Evaluation of Fluorescence-marked Gene Probes and Fourier Transform Infrared Spectroscopy as Novel Methods to Detect Beer Spoilage Bacteria

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Summary
A novel method using fluorescence-marked gene probes is introduced into beer analysis in the context of official food control. Two commercial test kits for the detection of Lactobacilli/Pediococci and *Megasphaera/ Pectinatus* beer spoilage bacteria were used and compared to classical plating techniques. The new method was found to be very sensitive and convenient for the identification and assessment of hygienic risks in beer samples from all stages of production. Besides that, Fourier transform infrared spectroscopy (FTIR) in combination with multivariate data analysis was evaluated to detect beer spoilage directly from the sample without prior inoculation. However, the correlations of the FTIR calibrations showed only low sensitivity so that this approach is not yet usable in food control.

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Zusammenfassung
In diesem Artikel wird eine neue Bestimmungsmethode mit fluoreszenzmarkierten Gensonden erstmals für die Bieranalyse im Rahmen der amtlichen Lebensmittelüberwachung beschrieben. Zwei kommerzielle Testkits zur Bestimmung von bierverderbenden Mikroorganismen der Gattungen *Lactobacillus/Pediococcus* und *Megasphaera/Pectinatus* wurden eingesetzt und mit den klassischen Platten-Techniken verglichen. Die neue Methode ist sehr empfindlich und zweckmäßig zur Identifizierung und Begutachtung von Hygienerisiken in Bierproben auf allen Herstellungsstufen. Daneben wurde Fourier-Transformations-Infrarot-Spektroskopie (FTIR) in Kombination mit multivariater Datenanalyse zum Nachweis von Bierschädlingen direkt aus der Probe ohne vorherige Anreicherung eingesetzt. Jedoch zeigten die Korrelationen der FTIR Kalibrierungen nur eine sehr geringe Detektionsempfindlichkeit, sodass diese Methode noch nicht in der Lebensmittelüberwachung einsetzbar ist.

Keywords: beer, microbiology, spoilage bacteria, DNA-hybridisation, Gene probe, FTIR, multivariate data analyis, PLS / Bier, Mikrobiologie, bierverderbende Bakterien, DNS-Hybridisierung, Gensonden, FTIR, multivariate Datenanalyse, PLS

1 Introduction

Sensory changes in beer, e.g. buttery aroma, rope, turbidity, fecal odour or even „rotten egg“ smell can be caused by various gram-positive and gram-negative bacteria[1–5]. Most frequently involved are lactic acid bacteria (*Lactobacillus* spp.), *Pectinatus* spp. and *Megasphaera* spp., the latter two being strictly anaerobic. In cases of beer spoilage, these bacteria are becoming more and more important due to improved technology that leads to a drastic reduction of the oxygen content in beer.

In official food control, as soon as sensory changes are eminent, the beer sample will be judged as „not fit for human consumption“. If it contains amounts of spoilage bacteria of over 10^5 cfu/ml, a sample will be considered as being produced under non-acceptable hygienic conditions (non-official limit agreed upon by food microbiologists in Baden-Württemberg, Germany). On the other hand, presence of *Escherichia coli* will not be tolerated, and coliforms in lower numbers will also be considered as not tolerable.

But it may be equally important to detect the presence of spoilage bacteria in lower counts, which have not yet produced sufficient amounts of metabolites to cause sensory change. Detection and identification may point to hygienic risks, sometimes down to the very stage of production where the problem is most likely to have arisen.

Our beer samples were taken in breweries – sometimes *after*, but frequently *before* bottling: during fermentation or ripening. Their continued „shelf life“ of weeks or even months demands timely detection. It may be instrumental in controlling hygiene status in the brewery. The monitored businesses are of small to medium size. Therefore they usually do not regularly analyse samples on their own behalf during or after the process – a cost-related problem. We also included a number of samples that were taken directly from the tap.

Besides the classical plating techniques, we evaluated two novel techniques in this study: fluorescence-marked gene probes using a commercially available test kit and Fourier transform infrared spectroscopy (FTIR).

FTIR in combination with multivariate data analysis (chemometrics) was previously described as capable to detect and identify bacteria in water, culture media and foods[6–10]. In our laboratory, FTIR is routinely used to screen alcoholic beverage samples for standard chemical parameters (e.g. alcoholic strength) in less than 2 minutes per sample[11–15]. We started the method development with the intent to gain microbiological parameters from the same spectra, meaning directly from the original beer sample without prior inoculation. In the current study, we compare for the first time results from classical microbiology with FTIR spectra using multivariate data analysis. Jack-Knifing[16] and genetic algorithms[17–19] were used to select specific wavelength ranges for groups of microorganisms that were used for subsequent Partial Least Squares (PLS) regression.

