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Enhanced Cultivation of Beer Spoilage Bacteria in Propagation Yeast by Enforced Yeast Suppresion

It is enormously important to be able to detect beer spoilage bacteria during the brewing process. These bacteria have the potential, for instance, to cause turbidity, acidity, and detrimental flavor changes in the product. It is extremely difficult to detect these beer spoilage organisms, in particular in the pure yeast culture or even in the yeast crop, as their growth is suppressed by the yeast. Furthermore, these bacteria are often only present as trace contaminants. In this study, a method was developed to more reliably and more quickly detect beer spoilage bacteria in pure yeast cultures than by previous methods. A natural antibiotic called Natamax® (Danisco, Niebull, Germany), which reliably kills the yeast cells but does not affect the bacteria, was added to the yeast samples. This made it possible to detect a significantly higher quantity of beer spoilage organisms than is possible without adding this antibiotic.

Descriptors: beer spoilage bacteria, lactic acid bacteria, Saccharomyces cerevisiae, yeast, natamycin, Natamax®

1 Introduction

Quality assurance plays a decisive role in every brewery. Besides technical chemical parameters, the microbiological safety of the beer is critical. If the microbiological purity of the beer is not ensured, this can lead to negative aroma changes, haze, acidification, right through to consistency changes [2, 4, 10]. For this reason, breweries constantly endeavor to subject sensitive areas of production to regular microbiological testing by means of stage-by-stage controls. In the field of brewery microbiology, a distinction is made between bacteria that are optimally adapted to the beer environment and can multiple within it without adaptation (obligate beer spoilage bacteria), and bacteria that can proliferate only after a certain adaptation phase, or only in certain beer types with reduced selective properties (potential beer spoilage bacteria) [3].

Many breweries use the traditional method of applying the product samples, rinse water samples, or wipe samples to several different culture media and incubating these at optimum temperatures for bacteria (28 ± 1 °C) in order to concentrate any contaminating bacteria [11, 14]. Depending on the type of sample, this kind of testing can take between 7 and 21 days, sometimes even longer, until the number of contaminants reaches the relevant detection limit (microscope: 10^5 cells/mL; real-time PCR (polymerase chain reaction): 10^2–10^3 cells/mL) and a reliable result can be obtained.

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These days larger breweries in particular employ PCR-based methods that provide a fast and reliable means of detecting selected harmful bacteria at low concentrations.

Regardless of the detection method used, the detection of beer spoilage microorganisms in culture yeast poses particular problems for breweries. In thick yeast slurry, e.g. in propagation yeast and especially in the yeast crop, beer spoilage bacteria can "hide" and be extremely difficult to detect amid the high number of yeast cells (e.g. 10^2 cells/mL bacteria per 10^8 cells/mL yeast). Vital yeast also suppresses the growth of bacteria, so the essentially small number of bacteria present in the sample will increase very little in the presence of the yeast cells, even under optimum conditions. This is why several growth media (for example NBB media (Doehler)) contain a yeast-repressing adding. However, the detection of beer spoiling bacteria remains difficult. Furthermore, another difficulty with yeast samples is that the contaminating bacteria cannot be mechanically concentrated, as is possible, for example, for bright beer tank samples using a membrane filter. Repeated incubation in specific culture media is therefore the only option available to conventional brewery microbiology. It is also not always possible to reliably detect beer spoilage bacteria using modern molecular biological methods as the necessary detection limits cannot be reached due to the suppressive effect of the brewing yeast.

The natural antibiotic Natamax® from Danisco is used to specifically kill yeast cells [7]. Natamax® contains natamycin, which is produced during fermentation by the bacterium *Streptomyces natalensis* and mixed with lactose. This agent is typically used in cheese manufacture or for other non-sterile foods where it provides a reliable means of killing undesirable yeasts and molds [1]. It is also used in other areas of the food industry such as in the production of fish and meat products. Natamycin inhibits endocytosis (the active uptake of membrane vesicles into the cell) in yeast cells by binding to ergosterol [9, 12, 17]. In contrast to other fungicidal agents such as filipin and nystatin, natamycin does not increase the permeability of the cytoplasmic membrane [17, 20]. Natamycin prevents the growth of yeasts and molds and causes these cells to die, though not due to the leakage of vital cell components as a result of an increase in membrane permeability. The precise mode of action of so-called polyene antimycotics is not yet fully understood but has been extensively discussed [6, 8, 12, 13, 16-20]. The growth of lactic acid bacteria (LAB) is not affected by the addition of natamycin [15].

