Simple Rapid Test for Establishing the Presence of Beer-Spoilage Organisms

VALUABLE TOOL | Constant monitoring of cleanliness of the operation is routine in a brewery. Microbiological checks are an important part of hygiene monitoring. Swab samples from plants and equipment and their surroundings were cultivated. Depending on microorganism exposure, these exhibit hazes or colour changes in culture media at various rates. Additional analyses of these samples require a lot of experience and are often imprecise. A rapid test detecting PCR products on a test strip could be useful in evaluating difficult samples. This test strip might work in a similar way to a pregnancy test strip.

MONITORING THE GENERAL STATE of hygiene of the whole filling plant and its surroundings is one of many microbiological standard analyses. Samples are taken with sterile swabs at various locations, particularly at critical ones, at predetermined time intervals. These are subsequently incubated at 27±1 °C in a non-selective nutrient medium. In most cases, this is NBB-B-AM (Döhler GmbH, Darmstadt). Due to the relatively low selectivity of the medium, trace contaminations of all sorts of bacteria and yeasts in the culture can be determined. A wide spectrum of organisms is thus found in the enrichments. Under a microscope, even for very experienced laboratory staff, identifications from the enrichments are possible to a limited degree only as this method is often not precise in view of biological diversity in such a sample.

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Further selective cultivation is also a possibility for narrowing down spoilage organisms but costs again valuable time and is thus impracticable in day-to-day operation. In other words, this type of hygiene monitoring provides information about microorganism exposure at certain locations on equipment, but an exact status of organisms enriched or whether they can spoil the product involves a lot of effort and expense when following the conventional process.

Biomolecular detection systems would be another option for further analyses. PCR (polymerase chain reaction) in particular can be a very useful tool. This reaction is a very accurate and sensitive method for determining specific organisms/groups of organisms or even organism-specific properties.

In terms of the hygiene monitoring process presented here, a PCR-based screening test for beer-spoilage *Lactobacillus sp.* and *Pediococcus sp.* would make sense because numerous microorganisms of these genera have indeed a product-spoiling potential and can establish themselves in biofilms relatively early on.

OVERVIEW OF ESTABLISHED DETECTION SYSTEMS*

Detection	detected are among others ...
BS** *Lactobacillus sp.* - *Pediococcus sp.*-screening	*L. brevis, L. casei, L. backi, L. paracollinoides, L. lindneri, P. damnosus, L. rossiae*
Megasphaera sp. - *Pectinatus sp.* -screening	*M. cerevisiae, M. sueciensis, P. cerevisiiphilus, P. frisingensis, P. haikarae*
Hop resistance screening	mainly hop-tolerant *Lactobacillus sp.* and *Pediococcus* sp.
Lactobacillus brevis	*Lactobacillus brevis*

*Detection systems used in this study are highlighted in light brown. Further species-specific detections are on the agenda (L. lindneri, L. backi, L. casei, L. rossiae, P. damnosus).
** BS = beer spoilers; Sauergut bacteria such as e.g. L. delbrueckii and L. amylolyticus that are not beer spoilers are not picked up.

Table 1

Case Study

In March 2015, the microbiological status of all plants and equipment in a partner brewery which had considerable problems with beer-spoiling bacteria in 2014 was analysed by the Weihenstephan Research Centre for Brewing and Food Quality at the TU Munich (TUM FZW BLQ). More than 100 swabs were taken at potentially critical locations at predefined time intervals and subsequently analysed in two nutrient media, NBB-B-AM and non-alcoholic wheat beer (micro inoculum broth) + cysteine hydrochloride (acronym: AfWB+MIB). When colour had changed or haze was noted in the enrichments after three days, the particular sample was analysed both under the microscope and using biomolecular methods at the Research Centre. The PCR-based control method is a published real time PCR system according to Brandl [1] used for carrying out group detection (screening) for beer-spoilage *Lactobacillus sp.* and *Pediococcus sp.*

This real time PCR control assay according to Brandl is designated as sLP600. Various real time screening systems for beer-spoilage bacteria are commercially available. Some systems make it possible to also identify individual species. This is generally done using melting curve analysis and/or multiplex genotyping in various fluorescence channels. In this study, the screening system according to Brandl referred to was used as control, without complex melting curve analysis.

