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Detection of beer spoilage bacteria *Pectinatus* and *Megasphaera* with acridinium ester labelled DNA probes using a hybridisation protection assay

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ABSTRACT

DNA probes specific for rRNA of selected target species were utilised for the detection of beer spoilage bacteria of the genera *Pectinatus* and *Megasphaera* using a hybridisation protection assay (HPA). All the probes were modified during synthesis by addition of an amino linker arm at the 5′ end or were internally modified by inserting an amine modified thymidine base. Synthesised probes then were labelled with acridinium ester (AE) and purified using reverse phase HPLC. The internally AE labelled probes were able to detect target RNA within the range of 0.016–0.0032 pmol. All the designed probes showed high specificity towards target RNA and could detect bacterial contamination within the range of ca. 5×10^2 1×10^3 CFU using the HPA. The developed assay was also compatible with MRS, NBB and SMMP beer enrichment media, routinely used in brewing laboratories.

1. Introduction

In recent years various new microbial detection methods have been developed for use in the brewing industry based on cell and microcolony visualisation and analysis of cellular and genetic content (Suzuki, 2011; Russell and Stewart, 2003; Strögards, 2000). PCR based methods in particular have been widely evaluated (Suzuki, 2011; Juvonen, 2009; Motoyama and Ogata, 2000; Homann et al., 2002; Asano et al., 2008; Ijima et al., 2008). However there are several problems in introducing these rapid detection methods to brewing laboratories due to expensive instrumentation and complexity of the methods, hence plate counting and enrichment remain the principal methods for detection of microbial contamination in breweries throughout the brewing process and in the final products (Campbell, 2003). The strictly anaerobic beer spoilage bacteria belonging to the genera *Pectinatus* and *Megasphaera* are most difficult to detect due to their anaerobic nature, but there is a need to detect even a single viable microorganism in the packaged products to avoid undesirable consequences at a later stage (Juvonen, 2009).

The current study is based on application of a basic nucleic acid hybridisation technique using acridinium ester (AE) labelled DNA probes. AE displays chemiluminescence characteristics when it reacts with alkaline peroxide to yield light with a peak wavelength around 440 nm, which can be measured using any standard luminometer (McCapra, 1976). AE can be covalently attached to primary amine containing compounds. In the case of oligonucleotide probes, an amine linker arm can be inserted at various places during synthesis (Arnold, 2000) and an oligonucleotide can be labelled with AE without any changes in its chemiluminescence property making it suitable for use in hybridisation assays in various formats (Nelson et al., 1995).

The hybridisation protection assay (HPA) format involves hybridisation of AE labelled probe with target nucleic acid, followed by a differential hydrolysis step which involves alkaline hydrolysis of free and unhybridised AE probes while the hydrolysis of hybridised AE probe is prevented as a result of intercalation of AE molecules in a nucleic acid duplex (Arnold et al., 1989). The final step involves measurement of the chemiluminescence signal, which is directly associated with the hybridised probes. The main advantages of the HPA are the simple in-solution protocol, high sensitivity, specificity and versatility (Nelson and Kacian, 1990).

The HPA has been widely used in clinical laboratories for detection of pathogens (Clancy et al., 2012; Brentano et al., 2011; Marlowe et al., 2003; Harper and Johnsons, 1990). It has also been used for detection of target amplified products from PCR (Mullis and Faloona, 1987; Nelson and McDonough, 1994) and in genetic mutation studies (Nelson, 1998; Dhingra et al., 1991). Applications of the HPA in basic nucleic acid research such, as studies of the DNA double helix (Becker et al., 1999; Majlessi and Becker, 2008) and nucleic acid hybridisation kinetics (Mazumdar et al., 1998) have also been documented. The HPA has been utilised for detection of food pathogens in contaminated food samples (Livezey et al., 2013; Clancy et al., 2012; Hogan, 2000) but comparative application of the assay is limited in the food industry and beverage industries (Mozola, 2000).

The present study aimed at development of a chemiluminescence based method for detection of beer spoilage bacteria belonging to the

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genera *Pectinatus* and *Megasphaera*. ATP bioluminescence is a method that has been routinely used in most major breweries for hygiene monitoring (Paradh et al, 2011; Ehrenfeld et al., 1996) and a Luminometer is readily available in medium sized and large breweries. Application of a luminometer based method for detection of brewery contaminants using a highly sensitive chemiluminescence based assay could be an easy and effective approach. For the present study, HPA formats were developed for detection of beer spoilage microorganisms belonging to the genera *Pectinatus* and *Megasphaera*. Species specific AE labelled DNA probes were designed that were complementary to 16S r-RNA of each bacterium and a single genus specific probe was developed for detection of all three species of genus *Pectinatus*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Strains of beer spoilage bacteria and other related bacteria were obtained from DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany), ATCC (American Type Culture Collection), VTT (Valtion Teknillinen Tutkimuskeskus Culture Collection, Finland), Heineken Brewery, Netherlands and ICBD (International Centre for Brewing and Distilling, School of Life Sciences, Heriot Watt University, Edinburgh, UK). The species and the strains, specific media and incubation conditions used in this study are shown in Table 1.

2.2. Isolation of total cellular RNA and quantification

Working cultures of *Pectinatus* and *Megasphaera* were obtained by inoculating 10 μl of pure culture onto PYF agar plates and incubating in anaerobic conditions under an atmosphere of N_2:H_2:CO_2 (80:10:10) for 4 days at 30 °C. A single colony was picked and inoculated into 10 ml of PYF broth then further incubated for 4 days under the same conditions.