Table 1: Yeast and bacterial strains used

Microorganism	Characterization	Culture collection number
Lactobacillus backi	Obligate beer-spoiling	TUM BP 120123021-2124
Lactobacillus brevis	Obligate beer-spoiling	TUM BP 120711011-2578
Lactobacillus casei	Potential beer-spoiling	TUM BP 120509129-2360
Lactobacillus harbinensis	Potential beer-spoiling	TUM BP 120906016-2993
Lactobacillus lindneri	Obligate beer-spoiling	TUM BP 120703011-2512
Pediococcus damnosus	Obligate beer-spoiling	TUM BP 121012022-3041
Saccharomyces pastorianus	Bottom-fermenting beer yeast	TUM 34/70

The aim of this study was to develop a method of reliably killing yeast cells in high concentrations without, however, adversely affecting the growth of beer spoilage bacteria to improve and speed up the their detection in yeast samples. Even if the subsequent enrichment medium contains a yeast-suppressing adding, the guarantee of 100 % dead yeast cells in a short time is advantageous for a secure identification. The objective was to ensure that the growth of the bacteria starts significantly earlier thanks to reduced competition from the yeast cells. Various test approaches comprising both culture and molecular biological methods were used to verify whether the detection of beer spoilage bacteria in yeast containing samples can be accelerated by adding Natamax®. Any time saving achieved would provide a practical benefit to the brewing microbiology lab and, ultimately, the brewery commissioning the testing who will receive the results more quickly and be able to react accordingly.

2 Material and Methods

2.1 Yeast and bacterial strains

The most frequently used bottom-fermented yeast *Saccharomyces* (S.) *pastorianus* TUM 34/70 and six different beer spoilage bacteria (4 obligate beer spoiling; 2 potentially beer spoiling) of the genera *Lactobacillus* (L.) and *Pediococcus* (P.) were used (Tab. 1). The yeast was obtained from the Yeast Center at the Research Center Weihenstephan for Brewing and Food Quality, where they are routinely checked for the presence of microorganisms using molecular biological methods. All bacterial strains used were isolated from beer samples, separated, identified using molecular biological methods, and preserved in a strain collection at – 80 °C in a cryobank.

After cultivating the yeast and bacterial strains in suitable culture media at 28 °C, isolation streaks of all the cultures were prepared and 10 individual colonies from each were combined to produce corresponding initial suspensions. These were tested for contamination using real-time PCR (see 2.4).

2.2 Cell count determination

The cell count determination was performed in triplicate and using a Thoma counting chamber (Bast, 1999). For each test, sterile wort was used to adjust the yeast cell count to 10^8 cells/mL, which is comparable to the cell count in a sample of thick yeast slurry. After counting, the bacterial suspensions were adjusted to the desired cell count (10^1, 10^2, 10^3, etc. cells/mL) using a 2 % saline solution.

2.3 Culture methods

Culture methods in brewery microbiology employ a variety of enrichment media that are tailored to the growth requirements of the type of microorganism being cultivated. Bottom- and top-fermenting brewing yeast is particularly well adapted to the wort medium, which is why liquid cultures are grown in sterile wort. To prepare the agar plates, 2 % agar-agar, and tetracycline to kill off undesired bacteria, were added to wort (wort agar).

For the beer spoilage bacteria, a detection medium was used that fulfils the most important nutrient and growth requirements of this small group of bacteria that is well-adapted to the beer environment (NBB®, detection medium for beer spoilage bacteria, Döhler). NBB® broth was used for liquid enrichment, and NBB® agar for quantitative analyses. The growth of the yeast cells was investigated in an aerobic atmosphere, and the growth of the bacteria in an anaerobic atmosphere, in each case at 28 °C.

2.4 Molecular biological methods

Extraction was performed using the InstaGene™ Matrix (Bio-Rad) as per the manufacturer's instructions for gram-positive microorganisms. The sample volumes were 1 mL for liquid samples, and 1 colony for agar plate samples. Identification and quantification of the bacteria was performed using the LightCycler® 480 II (Roche) and foodproof® beer screening kits (Biotecon Diagnostics). The beer spoilage bacteria were identified on the basis of a melting curve analysis. The quantity of DNA present in the sample was determined by means of the so-called cycle threshold (Ct) value, which identifies the point at which exponential growth of the PCR products begins. The lower this Ct value, the more DNA was present initially.