Together with the control system, potentially critical, blinded samples were analysed for the presence of *Lactobacillus sp.* and *Pediococcus sp.* using the Milenia Biotec PCR rapid test system. This test system also includes a PCR that is subsequently evaluated in a simple and rapid test using an antibody-based test strip.

In this case study, a second screening test was run, in addition to the screening test for beer-spoilage *Lactobacillus sp.* and *Pediococcus sp.* In this second test it was determined whether the microorganisms have the ability to tolerate hop bitter substances. When microorganisms tested have the required set of genes to remove hop bitter substances effectively from the cell, this is a compelling indicator for the ability of microorganisms to be able to indeed grow in beer. This test thus indicates the product-spoiling potential of the organism found. Fig. 1 is an overview of detection systems used in this study.

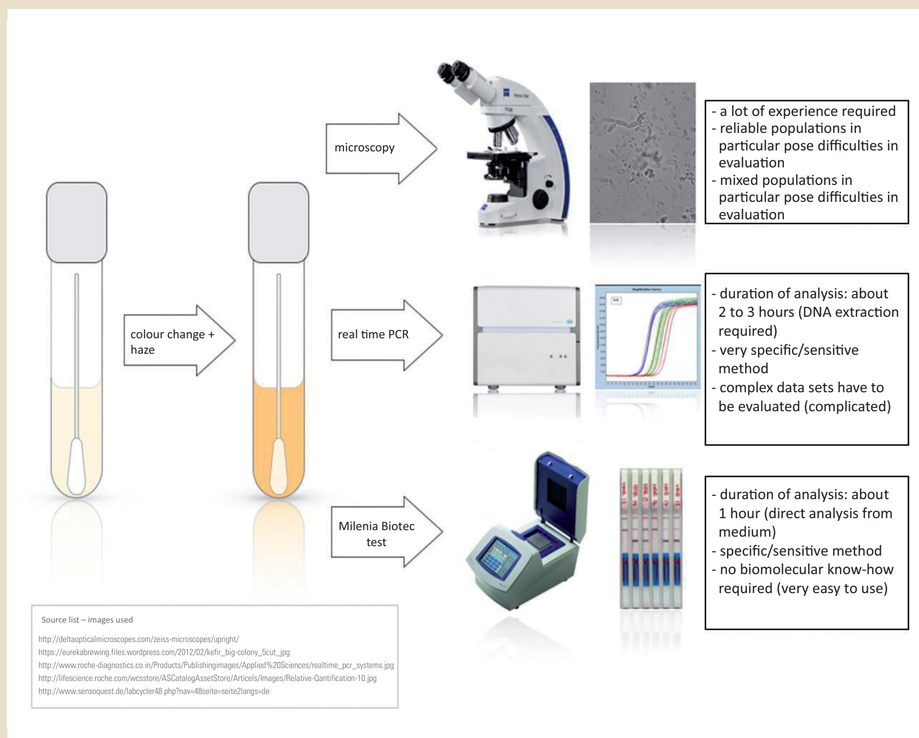

Fig. 1 Diagram of unit operations in the case study: swab samples were incubated on a non-selective nutrient medium; samples showing colour change or haze were examined under the microscope, analysed using RT-PCR and investigated together with the Milenia Biotec test

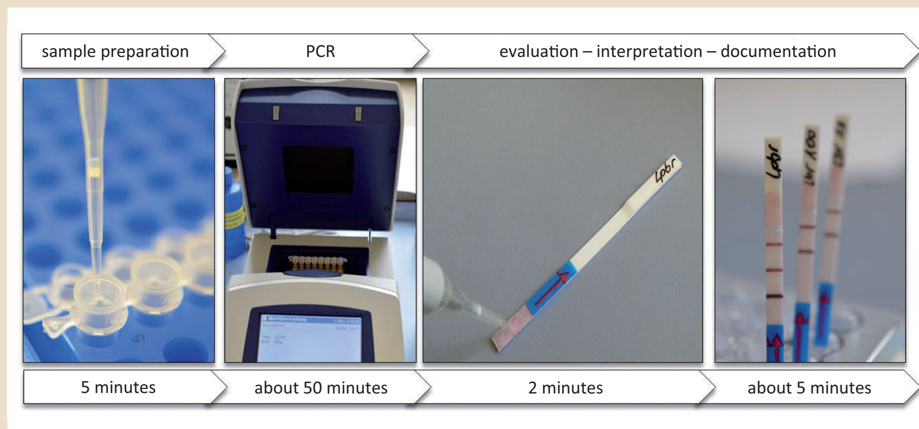

Fig. 2 Steps of the alternative rapid test comprise sample preparation, reaction in thermal cycler (PCR) and evaluation with test strips. Relevant samples can be screened in an overall analysis lasting about one hour

