. cerevisiae* var. *diastaticus* cells in 10^{12} cfu/ml culture yeast cells of G-tank G33 (pure and 1:20 diluted) with the QPP1SD real-time PCR kit**

2.5 DNA-extraction

For the DNA extraction out of yeast slurries, a modified protocol was developed and used for further experiments (CSY0100, GENIAL). Hence the spiked yeast samples were diluted with sterile Ringer solution. The dilution factor (pure and up to 1:50) depends on the culture yeast cell concentration which was determined by using the Neubauer improved cell counting chamber. From the dilution 1 ml was pretreated enzymatically, incubated at 65 °C for 30 min. and centrifuged for 5 min. at 15.500 x g. The supernatant was discarded, 250 µL QuickGEN lysis buffer was added to the cell pellet and incubated for 5 min. at 95 °C. After vortexing 2.5 µL was used for real-time PCR.

2.6 Real-time PCR

Multiplex real-time PCR assay was used in this study. The TaqMan® based PCR includes the detection of *Lactobacillus*, *Pediococcus*, *Megasphaera*, *Pectinatus*, *S. cerevisiae* var. *diastaticus* and an inhibition control in one assay (QPP1SD). The target sequences for all species are located in 16S r-DNA, IGS-spacer and STA1 genes. Primers and Probes were designed using Primer Express 3 Software (Thermo Fisher Scientific). All primers and probes were tested using Blast (NCBI, Basic Local Alignment Search Tool) for homologies with other sites or species. The TaqMan® PCR was performed as a ready to use system in PCR strips, lyophilized with primers, probes, inhibition control and lyticase. 17.5 µl Premix and 2.5 µl of DNA were added to a 20 µl end-volume. The PCR reactions were run under the following conditions: lyticase treatment for 15 min at 37 °C, initial denaturation for 15 min at 95 °C followed by 35–40 cycles of 95 °C for 10 sec and 63 °C for 20 sec. The PCR-assays were performed on the qPCR cyclers Mx3005p (Agilent Technologies) and MyGo Pro (IT-IS life science, LTF Labortechnik).

3 Results and Discussion

3.1 Inhibitory influences on real-time PCR-detection in yeast slurries

In this study it could be shown that there are a number of inhibitory factors which lead to false negative PCR-results of beer-spoiling bacteria and yeast in yeast slurries. The real-time PCR-analysis of *Lactobacillus brevis* (data not shown) and *S. cerevisiae* var. *diastaticus* is mainly influenced by PCR-inhibitors like high amounts of culture yeast cells, type of yeast slurry, progress of the autolytic process, autolytic by-products like proteins, fatty acids etc. and sampling methods.

To find out the inhibitory influence of the high cell amount of culture yeasts, samples were spiked with *S. cerevisiae* var. *diastaticus* or *L. brevis* (serial dilutions from 1x10^2 to 1x10^9 cfu/ml) and diluted 1:5, 1:10, 1:20, 1:50 with sterile Ringer solution. Pure G-tank sample (G33, Table 1) and 1:20 dilution showed no positive PCR-amplification in the specific channel for *S. cerevisiae* var. *diastaticus*

Table 1 **Protein amounts in different yeast tank samples**

Sample	Protein mg/ml
G31 (yeast storage tank)	23.0
G33 (yeast storage tank)	59.0
G34 (yeast storage tank)	22.5
G35 (yeast storage tank)	26.2
G36 (yeast storage tank)	52.8
G37 (yeast storage tank)	23.2
D1 (pitchable yeast tank)	6.2
D2 (pitchable yeast tank)	6.2
P3 (yeast propagators)	24.4
P4 (yeast propagators)	45.2
P5 (yeast propagators)	49.2