A 500 μl aliquot of 12–18 h grown culture containing approximately 10^8 cells/ml was mixed with 1000 μl of bacterial RNA stabilisation solution (Qiagen); the suspension was vortexed briefly and incubated at room temperature for 10 min. The suspension was then centrifuged at 12,000 rpm for 10 min and the supernatant was removed by pipetting. The pellets were quickly frozen in liquid nitrogen and stored at −70 °C till further use.

RNA extraction was carried out using an RNeasy® mini Kit (Qiagen) following the manufacturer's instructions. RNA was quantified using a UV spectrophotometer (SHIMADZU-UV-1650PC UV–VIS spectrophotometer). and the relationship of 1 absorbance unit at 260 nm = 40 μg/ml of RNA. Isolated RNA was checked for quality using denaturing MOPS gel electrophoresis in the presence of 1% formaldehyde (Sambrook and Russell, 2001).

2.3. Selection of probes and modification

The new probes targeting the 16S ribosomal RNA gene of *Pectinatus* and *Megasphaera* species were designed using LNA™ probe designer software (www.exiqon.com). The probes were analysed for specificity using the nucleotide BLAST search tool (http://blast.ncbi.nlm.nih.gov; Johnson et al., 2008) and the probe-match tool (http://rdp.cme.msu.edu/probematch/search.jsp; Kim et al., 2009). The probes used in the present study are shown in Table 2. The oligonucleotide probes were obtained from MWG Eurofins, UK. Each probe was modified in two ways; by addition of a C_5 amino linker arm at the 5′ end and an internal modification by insertion of an amine-modified thymidine base (Arnold, 1997, 2000). Probes labelled with DIG at the 5′ end were also obtained from MWG Eurofins, UK. All probes were obtained as freeze dried pellets which were reconstituted to a concentration of 50 pmol/μl using sterile deionised water and stored at −70 °C.

2.4. Labelling and purification of AE labelled probes

The succinimidyl derivative of the acridinium ester (9[[4-[3-[(2,5-dioxo-1-pyrrolidinyl) oxy]-3-oxopropyl] phenoxy] carbonyl]-10-methyl-acridinium trifluoromethane sulfonate) (Weeks et al., 1983) was obtained from Cambridge Biosciences. The working stock was obtained by dissolving AE in anhydrous DMSO to obtain a final concentration of 25 mM. The working stock was prepared freshly for each use and used immediately after preparation. The main AE stock was stored desiccated using silica beads in an airtight container at −70 °C.

The labelling was carried out as described by Arnold and Nelson (1999) and Mazumdar et al. (1998). After labelling free AE molecules were separated using a DyeEx spin column (Qiagen) according to the manufacturer's instructions and the probe was ethanol precipitated. The AE labelled DNA probe pellet was dissolved in 20 μl of 0.1 M sodium acetate (pH-5.0), 0.1% SDS and stored at −20 °C till further use.

Table 1
Bacterial species and strains used for the study.

Bacteria species	Culture collection strains	Media used	Incubation
Pectinatus cerevisiiphilus	ATCC 29359[a], DSM 20467	PYF	Anaerobic at 30 °C
Pectinatus frisingensis	VTT E 79100[a], DSM 6306[a]	PYF	Anaerobic at 30 °C
Pectinatus haikarae	VTT E 88330[a], DSM 16980	PYF	Anaerobic at 30 °C
Megasphaera cerevisiae	ATTC 43254, DSM 20461	PYF	Anaerobic at 30 °C
Megasphaera sueciensis	DSM 17042	PYF	Anaerobic at 30 °C
Megasphaera paucivorans	DSM 16981	PYF	Anaerobic at 30 °C
Lactobacillus brevis	ICBD culture collection strain[b]	MRS + 1% Sucrose	Anaerobic at 30 °C
Lactobacillus casei	ICBD culture collection strain[b]	MRS + 1% Sucrose	Anaerobic at 30 °C
Lactobacillus lindneri	ICBD culture collection strain[b]	MRS + 1% Sucrose	Anaerobic at 30 °C
Lactobacillis paracollinodes	ICBD culture collection strain[b]	MRS + 1% Sucrose	Anaerobic at 30 °C
Lactobacillus plantarum	ICBD culture collection strain[b]	MRS + 1% Sucrose	Anaerobic at 30 °C
Lactobacillus coryniformis	ICBD culture collection strain[b]	MRS + 1% Sucrose	Anaerobic at 30 °C
Pediococcus damnosus	ICBD culture collection strain[b]	MRS + 1% Sucrose	Anaerobic at 30 °C
Pediococcus inopinatus	ICBD culture collection strain[b]	MRS + 1% Sucrose	Anaerobic at 30 °C
Pediococcus pentosaceus	ICBD culture collection strain[b]	MRS + 1% Sucrose	Anaerobic at 30 °C
Zymomonas mobilis	ICBD culture collection strain[b]	UBA agar	Aerobic at 37 °C
Micrococcus kristinae	ICBD culture collection strain[b]	UBA agar	Aerobic at 37 °C
Escherichia coli	ICBD culture collection strain[b]	LB agar	Aerobic at 30 °C

PYF (peptone-yeast extract- fructose; medium 41, VTT Culture Collection, Finland), MRS medium (Oxoid, UK, medium CM0361), UBA agar (Universal beer agar; CM0651, Oxoid, UK), LB agar (Luria Bertani agar;CM1021, Oxoid, UK).

[a] Strains obtained from Heineken Brewery, Netherlands.

[b] Culture collection strain from the International Centre for Brewing and Distilling (ICBD), School of Life Sciences, Heriot Watt University, Edinburgh, UK.