Combination of PCR and "Pregnancy Test"

Most commercially available PCR-based detection tests for beer spoilers require both biomolecular know-how and, generally, familiarity with PCR. For these mostly real-time based systems, a complex interpretation of melting curves is required in some instances in order to be able to reliably identify certain microorganisms. Many brewers are reluctant to acquire those systems as they are costly and the method is also complex.

In the context of a project funded by AiF (Arbeitsgemeinschaft industrieller Forschungsvereinigungen – The German Federation of Industrial Research Associations), Milenia Biotec GmbH of Giessen, in cooperation with the Research Centre for Brewing and Food Quality at the TU Munich, has investigated this topic in more detail. The target was to design a tool allowing SME brewers to have easy access to the possibilities offered by molecular biology. The primary objective was to assure easiest pos-

OVERVIEW OF SAMPLES ANALYSED IN *LACTOBACILLUS* AND *PEDIOCOCCUS* SCREENING*

Results of analyses TUM FZW BLQ

No.	Microscopy	Real time PCR (sLP600, Brandl 2006)	Milenia test
54	lactic acid bacteria, short rods, yeast	–	–
55	lactic acid bacteria, yeast, mould fungus	+	+
57	short/long rods, yeast	+	+
58	yeast	–	–
59	yeast	–	–
60	short rods, yeast	–	+
79	short/long rods, yeast	–	–
80	short/long rods	–	–
82	short/long rods, partly motile	+	+
91	lactic acid bacteria, short/long rods, mould fungus	+	+
92	short rods, partly motile, yeast fungus	–	–
93	short/long rods, partly motile, yeast	+	+
95	short/long rods	–	–
96	short/long rods, little yeast	–	–
97	short/long rods, yeast, mould fungus	–	–
103	lactic acid bacteria, short/long rods, partly motile, yeast	+	+
104	short/long rods, yeast	–	–
105	short/long rods, yeast	+	+
6	little yeast	–	–
30	yeast	–	–
32	yeast	–	–
33	yeast	–	–
34	yeast	–	–
35	short rods	–	–
38	yeast	–	–
40	yeast	–	–
49	yeast	–	–
51	yeast	–	+
53	yeast	–	–
64	yeast	–	–
74	short/long rods, partly motile	+	+
75	short/long rods	–	–
76	short/long rods, partly motile	–	–
78	short/long rods	–	–
81	short/long rods, yeast	–	–
83	short/long rods, partly motile	+	+
84	short/long rods	–	+
85	lactic acid bacteria, short/long rods, yeast	+	+
86	short/long rods	–	–
87	short/long rods, partly motile, yeast	+	+
88	short/long rods, partly motile, yeast	–	–
89	lactic acid bacteria, short/long rods, partly motile, yeast	+	+
90	lactic acid bacteria, short/long rods, partly motile, yeast, mould fungus	+	+
98	short rods, mould fungus	–	–
100	short rods	–	–
101	short/long rods	+	+
102	lactic acid bacteria, short/long rods, partly motile, yeast	+	+
106	short rods, partly motile	–	–
107	short rods, yeast	–	–
108	short rods, yeast	+	+

**sample designation anonymised by number code: results of the Milenia PCR rapid test are highlighted in light brown. Results deviating from real time PCR (sLP600, Brandl 2006) are highlighted in dark brown. CT value = Threshold Cycle and defines the exact point in time (cycle) when amplification of the target sequence goes into the exponential phase in the RT-PCR*

