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Identification and characterization of a *Bacillus subtilis* strain HB-1 isolated from *Yandou*, a fermented soybean food in China

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ABSTRACT

A Gram-positive *Bacillus* strain HB-1 was isolated from *Yandou*, a traditional fermented Chinese soybean product. Comparative 16S rRNA sequence analysis suggested that HB-1 belongs to *Bacillus subtilis* group without further classification to the individual species. At the same time, Biolog classifying results suggested that HB-1 might be *Bacillus licheniformis*, one of the eight members in *B. subtilis* group. In order to determine HB-1 to the species level, the 16S–23S rRNA internal transcribed spacer (ITS) sequences were cloned and analyzed. The analyses indicated that the ITS sequences of HB-1 were 99–100% and 91–92% identical to that of *B. subtilis* strain 168 and *B. licheniformis* ATCC 14580, respectively. Thus, the isolated strain HB-1 could be identified as *B. subtilis*. In addition, neutral protease activity and γ-polyglutamic acid production of strain HB-1 were determined to be 43.4 ± 3.9 U/ml and 9.0 ± 0.6 g/l, respectively. Finally, total viable counts and sensory evaluations were also performed in *Yandou* prepared in the laboratory by mimicking natural fermentation process. Without inoculation of pure culture, the bacterial number reached a maximum of 8.9 ± 0.7 log 10 CFU/g after 48 h of incubation.

1. Introduction

Fermented soybean foods are produced using soybeans as the sole or main material, and they are widely consumed in countries such as China, Japan, Korea, Indonesia and India (Golbitz, 1995; Macfarlane et al., 1990). Through fermentation, anti-nutritional factors naturally found in soybeans are largely removed (Tsuji et al., 1995), resulting in increased nutritional values in the final products due to the high protein content and the existence of other beneficial compounds such as isoflavones, tocopherols, phospholipids, caffeic acid, ferulic acid and melanoidin (Hu et al., 2004; Juan & Chou, 2010; Li, Feng, Shen, Xie, & Li, 2007; Takahashi et al., 2005). There are many types of fermented soy foods worldwide, among the best known are *Douchi* (Chen et al., 2005), *Natto* (Takahashi et al., 1995), *Miso* (Murooka & Yamshita, 2008), *Doenjang* (Kim et al., 2009), *Thua nao* (Inatsu et al., 2006), *Tempeh* (Liem, Steinkraus, & Cronk, 1977), and *Hawaijar* (Jeyaram et al., 2008).

Traditional fermentation process depends on micro-organisms naturally existing in the raw materials. Among these microbes, most frequently isolated from fermented soy foods are *Bacillus* species, such as *Bacillus subtilis* natto strain in Japanese *Natto* (Tanaka & Koshikawa, 1977; Yoshida, 2006); *B. subtilis*, *Bacillus licheniformis*, and *Bacillus cereus* strains isolated from traditional Indian food *Hawaijar* (Jeyaram et al., 2008), although *B. cereus* has been known to contribute to food poisoning via exo-toxin production. Besides bacilli, another group of microbes commonly isolated from fermented soy foods are fungi. For example, *Mucor racemosus*, *Aspergillus oryzae*, *Aspergillus egyptiacus*, *Rhizopus oryzae* and *Rhizopus oligosporus* have been identified in the traditional Chinese soy food *Douchi* (Kasankala, Xiong, & Chen, 2012; Li et al., 2007). Also multiple *R. oligosporus* strains were isolated in *Tempeh*, a fermented soybean food from Indonesia (Murooka & Yamshita, 2008). Not surprisingly, almost all fermented soybean products are geographically different since no pure starter cultures are used in the manufacturing processes. The only exception is *Natto* which uses pure *B. subtilis* strain natto for industrialized production (Nishito et al., 2010). Due to growing food-safety concerns in our increasingly globalized economy, it has become urgent to classify resident microbes involved in various food-manufacturing processes.