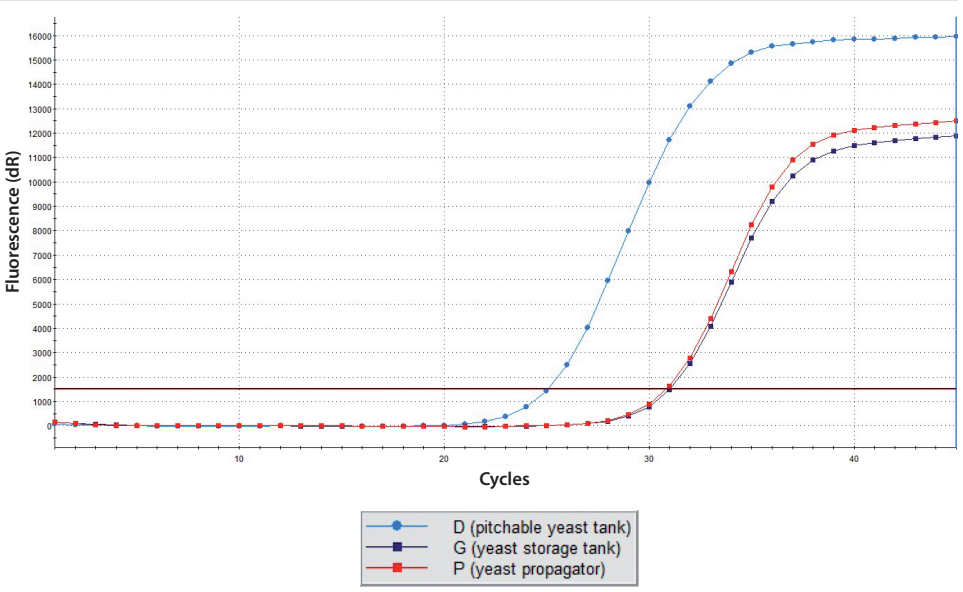

Fig. 2 **Correlation between protein amount of the sample and PCR amplification of *S. cerevisiae* var. *diastaticus*. The samples contain the following protein amounts: D1: 6.2 mg/ml, G35: 26 mg/ml, P5: 49.2 mg/ml**

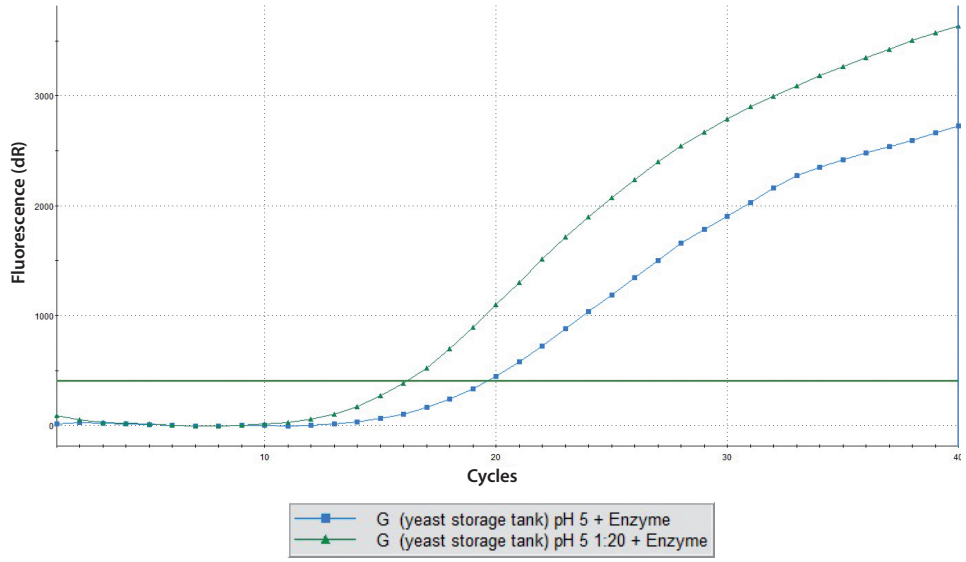

Fig. 3 **PCR amplification of 10^9 cfu/ml *S. cerevisiae* var. *diastaticus* cells (5×10^7 in a 1:20 dilution) in 10^{12} cfu/ml culture yeast cells of G-tank G33 (5×10^{10} in a 1:20 dilution)**

(Fig. 1). Dependent on the type of yeast sample D (pitchable yeast tanks), G (yeast storage tanks) or P (yeast propagators), the PCR-assays showed different PCR-results. These results showed, that not only the yeast cell amounts were responsible for the invalid PCR-results (Fig. 1), other chemical ingredients e.g proteins seem to play an additional role in PCR.

Proteins as degradation products during autolysis have been identified in this study as the main factor for real-time PCR-inhibition in yeast slurries. The protein amounts in authentic yeast slurry samples vary between 5–60 mg/ml (Table 1). Some factors which may cause such variations are the kind of sampling, harvest time and repitching. By inoculating the samples with dilutions of *S. cerevisiae* var. *diastaticus* cells a correlation between protein amount, matrix and PCR-inhibition could be shown (Table 1, Fig. 2). High amounts of proteins and matrices like G-tank lead to reduced PCR-sensitivity. Depending on the G-tank sample the PCR-result may be positive (Fig. 2, G35) or negative (Fig. 1, G33). Without removal of these components no positive PCR-result was obtained in the G-tank sample G33. For this purpose an enzymatic pretreatment of the sample is added. Different enzyme concentrations (5 U–20 U) and several incubation temperatures (37 °C–65 °C) were tested to get an optimized DNA-extraction protocol (see material and methods). Together with the sample dilutions which are in accordance to the yeast cell amount and type of culturing yeast, the enzymatic pretreatment showed significant improvements in QPP1SD-PCR (Fig. 3).