Table 2

OVERVIEW OF SAMPLES ANALYSED IN HOP RESISTANCE SCREENING*

	Results of analyses TUM FZW BLQ (presence of Hor A or Hor C gene)		
No.	**Microscopy**	**Real time PCR**	**Milenia test**
55	lactic acid bacteria, yeast, mould fungus	-	-
57	short/long rods, yeast	+	+
82	short/long rods, partly motile	-	-
91	lactic acid bacteria, short/long rods, mould fungus	+	+
93	short/long rods, partly motile, yeast	+	+
103	lactic acid bacteria, short/long rods, partly motile, yeast	+	+
105	lactic acid bacteria, short/long rods, yeast	+	+
51	yeast	-	-
74	short/long rods, partly motile	-	-
83	short/long rods, partly motile	-	-
85	lactic acid bacteria, short/long rods, yeast	+	+
87	short/long rods, partly motile, yeast	+	+
89	lactic acid bacteria, short/long rods, partly motile, yeast	+	+
90	lactic acid bacteria, short/long rods, partly motile, yeast, mould fungus	+	+
101	short/long rods	-	-
102	lactic acid bacteria, short/long rods, partly motile, yeast	+	+
108	short rods, yeast	-	-

** sample designation anonymised by number code: results of the Milenia PCR rapid test are highlighted in light brown*

Table 3

sible handling in line with ease of handling a pregnancy test in a broader sense.

How Does the Test Work?

In all steps of the test, the objective of easy handling in all unit operations has to be assured. Even without having biomolecular know-how and without having background knowledge of "PCR", every user should be in a position to carry out and evaluate the test. The steps of detectability are thus very simple (Fig. 2).

In a first step, the reaction mixture is prepared. Two solutions are mixed at a defined ratio and subsequently transferred into reaction containers. The sample to be analysed is then added.

PCR proper takes place in a thermal cycler that is easy to operate. In contrast to established RT-PCR, fluorescences need not be read out when using this equipment. This is reflected in the purchase price. Evaluation can begin after about 60 minutes. Part of the sample is placed on the test strip that is subsequently placed vertically in a running buffer. Results can be read after five to ten minutes. Simply the presence of test lines will decide whether or not beer spoilers were found in the sample analysed.

All steps of the test are identical for all analyses. Only one of the starting solutions used at the beginning of the process is changed out. Table 1 provides a short overview of established test methods.

Analysis as such is based directly on the different media in most cases. DNA extraction is not required in contrast to established real-time PCR systems. If required, even yeast-containing samples can be analysed directly. In this study, direct analysis was done with NBB-B-AM and AfWB+MIB, a special nutrient medium developed by the BLQ Research Centre.

Results

Haze or change of colour was noted in the detection medium in about 50 of more than 100 swab samples. These 50 samples were analysed in more details. Both microscopic and the biomolecular tests described were carried out.

Cells were subdivided into yeasts, mould fungi, short and long rods, motile cells and lactic acid bacteria microscopically. Typical cell morphologies of *Lactobacillus sp.*, *Pediococcus sp.*, *Lactococcus sp.* and *Leuconostoc sp.* were lumped together as lactic acid bacteria. Short and/or long rods were found in 37 of the 50 samples. It would be difficult

RESULTS FROM THE THREE ABERRANT SAMPLES FROM *LACTOBACILLUS AND PEDIOCOCCUS*-SCREENING

No.	Microscopy	Sequencing
60	short rods, yeast	*L. harbinensis*
51	yeast	*L. rossiae*
84	short and long rods	*L. rossiae*

Table 4

to reliably identify the species or even the genus.

Real-time PCR detected unambiguous *Lactobacillus sp.* and *Pediococcus sp.* beer spoilers in 16 samples. On the other hand, the Milenia Biotec PCR rapid test found unambiguous *Lactobacillus sp.* and/or *Pediococcus sp.* beer spoilers in 19 samples. When comparing the two PCR methods, it can be seen that the "pregnancy test strip" also detected all 16 clearly positive results of RT-PCR and marked them positive. Table 2 provides information about *Lactobacillus* and *Pediococcus* detection.

It is very important that brewers know whether potential beer-spoilage *Lactobacillus* or *Pediococcus* species are present in a non-selective enrichment. However, it is in most cases not very important to know to which phylogenetic group (genus) an organism belongs to predict its product-spoiling capability.