Yandou, meaning salty soybeans in Chinese, is a traditional snack favored by the locals in the Jiangsu province of southern China. *Yandou* is made from boiled soybeans without inoculation of any starter microbes, and currently no information is available regarding its microbial ecology. In this study, *Yandou* samples were collected and microorganisms were isolated using bacterial cultivation method.

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Biolog system and sequence analyses of 16S rRNA and 16S–23S rRNA internal transcribed spacer (ITS) regions were performed to identify the isolated microbes. One isolate was further characterized for its ability to secrete neutral protease and the synthesis of γ-polyglutamic acid (γ-PGA). The strain isolated in this work might be a good candidate for commercial production of *Yandou* in the future.

2. Materials and methods

2.1. Growth conditions

Luria Bertani (LB) medium was used for general bacteria cultivation (Sambrook, 2001), with ampicillin (100 μg/ml) added for selection of *Escherichia coli* transformants. Casein agar was used for evaluation of proteolytic activities of isolated bacteria: KH_2PO_4 (0.036%, w/v), $MgSO_4{\cdot}7H_2O$ (0.05%), $ZnCl_2$ (0.0014%), $Na_2HPO_4{\cdot}7H_2O$ (0.107%), NaCl (0.016%), $CaCl_2$ (0.0002%), $FeSO_4$ (0.0002%), casein (0.4%) and agar (1.5%); and the final pH was adjusted to 6.5–7.0 (Sambrook, 2001). Seed medium contained peptone (1%), glucose (2%), K_2HPO_4 (0.1%) and $MgSO_4{\cdot}7H_2O$ (0.05%); and the final pH was 7.0. For fermentation and enzyme production, the medium contained maltose (2%), yeast extract (4%) and $CaCl_2$ (0.03%), with a final pH of 7.0 (Cromwick & Gross, 1995). γ-PGA producing medium contained glucose (4%), yeast extract (0.8%), K_2HPO_4 (0.15%), $MgSO_4{\cdot}7H_2O$ (0.05%), $(NH_4)_2SO_4$ (0.5%) and L-glutamic acid sodium (3%), with a final pH of 7.5. Unless specified otherwise, all cultures were incubated at 30 °C.

2.2. Microbial analysis

We collected *Yandou* and other soybean samples from one household located in the suburb of the city of Xuzhou, Jiangsu province. Each time, 10 g of sample was transferred into 30 ml sterile LB broth, shaken vigorously, and held in boiling water (100 °C) for 20 min to kill all vegetative microbial cells. After cooling down to room temperature, an aliquot of the supernatant was serially diluted and plated on L-agar plates. Colony morphology was recorded after incubation at 30 °C for 24 h, and Gram-staining was performed to help identify individual colonies. Controls without boiling treatment were also included for each sample.

2.3. Sequence analysis of 16S rRNA gene and the 16S–23S rRNA intergenic transcribed spacer region and flanking regions

The isolated bacterial strain was grown in L-broth at 30 °C overnight, from which chromosomal DNA was extracted using the Genomic DNA Purification Kit (Invitrogen) according to the manufacturer's instructions.

16S rRNA gene sequence was amplified using primers 27f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492r (5′-GGT TAC CTT GTT ACG ACT T-3′) (Lane, 1991). PCR was performed in a 50-μl reaction system using 10 ng of bacterial genomic DNA as template, according to a previously described procedure (Sambrook, 2001). The 16S–23S rRNA ITS regions were amplified using primers L516SF (5′-TCG CTA GTA ATC GCG GAT CAG C-3′) and L523SR (5′-GCA TAT CGG TGT TAG TCC CGT CC-3′) (Xu & Cote, 2003). PCR was performed in a 50-μl reaction with 10 ng of bacterial genomic DNA according to a protocol detailed elsewhere (Xu & Cote, 2003).