2 Materials and Methods

2.1 Fluorescence-Marked Gene Probes

From 2004 till 2006, a total of 124 tested beer samples were included in this study. A combination of classical microbiological plating methods (Tab. 1) and sensory inspection (turbidity, odour, taste) was used to screen samples for potential presence of spoilage bacteria. It was found that in every case of severe sensory change as well as in almost all cases of increased bacterial count on NBB- and/or MRS-medium the microorganisms concerned could be identified as typical beer spoilage bacteria by the so-called VIT-method (Vermicon Identification Technology). Already in use in the brewing industry[20], this method works on a DNA-hybridisation principle. Two kits exist: one for detection of Lactobacilli/*L. brevis* and Pediococci and another for the detection of *Megasphaera/Pectinatus* spp.

Tab. 1 Classic microbiological methods

Groups of microorganisms	Agar	Incubation
Total beer spoilage bacteria (gram positive and -negative)	Nutrient Beer Broth-Agar (NBB); poured-plate-technique	30 °C, 72–96 h, anaerob
Lactic acid bacteria	Lactobacillus-Agar after de Man, Rogosa and Sharpe (MRS); surface-plate-technique	25 °C, 72 h, anaerob
Coliforms and E. coli	Chromocult-Coliform-Agar (COFO); surface-plate-technique	37 °C, 24 h, aerob

Tab. 2 Results of beer analysis using Fluorescence-Marked Gene Probes

Spoilage bacteria	Beer samples tested positive	Frequency in beer samples* (total n = 124)
L. brevis	17	14%
Megasphaera spp.	23	19%
Pectinatus spp.	17	14%

* (due to a mix of spoilage bacteria in some samples, figures overlap)

The beer samples are placed on the slides supplied with the kit, the VIT-solutions are added, and the slide is incubated. The marked gene probes enter the bacteria and bind to their specific matching signatures in the genetic material of the cells. Following this, a washing step removes all unbound gene probes from the cells. Identification is achieved by fluorescent markers i.e. by visualisation under the specially adjusted microscope. The assay is very specific. It is possible to detect bacteria even if they are affected in such a way that classic cultural detection is not successful[20].

2.2 Fourier Transform Infrared Spectroscopy

To gain a sufficient data set for calibration of the analyzer in the range between 10^0 to 10^7 cfu/ml, we have inoculated beer samples with stock cultures of the different groups of microorganisms (n = 498). Simultaneously to the measurement of the inoculated material with FTIR, we conducted a sterile sampling of part of the material for cultural determination with classical microbiological plating techniques (Tab. 2). After incubation, the plates were analyzed and the bacterial counts determined.

Furthermore, the inoculated beer samples were incubated over night at different temperatures (+8 °C, +25 °C, +30 °C, +37 °C) and again measured using FTIR and classical microbiology.

The FTIR spectra were measured with the Winescan FT 120 (*Foss,* Hamburg, Germany). The instrumental details were previously described[11]. The spectra were preprocessed using multiplicative scatter correction (MSC). The partial least squares (PLS) regression including selection of significant wavelength using jack-knifing was conducted with the software package „The Unscrambler" V9.2 (*Camo Process AS*, Oslo, Norway). The validation was done using "full cross-validation" in every case.

Besides that, the software Winescan FT 120 (*Foss*, Hamburg, Germany) was used for wavelength selection (unknown algorithm by *Foss*). We also used genetic algorithms (GA) for wavelength selection with the software PLS Toolbox 3.5 (*Eigenvector Research*, Manson, USA) for Matlab 7.0 (*Mathworks*, Natick, USA). We also evaluated the spectra with support vector machines (SVM) using the software DTREG (*Phillip H. Sherrod*, Brentwood, USA) with the radial basis function (RBF).

3 Results and Discussion

3.1 Fluorescence-Marked Gene Probes

The assay using fluorescence marked gene probes is a commercially available test-kit, which we were able to use without problems in our laboratory. The results of 124 beer samples are shown in Table 2. It was confirmed that most of the isolated bacteria belonged to the lactic acid variety – Lactobacilli and Pediococci – with *Lactobacillus brevis* most prominently figuring among them. However, some samples showed a mix of gram-positive and gram-negative spoilage bacteria (gram-negative counts being mostly around 10^3 cfu/ml), and in one case a monoculture of *Megasphaera cerevisiae* could be found. The contamination, having occurred at the beginning of the bottling process, was confirmed by the brewery. This gram-negative, strictly anaerobic bacterium has been described by *Narziß*[21] as being more and more frequently identified as the cause of beer spoilage cases in recent years. *Sakamoto* and *Konings*[1] estimate that *Megasphaera* is responsible for 3 to 7% of all cases of beer spoilage. The detection of *Megasphaera* during the bottling process has been a singular experience for us, yet we have also found the bacterium in a beer sample that was taken directly from the tap and in a sample of bottled beer from a retailer.