The ability of an organism to tolerate hop bitter substances in its medium is a clearly better indicator of its beer-spoiling capability. Beer-spoiling bacteria need special genes in order to be able to effectively remove xenobiotics (foreign substances) effectively from their cells via special transport systems. This ability (among others) provides these microorganisms with the potential to adapt to the medium beer and even grow in it. In another screening test, coinciding positive findings from *Lactobacillus* and *Pediococcus* screening were additionally tested for the presence of hop resistance genes (HOR screening). Again, an in-house RT-PCR system developed at the BLQ Research Centre served as control. Table 3 summarises the results of these analyses. This investigation proved that there were no discrepancies between the two test systems. Ten of 17 samples analysed contained bacteria having corresponding hop resistance genes. In other words, organisms that could potentially spoil the final product were found at ten locations on the equipment.

Discrepancy Analysis

Compared to hop resistance screening, *Lactobacillus* and *Pediococcus* screening yielded a total of three aberrant results. Samples 60, 51 and 84 were identified as being positive in the Milenia Biotec PCR rapid test whereas they were not classified as positive in the control assay sL600 according to Brandl.

FOURFOLD TABLE – COMPARATIVE OVERVIEW OF THE RESULTS FROM LACTOBACILLUS AND PEDIOCOCCUS SCREENING AND FROM HOR SCREENING*

		Milenia (LB-PC screen)		**Sum**	
		+	-		
RT-PCR (sLP600, Brandl 2006)	+	16	0	16	
	-	3	31	34	Concordance index 0.91
Sum		19	31	50	
		Milenia (HOR)		**Sum**	
		+	-		
RT-PCR (TUM FZW BLQ Hor A or Hor C gene)	+	10	0	10	
	-	0	7	7	Concordance index 1
Sum		10	7	17	

**the concordance index calculated is also listed (0.81-1 corresponds to "almost exact agreement" between the method being established and the control method)*

Table 5

In view of the discrepancy, these samples were analysed further by sequencing PCR product from the Milenia Biotec PCR rapid test (Table 4 shows the results).

The table shows that the Milenia Biotec PCR rapid test did not generate false positive results. The species *Lactobacillus rossiae* (common organism in wheat beer) was detected in samples 51 and 84. *Lactobacillus harbinensis*, also a potential beer-spoiling lactic acid bacterium, was found in sample 60. These two organisms do not belong to the target spectrum of sLP600 screening because, when the test was developed, neither *L. rossiae* nor *L. harbinensis* were known to be potential beer spoilers.

Summary

Results obtained with the Milenia Biotec PCR rapid test were largely identical to those of control test systems. *Lactobacillus* and *Pediococcus* screening from Milenia Biotec could identify three additional samples as being contaminated compared to the corresponding control system. The detection method developed for classification of potential product spoilers using determining hop resistance genes and the control test yielded identical results. No discrepancies were found in 17 samples analysed (see Table 5).

The data generated shows that both *Lactobacillus* and *Pediococcus* screening as well as hop resistance screening are suitable alternative tools for detection of beer-spoilage microorganisms in hygiene samples. As this method is easy to handle and evaluate, it can open up the way also for smaller and medium-sized breweries to classify hygiene samples more accurately in terms of their spoiling potential. In summary, this study showed that PCR analysis can play an important part in hygiene management. The detection system presented in this article can assist microbiological step-by-step monitoring at many locations and offer smaller brewers an opportunity of being able to use molecular biology as a valuable routine tool.

A Word of Thanks

The project KF2271416MD3 was funded via AiF (Arbeitsgemeinschaft industrieller Forschungsvereinigungen – The German Federation of Industrial Research Associations) in the context of ZIM (The Central Innovation Programme for SMEs) by the German Federal Ministry of Economics and Energy, based on a decision by the German Federal Parliament.

We would also like to thank everyone who has been fund-aided by this project and who has made important contributions to project development: *Dr. Gertrud Eberle-Adamkiewicz*, *Susanne Mauracher*, *Lena Bender*, *Dr. Ralf Dostatni* (all Milenia Biotec, Giessen), Siglinde Huber (TUM FZW BLQ). ■

Source

1. Brandl, A:. "Entwicklung und Optimierung von PCR-Methoden zur Detektion und Identifizierung von brauereirelevanten Mikroorganismen zur Routine-Anwendung in Brauereien", Dissertation TU München, Freising-Weihenstephan, 2006.