All PCR products were separated by electrophoresis on 0.8% agarose gel and purified using a QIAquick PCR Purification Kit (Qiagen). Eluted DNA fragments were subsequently cloned into pGEM®-*T Easy vector* (Promega) according to the manual of the supplier. All clones were sequenced with M13 universal primers and the sequences were deposited in GenBank database. Classifier of the Ribosomal Database Project II (RDP II) was used to classify the bacteria (Wang, Garrity, Tiedje, & Cole, 2007). Sequences alignment was performed via NCBI BLAST algorithm and phylogenetic trees were constructed using MEGA 5 program (Tamura et al., 2011).

2.4. Biolog microbial identification

Biolog GN MicroPlate (Biolog, Inc.) was used to evaluate substrate utilization patterns of the isolated strain HB-1. The experiment was performed according to the supplier's instructions. Briefly, bacterial cells were grown on Biolog Universal Growth agar supplemented with 5% sheep erythrocytes (BUG-S) at 28 °C for 24 h, harvested and resuspended in sterile saline solution to required optical density. 150 μl/well of bacterial suspension was then used to inoculate the MicroPlates, followed by incubation at 28 °C. Analysis results at various time points were recorded by the instrument (Garland & Mills, 1991; Smalla, Wachtendorf, Heuer, Liu, & Forney, 1998).

2.5. Determination of proteolytic activity

Folin phenol method was used to assess neutral protease activities of bacteria cultures (Lowry, Rosebrough, Farr, & Randall, 1951). Bacteria were cultivated in 100 ml enzyme production medium with vigorous shaking (150 rpm) at 30 °C. At different time points, a 1-ml sample was taken out from the culture, centrifuged at 10,000 rpm at 4 °C for 5 min and the supernatant was used in subsequent protease activity test.

2.6. Productivity of γ-PGA

Isolated bacteria were grown in seed medium at 30 °C overnight, and 2 ml of the culture was transferred to 100 ml γ-PGA producing medium. After 48 h cultivation at 30 °C with shaking at 220 rpm, the cells were removed by centrifugation at 8000 × *g* at 4 °C for 10 min. The supernatant was first treated with proteinase (5 μg/ml) at 30 °C for 1 h, then mixed with 4 volumes of cold 95% ethanol and left overnight at −20 °C. After spinning at 15,000 × *g* at 4 °C for 20 min, the pellet was dissolved in water. This precipitation step was repeated twice, and the exopolymer was finally resuspended in 10 ml ddH_2O.

Hydrolyzing method was used to determine the γ-PGA content. Briefly, the exopolymer solution was treated with 6 M HCl at 110 °C for 24 h to completely release glutamatic acid. Subsequently, the pH of the solution was adjusted to 7.0, and the content of glutamatic acid was determined using a biosensor (SBA-40C, Shandong Biology Institution) (Do, Chang, & Lee, 2001; Shi, Xu, & Cen, 2006).

2.7. In-lab Yandou fermentation

To determine the viable counts of bacterial population in actual fermentation process, raw soybeans (collected from the same household in Xuzhou) were wrapped with sterile gauze, boiled for 20 min, and incubated in sterile glass petri dishes (90 mm) at 30 °C. At every 24 h, soybean samples were withdrawn and suspended in 5 ml sterile phosphate buffered saline (PBS). The numbers of viable bacterial cells were then monitored by colony formation on LB plates.

In a separate process, isolated HB-1 was used as the starter microbe in making *Yandou* in the laboratory. 150 g of raw soybeans (collected from the same household in Xuzhou) were washed thoroughly and soaked in water for 12 h at room temperature. The soaked soybeans were then autoclaved at 121 °C for 15 min, and 3 ml exponentially growing bacterial culture (OD_{600} = 0.6) was added to start the fermentation. After incubation at 30 °C for 72 h, *Yandou* products were evaluated by a panel of 10 judges using a score range of 1 (poor) to 5 (excellent). The evaluated sensory variables include appearance, slime, smell, texture, and color.