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Development of a propidium monoazide-polymerase chain reaction assay for detection of viable *Lactobacillus brevis* in beer

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ABSTRACT

The spoilage of beer by bacteria is of great concern to the brewer as this can lead to turbidity and abnormal flavors. The polymerase chain reaction (PCR) method for detection of beer-spoilage bacteria is highly specific and provides results much faster than traditional microbiology techniques. However, one of the drawbacks is the inability to differentiate between live and dead cells. In this paper, the combination of propidium monoazide (PMA) pretreatment and conventional PCR had been described. The established PMA-PCR identified beer spoilage *Lactobacillus brevis* based not on their identity, but on the presence of *horA* gene which we show to be highly correlated with the ability of beer spoilage LAB to grow in beer. The results suggested that the use of 30 μg/mL or less of PMA did not inhibit the PCR amplification of DNA derived from viable *L. brevis* cells. The minimum amount of PMA to completely inhibit the PCR amplification of DNA derived from dead *L. brevis* cells was 2.0 μg/mL. The detection limit of PMA-PCR assay described here was found to be 10 colony forming units (CFU)/reaction for the *horA* gene. Moreover, the *horA*-specific PMA-PCR assays were subjected to 18 reference isolates, representing 100% specificity with no false positive amplification observed. Overall the use of *horA*-specific PMA-PCR allows for a substantial reduction in the time required for detection of potential beer spoilage *L. brevis* and efficiently differentiates between viable and nonviable cells.

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Introduction

Limited ranges of bacteria are capable of spoiling beer owing to the presence of ethanol (0.5–10% w/w), high carbon dioxide content (approximately 0.5% w/v), relatively low pH (3.8–4.7), extremely reduced concentration of oxygen (<0.1 ppm), lack of nutrition and the antibacterial effects of hop bitter compounds. Among the most problematic beer spoilers are several species of the gram-positive genera lactobacilli and pediococci.[1,2] *Lactobacillus brevis* appears to be the most frequently isolated beer spoilage *Lactobacillus* species in beer and breweries. More than half of the bacterial incidents were caused by this species.[1] It is one of the best-studied beer spoilage bacteria and grows optimally at 30 °C and pH 4–6.

Current methods of detecting beer spoilage bacteria are time-consuming. Therefore, the brewer requires a rapid, accurate method as a quality control tool for screening samples before release into the marketplace. To shorten the detection time, several molecular methods have been developed for the detection of beer spoilage bacteria particularly *L. brevis*, based on techniques such as the polymerase chain reaction (PCR).[3,4] However, one of the drawbacks is the inability to discriminate between live and dead cells due to the persistence of DNA after cell death. Ethidium monoazide (EMA) and propidium monoazide (PMA) were applied prior to PCR analysis to circumvent this problem, allowing a live/dead discrimination of bacteria.[5–8] The intercalating dye can enter bacteria with damaged cell membranes and covalently bind to genomic DNA upon exposure to light. The bound DNA cannot be amplified by PCR, thus preventing the detection of dead cells.[8] Although EMA/PMA-PCR has been known for several years, its applications in the brewing industry are scarce.

Beer spoilage *L. brevis* is generally resistant to hop compounds and thus can spoil beer.[1,2] It is thought that *L. brevis* undergoes a multi-factorial hop adaptation process involving changes in metabolism and morphology, as well as the more energy-dependent multidrug transporter, hop-efflux mechanisms.[9] The known beer spoilage-specific genetic markers for these bacteria are *hitA*,[10] *horA*,[11] and *horC*,[3,12] with *hitA* and *horC* recently being shown to be less well associated with ability to spoil beer.[4] Another beer spoilage related gene, *bsrA*, was recently found to be a marker for predicting beer spoilage ability of *Pediococcus* isolates.[13] The wide and exclusive distributions of *horA* in various beer spoilage *L. brevis* isolates indicate the possibility of species-independent detection of beer spoilage *L. brevis* with the genetic marker.[4,14] The hop resistance gene, *horA*, was originally identified on a 15.0 kb plasmid, designated as pRH45.[2] This plasmid was carried by a strong beer spoilage *L. brevis* strain ABBC45. pRH45 was initially recognized as a plasmid, the copy number of which multiplied with the hop adaptation of *L. brevis* ABBC45.[2] Consequently, the aim of this study was to investigate the applicability of PMA-PCR targeting the *horA* gene to discriminate between viable and nonviable *L. brevis* not to amplify other bacteria.

Materials and methods

Bacterial strains

A list of the bacterial species tested is provided in Table 1, with the strains comprising 13 *L. brevis* and 5 non-lactic acid bacteria (5 species). All these strains employed in this study were isolated and stored in our laboratory previously.[15] Among them, the lactic acid bacteria (LAB) were grown anaerobically in de Man Rogosa Sharpe (MRS) broth (Oxoid, UK) at 26 °C for 5 days, while the non-LAB were incubated at 37 °C and maintained in Luria-Bertani (LB) broth (Oxoid, UK) for 24 h.

The beer spoilage ability was investigated using the traditional "growth in beer test" described as Deng et al.[16] Approximately 10^2 cells mL^{-1} of each strain were inoculated onto the apical surface of commercial bottled lager beers (filter-sterilized, 4.5% vol/vol alcohol, pH 4.8, around 9 bitterness units) under sterile conditions at room temperature. Bottle headspaces were flushed with CO_2 at a flow rate of 120 mL/min for approximately 3 min to remove the air. These bottles were then tightly recapped with metal lids and incubated at 26 °C and examined regularly for visible growth for up to 1 month. Bacteria capable of growing in either beer were considered to be beer-spoilers. The ability of these 18 isolates to grow in beer was recorded in Table 1 for direct comparison with the results on presence or absence of *horA* gene.

Inactivation of bacterial cells

The bacteria were heated at 65 °C in a water bath for 30 min. The resulting heat-treated samples were cooled to room temperature and the absence of viable cells determined by the passive dye exclusion method[16] using a Live/Dead *Bac*Light bacterial viability kit (Molecular Probes, USA). Two fluorescent dyes SYTO 9 and propidium iodide (PI) were used following the manufacturer's instructions to evaluate cell membrane integrity in this kit. Cell samples were stained with the mixture of SYTO 9 (5 μM final concentration) and PI (30 μM) in 0.5 M sodium phosphate buffer at pH 7.0, and incubated in the dark at room temperature for 20 min. The stained cells were analyzed under the Guava easyCyte 8HT flow cytometer (Guava Technologies Inc., USA) using blue line excitation at 488 nm. Results are expressed as the number of viable cells per milliliter of the samples.

DNA isolation and PCR assays

Genomic DNA were extracted from bacterial strains by using the TIANamp Bacteria DNA kit (Tiangen Biotech, China) according to the manufacturer's instructions. The primer pairs specific to *horA* were designed as described by Haakensen et al.[4] The sequences of forward and reverse primers are 5′-ATCCGGCGGTGGCAAATCA-3′ and 5′-AATCGCCAATCGTTGGCG-3′ respectively, and amplify a 335-bp segment in the conserved region of the *horA* gene.[15]

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