2.5 Experimental

Determination of the exposure time

In a triplicate series of tests, 50 ml of yeast suspension (10^8 cells/mL) was added to an Erlenmeyer flask and mixed with *L. brevis* (10^2, 10^3 and 10^4 cells/mL) and 5 g/L Natamax®. A parallel sample without Natamax® was prepared. The samples were stored in an aerobic atmosphere at 20 °C.

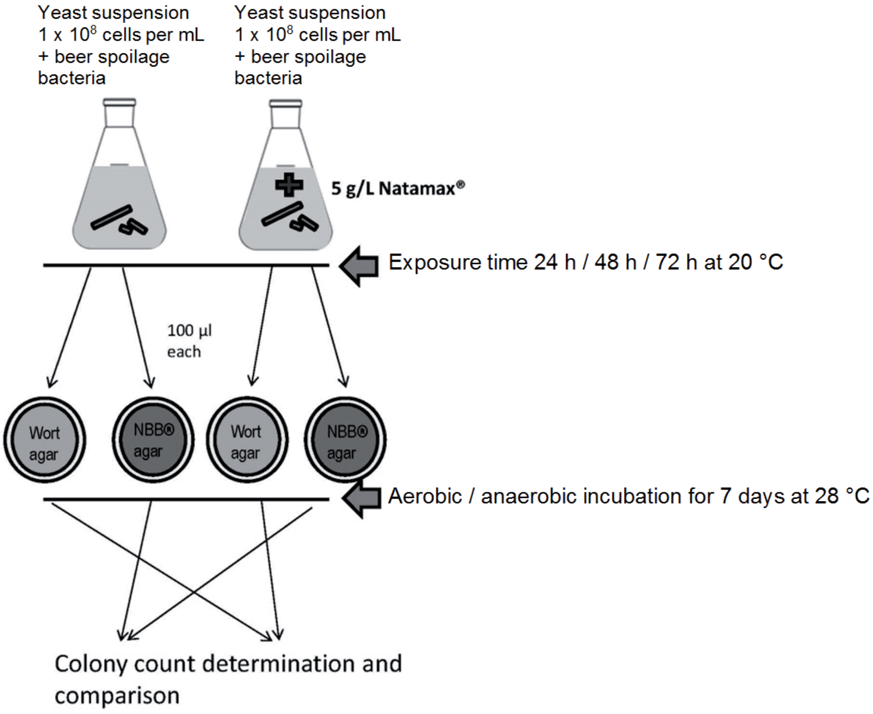

Fig. 1 **Schematic drawing of cultural approach**

After 24 h, 48 h and 72 h, 100 µl of each test solution was smeared onto wort agar and NBB® agar using a spatula. The wort agar plates and NBB® agar plates were evaluated after 7 days of aerobic or anaerobic incubation at 28 °C. At the same time, the required exposure time for Natamax® to reliably kill the 10^8 cells/mL of yeast cells without reducing the bacterial cell count was also compared.

Culture method

Six different strains of beer spoilage bacteria were inoculated in yeast suspensions with cell counts of 10^1 and 10^3 cells/mL in each case with and without 5 g/L antimycotic. An exposure time of 48 h was selected. 100 µL of each suspension was then smeared onto wort agar to determine the yeast concentration, and 100 µL of each suspension as well as 2 dilutions (1:10 with sterile water) onto NBB® agar to determine the bacterial concentration. The agar plates were then incubated aerobically (for wort agar) and anaerobically (for NBB® agar) for 7 days at 28 °C. If the addition of the antimycotic suppresses the growth of the yeast cells and promotes the growth of the inoculated bacteria, the bacterial cell count in the suspension with added Natamax® ought be higher than in the comparison sample, and the yeast cell count lower (Fig. 1).

