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Isolation and identification of gas-producing spoilage microbes in fermented broad bean paste

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ABSTRACT

This study aimed to isolate and identify the spoilage microbes which could produce gas in fermented broad bean paste and to provide an effective method to restrain their growth in media. The gas-producing spoilage microbe was isolated in anaerobic condition and identified as *Bacillus licheniformis* FHYM 8. *B. licheniformis* FHYM 8 was resistant to high temperature, low pH and high salinity condition since it could survive after treatment at 100 °C for 10 min and when the pH value was as low as pH3.8. The inhibitory salinity for *B. licheniformis* FHYM 8 was 15% in aerobic condition while 11% of salinity could completely inhibit its growth in anaerobic condition. Interestingly, *B. licheniformis* FHYM 8 showed the ability to produce gas when the broad bean paste was sterilized at 66 °C while no gas was produced when *B. licheniformis* FHYM 8 was inoculated in broad bean paste after the heat treatment process, indicating that heat treatment was the necessary step for *B. licheniformis* FHYM 8 to produce CO_2 in broad bean paste. The inhibition analysis showed that nisin with a minimal inhibitory concentration of 0.02 mg mL^{-1} was efficient in inhibiting the growth of *B. licheniformis* FHYM 8 in MRS media when the salinity was 8%. This will provide guidance to broad bean paste manufacturers to further adopt efficient strategies to inhibit the growth of gas-producing microbe in broad bean paste.

1. Introduction

Broad bean paste is a Chinese traditional aliment which is usually manufactured via fermentation by *Aspergillus oryzae* and various groups of microorganisms with broad beans and chopped chilies as raw materials (Lang & Jian-quan, 2008). The production of broad bean paste is consisted of two stages. The first stage is the steeping and boiling of broad beans in water for production of fermentable sugars and other nutrients. In the second stage, traditionally cultivated microbes are added to start the fermentation while chopped chilies are used as flavor enhancer. After fermentation, the broad bean paste is treated at 66 °C for 2 days when most of the typical flavor compounds in broad bean paste were formed. This heat treatment process can also kill or inactivate part of the microbes existed in broad bean paste before packaging. The high temperature used in normal commercial pasteurization process will cause unpleasant flavor in broad bean paste.

Broad bean paste has a low pH value around pH4.2–5.2 and a relatively high salinity around 10–12% (w/v), which is not suitable for growth of most bacteria. In recent years, salt of high concentration is considered harmful to human health (Alderman, 2000). Therefore, reducing the salinity of broad bean paste while maintaining the product quality will not only benefit the human health but also reduce cost for manufactures. However, the growth of spoilage microbes was observed in broad bean pastes when the salinity was lowered. The most severe problem occurred was the aerogenesis defect accompanied with strong sour taste. Microbial spoilage of food will increase waste, reduce consumers' confidence and cause economic losses (Snyder, Churey, & Worobo, 2016). Therefore, identifying and restraining the gas-producing spoilage microbes in fermented broad bean paste are highly demanded.

The aerogenesis defect is a common problem in fermented food industry and this problem was mostly occurred in cheese and soybean sauce industries (Liu et al., 2013; O Sullivan, Cotter, Giblin, Sheehan, & McSweeney, 2013). The aerogenesis defect was defined as the production of gas by microbes in fermented food which will

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negatively influence the food quality. Previously researches showed that the aerogenesis defect was usually caused by contamination of microbes which could produce gas (Sheehan, 2011). The aerogenesis defect in cheese production process was thoroughly studied and the gas producing microbes have been identified in recent years. In the early stage of cheese fermentation, the *Lactococcus* and *Leuconostoc* species existed in cheese were reported to be able to produce CO_2 (Mullan, 2000) while the *Kluyveromyces*, *Debaryomyces* and *Candida* species in yeast were also served as aerogens to contaminate the cheese (Sheehan, Wilkinson, & McSweeney, 2008). In the cheese ripening, propionic acid bacteria, butyric acid bacteria and salt tolerant *Lactobacilli* species were the main cause of gas production in cheese (Daly, McSweeney, & Sheehan, 2010; White, Broadbent, Oberg, & McMahon, 2003). In some kinds of cheese, *Bacillus* spores were the main contaminators and they could survive in the high temperature treatment (Sheehan, 2011). This phenomenon becomes more frequent especially when the storage temperature was higher than 20 °C and the salinity was lower than 12% (Sheehan, 2011). To prevent the gas production in cheese manufacturing and storage, enzymes (such as lysozymes), bacteriocins (such as nisin and reuterin) and preservatives (such as nitrate) were often added into cheese (McSweeney, 2007; Sheehan, 2011). Similar to cheese production, the manufacture of broad bean paste is a combinational effect of various groups of microbes. The bacteria community in fermented broad bean paste mainly consisted of *Bacillus* species, especially *B. subtilis*, *B. amyloliquefaciens* and *B. methylotrophicus*. Some *Bacillus* species were reported to be able to produce gas in anaerobic condition (Ray & Bhunia, 2007, Chapter 4). Therefore, gas-producing microbes might be the cause of aerogenesis defect in fermented broad bean paste.