3.2 DNA extraction and real-time PCR (QPCR)

Currently no DNA extraction method is known that is suitable for a sensitive real-time PCR detection of microbial DNA in yeast slurries without preenrichment. Especially for top fermenting yeast slurry samples the enrichment of "*Saccharomyces* wild yeast" is an issue due to currently missing reliable selective preenrichment media.

Yeast slurries are known to contain substances that may be carried along with DNA through sample processing und inhibit the real-time PCR analysis [11]. The real-time PCR was found to be more sensitive to yeast slurry constituents than to wort or filtered beer [12].

In our study different DNA-extraction methods like column-based systems (Qiagen, Macherey-Nagel) or glass-beads were tested (data not shown). These methods suggest high sample dilutions up to 1:50 to minimize the culture yeast cell concentration associated with loss of sensitivity for bacterial and spoilage yeast DNA. They are time-consuming, labor-intensive and not suitable for brewery routine analysis. Other procedures use low speed-centrifugation of yeast cells prior to cell lysis to enhance PCR detection of bacterial contaminations. In this case flocculation in which microbial cells spontaneously aggregate together is a common phenomenon in microbiology. It has also been reported to occur between brewery contaminating bacteria and brewer`s yeast [13].

Additionally these methods ignore the strong influence of autolytic by-products which are the main reasons for inhibition and false negative PCR-results (Fig. 1, G33). This study revealed that not

only the amount of culture yeast cells but also autolytic by-products and the pH-value [10,14,15] of the samples are the main inhibitors for PCR, which have to be minimized before and/or during DNA-isolation. To overcome this inhibition process and loss of sensitivity, a new DNA-extraction method with an enzymatic pretreatment of the sample to remove autolytic by-products is developed and validated (CSY0100, GEN-IAL). Concentrations of culture yeast cells (10^5–10^{12} cfu/ml) in relation to *S. cerevisiae* var. *diastaticus* cells (10^2–10^9 cfu/ml) were tested concerning PCR-inhibition and detection limit (Table 2). It could be shown that the sample dilution factors correlate with the culture yeast cell amount and had to be adapted (Fig. 4, Table 2). For samples with culture yeasts >10^9 cfu/ml a dilution of 1:20 is recommended. Additionally a modified lysis buffer which captured out the inhibition components and buffered the raising pH value caused by autolysis was developed. Optimized incubation steps for an efficient cell lysis, lyticase treatment and evaluation of a special protocol for DNA-isolation of bacteria and yeast DNA in one assay was performed. For real-time PCR detection a multiplex PCR kit is used which detects the beer spoiling bacteria *Lactobacillus*, *Pediococcus*, *Pectinatus*, *Megasphaera* and the spoilage yeast *S. cerevisiae* var. *diastaticus* in one PCR-assay (QPP1SD, GEN-IAL). For differentiation, the species were detected in four fluorescence channels. For easier handling and much less contaminations by too much pipetting, cell lysis and real-time PCR occur simultaneously in the PCR cycler and the QPP1SD-PCR assay is ready-to-use freeze-dried in PCR-wells.

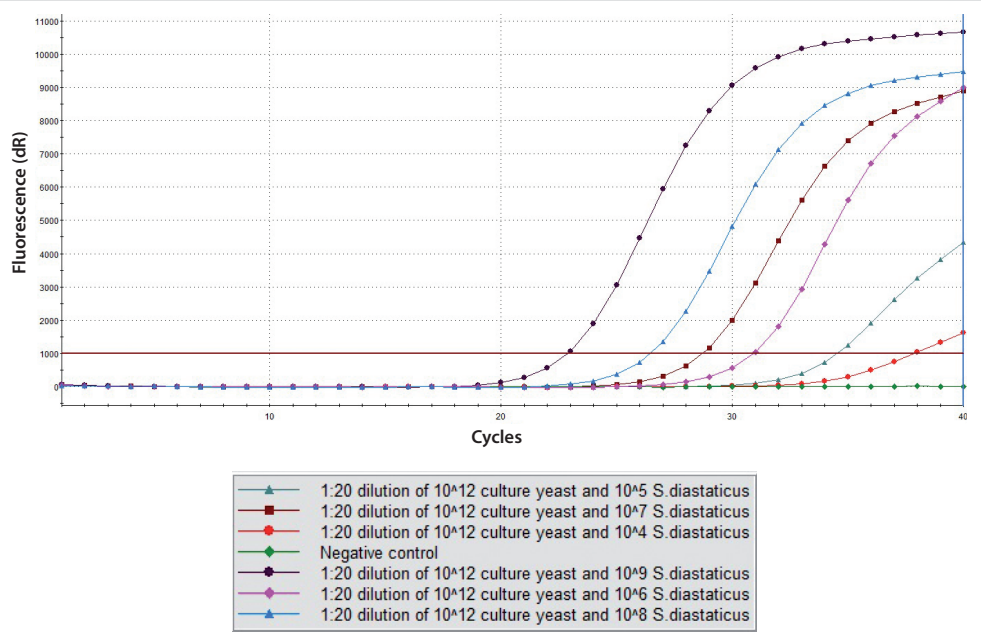

Fig. 4 **Effect of culture yeast cell concentration of 10^{12} cfu /ml on *S. cerevisiae* var. *diastaticus* PCR detection limit (QPP1SD-PCR)**