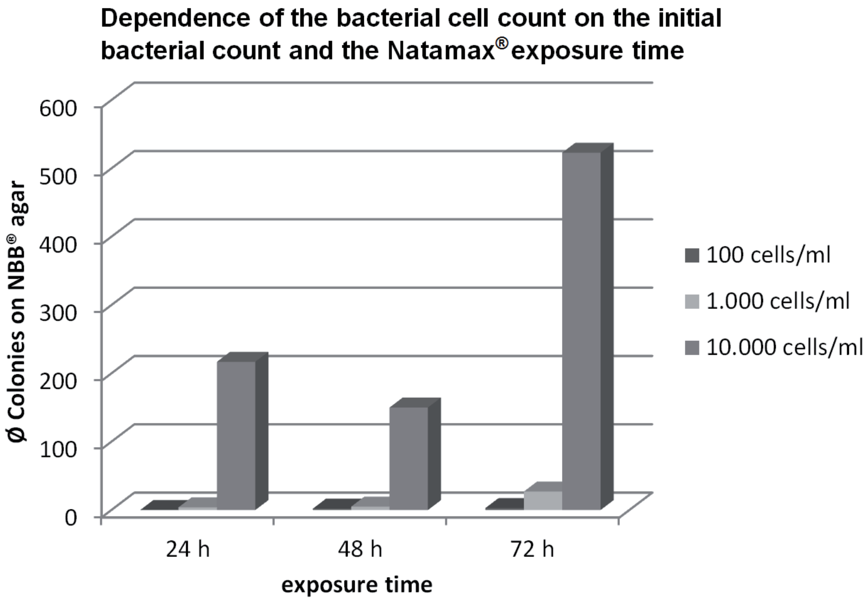

Fig. 2 **Change in cell count of L. brevis depending on the initial bacterial count and exposure time to Natamax®**

PCR method

As the PCR method is often used nowadays in brewery microbiology to test for the absence of bacteria in product, a further real-time PCR-based test was performed in this study. Instead of smearing the bacteria/yeast suspension onto culture mediums after the exposure time, 1 mL of each sample underwent molecular biological analysis. Comparing the Ct values provides information on the growth of the used bacteria.

3 Results and Discussion

Effect of exposure time

Following incubation on wort agar, all samples to which no Natamax® had been added were overgrown with yeast, thus making it impossible to determine specific cell counts. As a result of adding 5 g/L of the antibiotic and after an exposure time of just 24 h, no yeast colonies were identifiable after aerobic incubation on wort agar.

To detect the beer spoilage bacteria, 100 µl was taken from the same samples after 24 h, 48 h and 72 h, smeared onto NBB® agar, and incubated under anaerobic conditions at 28 °C (Fig. 2).

As the exposure time to the antimycotic increases, so too does the number of colonies on the NBB® agar. An increase in colonies depending on the initial bacterial count used was also observed. The low recovery rate (e.g. initial bacterial count: 10^4 bacteria/mL; bacterial count after 48 h: 150 colonies / 100 µL = 1.5×10^3 cells/mL) is explained by the exposure of the bacterial cells to stress, triggered by the yeast-containing medium, and by the fact that one colony on agar does not necessarily correspond to one cell, but usually several cells in a conglomerate.

While the initial bacterial count of 10^4 cells/mL gave the best results, this scenario is not representative, however, of the problem to be solved in practice. The addition of antibiotic is intended, in particular, to provide a faster and more reliable method of detecting trace contaminants, so bacterial cell counts of 10^1 and 10^3 cells/mL were selected in subsequent tests.

Test approach using cultures

With an initial cell count of 10^3 cells/mL, no significant increase in cell count as a result of adding the antimycotic could be determined for the *L. brevis* bacterium as the agar plates were completely overgrown in both preparations. *L. casei*, on the other hand, could no longer be cultivated in either test preparation (with or without Natamax®) after a 48 hour exposure time. For the four other species, a significant increase in the bacterial counts could be achieved by adding Natamax® (Fig. 3).

When using only 10 cells/mL, *L. brevis* again grew the fastest and overgrew the corresponding agar plates in both the preparations with and without the addition of Natamax®. *L. casei* and *P. damnosus* could not be cultivated under the selected conditions. The final bacterial count of *L. backi* was not significantly different for the preparations with or without antibiotic. A significant increase in cell count was observed for the *L. harbinensis* and *L. lindneri* bacteria (Fig. 4).