3.2 FTIR

The results of PLS regression show that a correlation exists between bacteria counts determined with classical plating techniques and the FTIR spectra with correlation coefficients between 0.21 and 0.74 (Tab. 3). It is interesting to note that the correlation depends on the specificity of the classic microbiology. More specific methods lead to higher

Tab. 3 Results of beer analysis using FTIR: Correlation Coefficient (R^2) and Root Mean Square Error of Prediction (RMSEP) of PLS regression using different methods for variable selection and cross validation

	Complete FTIR spectrum		Jack-Knifing Variables		Winescan-Variables		GA-Variables		SVM-Variables	
***log_{10}* cfu/ml**	R^2	RMSEP	R^2	RMSEP	R^2	RMSEP	R^2	RMSEP	R^2	RMSEP
E. coli	0.72	1.34	0.74	1.26	0.48	1.64	0.61	1.59	0.46	1.97
Coliforms	0.68	1.55	0.69	1.54	0.65	1.63	0.68	1.53	0.58	1.67
Total beer spoilage bacteria	0.49	1.58	0.46	1.58	0.37	1.66	0.21	1.73	0.35	1.66
Lactic acid bacteria	0.43	1.39	0.39	1.39	0.37	1.44	0.28	1.49	0.27	1.48

correlation, e.g. *E. coli* and Coliforms showed the highest correlation coefficients and lower RMSEP values, than lactic acid bacteria or beer spoilage bacteria. However, none of the methods for interpretation of the FTIR spectra was able to predict the microbiological counts with sufficient accuracy. The RMSEP values, which may be interpreted as the measurement uncertainty of the multivariate assay, were with $10^{1,26}$ to $10^{1,97}$ in ranges significantly above the classical microbiological methods. If we reduce the cut-off values so much, that false-negative samples would be excluded, we get so much false-positive samples (up to 60 %) that the use of FTIR screening prior to microbiological plating does not lead to a significant labour saving enhancement. A further disadvantage is the computing requirement for the multivariate statistic methods. So far, genetic algorithms and support vector machines are not integrated in the software of the FTIR device manufacturer, so that they must be calculated "offline" after export of the spectra.

3.3 Observation about microbiological spoilage of beer samples

During our inoculation experiments of beer samples, to gain a data set for FTIR calibration, we have made the observation that the proliferation of microorganisms in beer is extremely difficult. During incubation of inoculated beer at cold storage (+8 °C) the bacterial counts stayed constant, whereas at increased temperatures (+25, +30, +37 °C) the microorganisms were killed. Only in particular cases an increase of bacteria was possible with special cultures isolated from beer samples, which obviously appear to be adapted to the nutritional conditions in the beer media. These results suggest that a microbiological spoilage of beer can be exclusively explained by biofilm-contamination of the beer, for example in hoses, dispensing equipment or cooling units due to lacking hygiene regiment.

In the practise of governmental food control in the German federal state of Baden-Württemberg, a relative high quota of microbiologically conspicuous beer samples was detected in the last years (i.e. 26% in 2005, and 19% in 2006[22)]). Only by using a regular cleaning and disinfection of all parts of equipment that come into contact with the beer such problems can be excluded.

4 Conclusions

The method applying fluorescence-marked gene probes is routinely used in our laboratory since 2004 in the context of governmental food control. It has significantly enhanced our capabilities in microbiological evaluation of beer samples as well as the confidence of the results to stand up in court in cases of hygiene deficiencies in breweries.

The gene probe method is convenient (no enrichment procedures are necessary), but time-consuming. We found the method – combined with careful screening – to be a useful tool for the identification and assessment of hygienic risks in beer samples from all stages of production. In most cases, our information about the detection of spoilage bacteria helped the breweries to set up a satisfying hygienic regime: most follow-up samples that have reached us so far have been tested negative for spoilage bacteria or the count was so low that it could be safely neglected.

In contrast, the FTIR method proved to be less useful for the microbiological screening of beer samples. Hopefully, the next generation of FTIR analyzers with a higher sensitivity in combination with more advanced statistical treatment might be better suitable for our purposes.

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