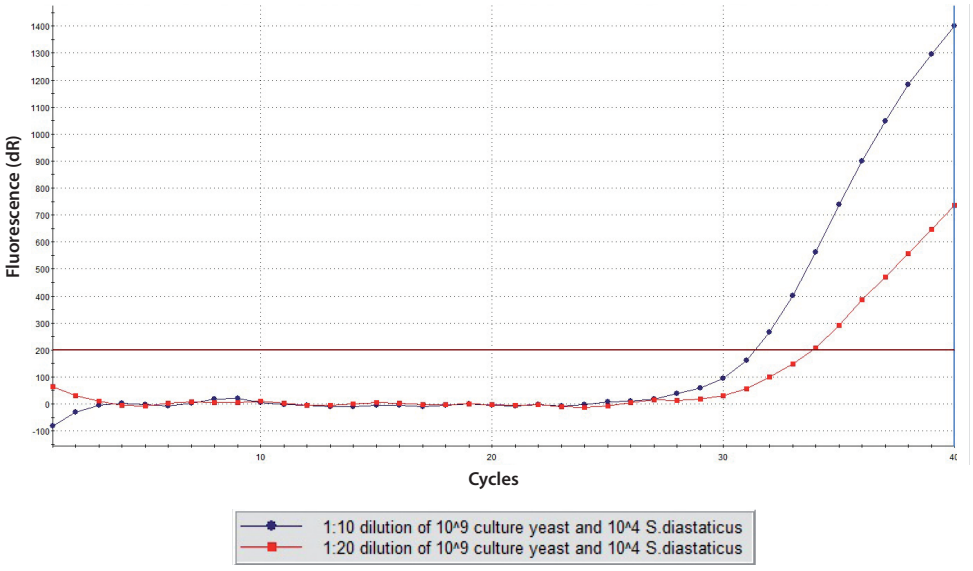

Fig. 5 **Influence of the dilution factor on PCR amplification of *S. cerevisiae* var. *diastaticus* (10^4 cfu/ml) according to culture yeast cell concentration in the sample. The 1:10 dilution is more efficient for 10^9 cfu/ml culture yeast cells than 1:20**

To determine the sensitivity of the modified DNA-extraction method (CSY100) combined with the multiplex PCR system QPP1SD, yeast slurry samples (10^7–10^{12} cfu/ml culture yeast) were artificially contaminated with serially diluted *S. cerevisiae* var. *diastaticus* and *L. brevis* cells ($1x10^1$–$1x10^{10}$ cfu/ml). The detection limit on cfu-level is 5×10^2–1×10^3 cfu/ml according to species, matrix (G, D or P tank), culture yeast cell counts and sample dilution factors (Fig. 4, Table 2). Depending on the culture yeast cell concentration the sample dilution factor plays an important role. A dilution of 1:20 is recommended for culture yeast cell concentrations higher than 10^9 (Fig. 5). PCR with dilution series (triple) on several days on different PCR-machines shows high reproducibility of the QPP1SD PCR-assay.

Table 2 **Detection limit of *S. cerevisiae* var. *diastaticus* real-time PCR according to culture yeast cell concentration**

Culture yeast (cfu/ml)	*S. cerevisiae* var. *diastaticus* Detection limit (QPP1SD-PCR, cfu/ml)
10^{12}	10^5
10^{11}	10^4
10^{10}	10^3
10^9	10^3
10^5–10^8	10^2 (data not shown)

4 Conclusion/Summary

In this study a reliable DNA-extraction method (CSY0100) for the isolation of *S. cerevisiae* var. *diastaticus* and *Lactobacillus brevis* in yeast slurries up to a culture yeast cell count of 10^{12} cfu/ml was developed and validated. It could be shown, that high culture yeast cell counts and autolytic by-products in the yeast samples lead to false negative real-time PCR-results due to inhibition. Enzymatic pretreatment, adapted dilutions of the yeast sample according to cell count and tank and a modified cell lysis buffer overcomes the inhibition and leads to QPP1SD-PCR detection limits of e.g.10^3 cfu/ml *S. cerevisiae* var. *diastaticus* in 10^9 cfu/ml culture yeasts without preenrichment. For detection limits < 10^3 cfu/ml *S. cerevisiae* var. *diastaticus* preenrichment in appropriate media is recommended.

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