Test approach using real-time PCR

Having demonstrated using the culture detection method that a higher bacterial count could be detected by adding Natamax®, the next step was to perform verification using PCR. Because conventional PCR systems require a minimum bacterial count of 100–1000 bacteria for reliable detection, and since it was necessary to maintain the same basic cultivation conditions as in the preceding tests, an initial bacterial count of 100 cells per milliliter was used. The Ct values obtained are shown in tables 2 and 3 (see page 146), where a Ct value of 40 represents the maximum possible Ct value for the PCR system used. A Ct value of 40 means that the quantity of DNA present in the sample is insufficient to be detected.

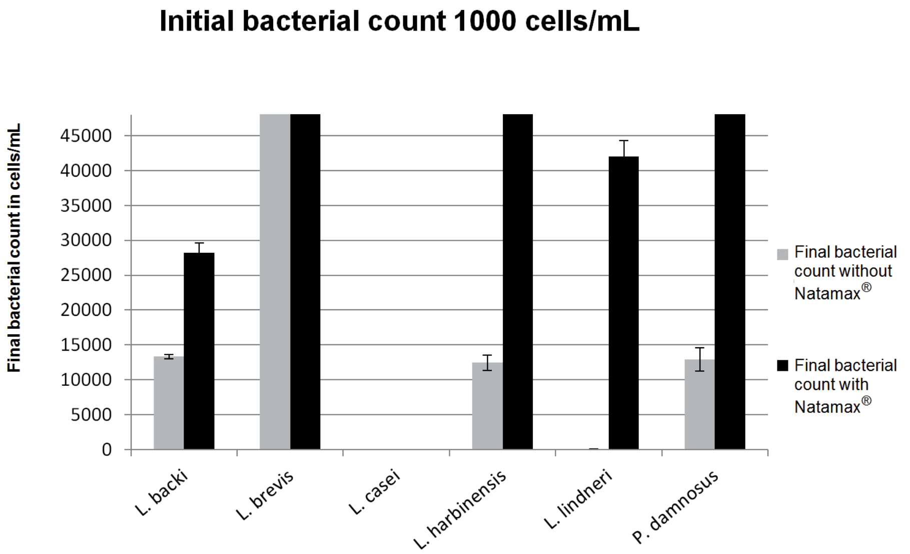

Fig. 3 Final bacterial count in the samples with and without the addition of Natamax® for an initial bacterial concentration of 103 cells/mL

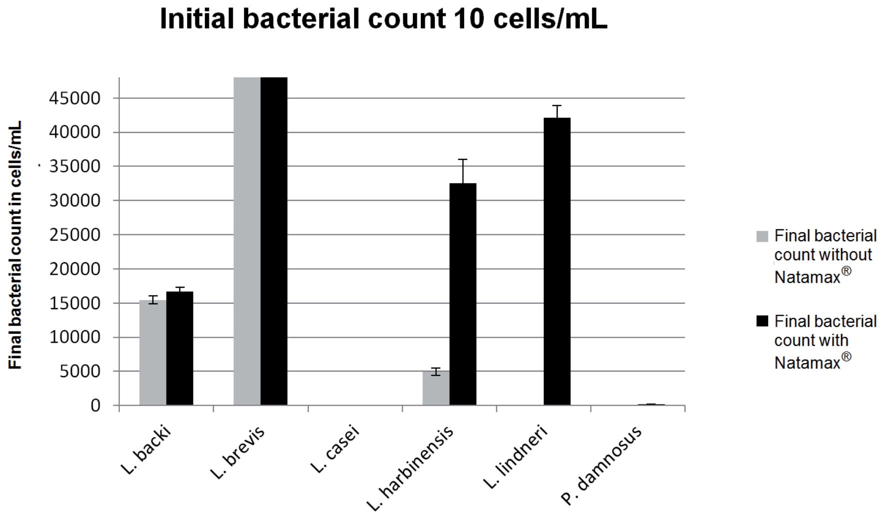

Fig. 4 Final bacterial count in the samples with and without the addition of Natamax® for an initial bacterial concentration of 101 cells/mL