In this study, to elaborate the reason for the aerogenesis defect in fermented broad bean paste, the potential microbes which caused the aerogenesis defect were isolated through culture dependent method and Durham tube test and identified by combining the traditional identification method and the molecular analysis. The physiological and biochemical properties of the gas-producing spoilage microbe were characterized while its ability to produce gas in broad bean paste was also verified. Finally, the inhibitory effect of bacteriocins and preservatives on the growth of gas-producing spoilage microbe in media was tested.

2. Materials and methods

2.1. Bacteria isolation from aerogenic broad bean paste

The aerogenic broad bean paste samples were prepared for isolation by mixing 10 g broad bean paste sample in 100 mL sterile distilled water with agitation for 30 min at 37 °C. The sample solution was diluted for 10^3 times and spread on 6 kinds of plates containing different media agar, including Tomato Juice Agar (TJA), De Man, Rogosa, Sharpe (MRS) agar, M17 agar, Luria-Bertani (LB) agar, Robertson's Cooked Meat (RCM) agar and nutrient broth agar. The anaerobic cultivation of bacteria strains in liquid media was conducted in anaerobic bottles while the anaerobic growth of bacteria strains on agar plates was performed by putting agar plates in anaerobic boxes (80% N_2, 10% H_2, 10% CO_2) (Sanbao, Jiangsu, China). After static aerobic and anaerobic cultivation at 37 °C for 72 h, the number of bacterial colonies and morphology of different colonies were recorded. The colonies obtained were classified according to their characteristics. Durham tube tests were used to select the bacteria strains which can produce gas as previously reported (De Jonghe et al., 2010). To successfully cultivate the microbes with small number, enrichment cultivation was performed. The broad bean paste sample (1 g) was mixed evenly in 10 sterile distilled water with glass beads. 200 μL sample solution was transferred into 20 mL fresh liquid media (the six media used above without agar) and cultivated in anaerobic conditions at 37 °C for 48 h. The obtained bacteria solution was then spread on six media agar plates and cultivated at 37 °C for 72 h. The colony morphology and gas-producing ability of the bacteria strains were recorded.

2.2. Morphological and physiological analysis

The gas-producing spoilage microbes were streaked on an neopeptone agar medium plates containing 40 g L^{-1} glucose, 10 g L^{-1} neopeptone and 20 g L^{-1} agar for identification and morphology characterization according to Bergry's Manual of Systematic Bacteriology (Paul De Vos, Garrity, & Jones, 2009). The screened microbes were stained with gram and endospore stain. The shape and spore-forming ability of the single colony were observed by optical microscopes (Olympus, Tokyo, Japan) and JEM-2100 Transmission electron microscope (TEM) (JEOL, Japan). The cultivated bacteria cells were centrifuged and washed with 10 mM PBS buffer (pH7.4) three times. The washed cells were then harvested by centrifugation, fixed with 5% (w/v) glutaraldehyde and postfixed with 1% OsO_4. After gradient dehydration, embedding, ultrathin section, uranium and aluminum staining, the samples were detected in a JEM-2100 Transmission electron microscope (JEOL, Japan). The physiological properties of spoilage bacteria were also analyzed. The activity of catalase was indicated by the appearance of bubbles within 15 s by adding 500 μL 30% (w/v) hydrogen peroxide solution into the bacteria culture which was incubated at 37 °C for 24 h in vitro (Baureder, Barane, & Hederstedt, 2014; RE & FJ., 1965). To determine the starch hydrolyzing ability, the spoilage microbes were grown on starch hydrolyze media agar plate and incubated at 37 °C for 72 h. The formed transparent zones were used as indicator of starch utilizing ability when 500 μL of 0.05 M iodine solution was added (Hamouda, Marzouk, Abbassy, Abd-El-Haleem, & Shamseldin, 2015). The ability of degrading gelatin was indicated by the presence of gelatinase in the vegetable cells. The spoilage microbes were inoculated in nutrient medium with gelatin as sole carbon source and cultivated at 37 °C for 48 h. The cultures became liquid at 20 °C after cultivation indicated that the strains were able to produce gelatinase (Hamouda et al., 2015). Voges-Proskauer (V-P) test was performed to determine the ability of the isolates to decarboxylate pyruvic acid into acetyl methyl carbinol (Barry and Feeney, 1967). The spoilage microbes were cultivated in Voges-Proskauer (VP) medium at 37 °C for 24 h and vibrated vigorously after addition of 40% (w/v) NaOH solution. The appearance of red within 2–10 min in tube indicated that the bacteria strain was V-P test positive. The utilizing ability of spoilage microbes towards different saccharides was conducted by inoculation of spoilage microbe (1%, v/v) in sugar hydrolyzing culture containing saccharides, such as glucose, lactose, sucrose, maltose, xylose, rhamnose and mannose, respectively. The gas-producing ability of microbes was evaluated using Durham tubes (Durham, 1898). After incubation at 37 °C in MRS and M17 media under aerobic and anaerobic conditions, changes in Durham tubes and Bromothymol Blue were recorded.

2.3. Molecular characterization

The genome DNA of the spoilage microbes was extracted by CTAB method (Arcuri, El Sheikha, Rychlik, Piro-Métayer, & Montet, 2013). The amplification of 16s rDNA was performed by PCR method as previously reported (Youssef et al., 2009) using primers listed in Online Resource 1. The PCR products of 16s rDNA were sequenced (Sangon, Shanghai, China) and identified through BLAST algorithm. After that, the published sequences of 15 different strains were obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank) and the phylogenetic tree was established using