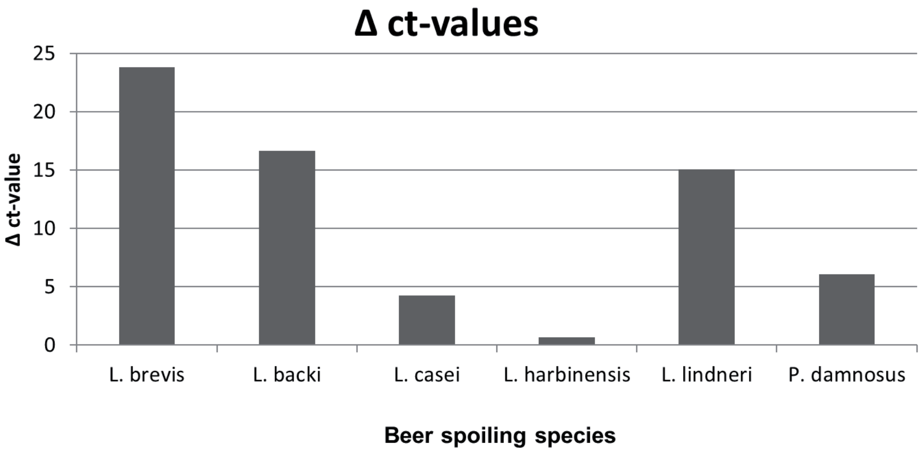

Fig. 5 Delta Ct values (= Ct value without Natamax® – Ct value with Natamax®)

Table 2 Ct values for the series of tests without the addition of Natamax®

Bacterial strain	Original cell amount [cells/mL]	Ct value 1 without Natamax®	Ct value 2 without Natamax®	Ct value 3 without Natamax®	Average Ct value	Standard deviation	Confidence interval
L. brevis	100	40.0	40.0	40.0	40.0	0.0	0.0
L. backi	100	33.2	31.2	40.0	34.8	3.8	4.3
L. casei	100	40.0	40.0	40.0	40.0	0.0	0.0
P. damnosus	100	40.0	40.0	31.1	37.0	4.2	4.8
L. harbinensis	100	32.7	31.8	32.3	32.3	0.4	0.5
L. lindneri	100	40.0	23.5	31.5	31.7	6.7	7.6

Table 3 Ct values for the series of tests with the addition of Natamax®

Bacterial strain	Original cell amount [cells/mL]	Ct value 1 with Natamax®	Ct value 2 with Natamax®	Ct value 3 with Natamax®	Average Ct value	Standard deviation	Confidence interval
L. brevis	100	15.5	16.5	16.6	16.2	0.5	0.6
L. backi	100	17.7	18.5	18.1	18.1	0.3	0.4
L. casei	100	40.0	34.0	33.2	35.7	3.0	3.4
P. damnosus	100	29.2	31.8	31.9	31.0	1.3	1.4
L. harbinensis	100	32.3	30.4	32.2	31.6	0.9	1.0
L. lindneri	100	16.8	16.7	16.5	16.7	0.1	0.2

As can be seen from tables 2 and 3 and figure 5, the results from the tests using culture enrichment (Fig. 3 and 4) could be verified. The DNA of all beer spoilage organisms tested could be detected in higher concentrations by adding Natamax®. All Ct values were lower than for the test samples with no antibiotic added to them. While the Ct values for the *L. casei*, *L. harbinensis* and *P. damnosus* samples were quite high, they were still within the detectable range. This was not possible without adding Natamax®.

4 Conclusion

As a result of using the natamycin-containing antibiotic, the cultured yeast was able to be reliably killed at a concentration of 10^8 cells/mL after 48 h. The addition of Natamax® proofed to be a practical tool for a secured and quicker identification of beer-spoiling bacteria in yeast samples. Especially the addition before the cultivation in a growth medium can speed up the identification of bacteria since 100 % dead yeast cells can be guaranteed. The growth of the most relevant beer spoilage organisms was not affected by the addition of Natamax® (Fig. 3). A contact time of 24 h with Natamax® led to a significantly higher number of detectable beer spoilage bacteria on agar plates. These results could be confirmed in PCR tests. Why some *Lactobacillus* strains were not detected in significantly higher number under the natamycin influence should be investigated in further studies. Also, a broader range of beer spoiling species and strains could be evaluated. However, adding 5 g/L Natamax® to conventional yeast samples enables trace contaminants to be detected more quickly and reliably. This does not impose any significant additional effort in everyday brewery laboratory practice, but leads to an optimized quality assurance process.

5 Notes

The authors declare no competing financial interest.

6 Literature

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