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BOX-PCR and PCR-DGGE analysis for bacterial diversity of a naturally fermented functional food (Enzyme®)

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ABSTRACT

Enzyme® product is a popular macrobiotic food in Asia, which is naturally fermented using vegetables, fruits, plant leaves, and flour as raw materials. To study the bacterial diversity in a commercial available Enzyme® product, culture-dependent BOX-PCR analysis and culture-independent PCR-DGGE analysis were used simultaneously. The amount of the cultivable bacteria in the product is about 2.5×10^7 CFU/g. A total of 329 isolated strains were grouped into six fingerprint types according to BOX-PCR analysis. Four dominant species were identified among the six groups, which are *Bacillus coagulans* (60.2%), *Lactobacillus plantarum* (23.7%), *Lactobacillus oris* (8.2%), and *Staphylococcus epidermidis* (7.9%). Other five unculturable species, which were not cultivated by the condition used in this study, were identified according to PCR-DGGE analysis. Among them, *Phyllobacterium myrsinacearum*, *Rhodococcus erythropolis*, and *Acidovorax konjaci* were frequently found to be associated with plant materials. Therefore, combinatory application of culture-dependent and -independent techniques allowed us to unravel the microbial diversity in a naturally fermented health-promoting food product.

1. Introduction

Traditional fermented foods are mainly produced from milk, meats and corns in Western countries, while from vegetables or other plant raw materials in Asia. The naturally fermented foods in China include soybean pastes, pickled bean curd, *douchi*, and pickled vegetables. Japan also has various such traditional fermented foods, which include black rice vinegar, *shoyu*, *miso*, *natto*, and *tempeh* (Murooka & Yamshita, 2008). These foods often experience long-time fermentation processes, contain complex microbial community, and are suggested to have multiple health-promoting functions. Nowadays, commercial Japanese Enzyme® products become increasingly popular as a functional food in Asia like

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Shimamoto Enzyme®, Manda Koso Enzyme®, and Ohtaka Enzyme® (Ashida, Okimasu, & Amemura, 2002; Kim, Hwang, & In, 2003; Lokaewmanee, Yamauchi, & Thongwittaya, 2012). It is well known that Enzyme® product can promote longevity and confers many beneficial health effects to human such as improvement of gastrointestinal function, protection against oxidation, reduction in serum cholesterol, and stimulation of immune systems (Ashida, Takei, Takagaki, Matsuura, & Okimasu, 2006; Lokaewmanee et al., 2012). Enzyme® product is usually produced through natural fermentation for 2–3 years using the mixture of various vegetables, fruits, flour, and other plants as raw materials. The brief fermentation process is as follows: all materials are mixed and thrown into barrels, which are incubated statically for 1–2 years, then the fermented products are concocted and poured back into barrels again, which are incubated statically further for a year. The fermentation temperature in each barrel is adjusted by hand work and no heating process is required. The resulted products not only contain various nutrient elements from plant materials, but also contain other health-promoting factors derived from microbial fermentations.

Many studies have been carried out to characterize the microbial communities in the naturally fermented foods and isolate unique probiotic microbes for applications in food industry. Based on culture-dependent method, 260 strains were isolated from a Chinese *douchi* product and clustered according to their morphological and biochemical characteristics. Fifty-six representative strains among them were identified as 14 species of lactic acid bacteria (LAB) (Liu et al., 2012). In a traditional fermented food in Taiwan, *sian-sianzih*, 184 cultivable bacterial strains were identified and they belonged to five predominant LAB genera based on analysis of restriction fragment length polymorphism (RFLP) and sequence analysis of 16S rDNA. Moreover, one *Weisella hellenica* strain with antibacterial activity was identified and behaved great potentialities in food industrial application (Chen et al., 2011). For two Japanese traditional fermented fish products, single-strand conformation polymorphism analysis (SSCP) of V3 region in 16S rDNA was used to study the bacterial diversities and identify their predominant viable bacterium (An, Takahashi, Kimura, & Kuda, 2010). Therefore, naturally fermented foods contain complex mixture of microorganism and some strains isolated from these traditional foods could be commercialized as probiotic products. One of the examples is the *Lactobacillus plantarum* L137 isolated from *Burong Isda*, a traditional fermented food made from rice and fish in the Philippines, which can augment acquired immunity and modulate immune system of host (Arimori et al., 2012; Hirose, Murosaki, Yamamoto, Yoshikai, & Tsuru, 2006) and has been used as probiotics in Japan (Murooka & Yamshita, 2008; Olympia, Ono, Shinmyo, & Takano, 1992).

The low-density microorganisms in naturally fermented foods can hardly be revealed by culture-dependent analysis, because some of them cannot be cultivated by a medium, and others might be even unculturable. Alternatively, culture-independent molecular biological techniques can be used to identify these microorganisms from the DNA directly extracted from the food sample. These techniques include denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and pyrosequencing (Kim et al., 2011; Nam, Lee, & Lim, 2012). Of these techniques, 16S rDNA PCR-DGGE analysis is most widely used to study the microbial diversity in traditional fermented food. Fermented foods that have been analyzed by PCR-DGGE include Chinese Zhenjiang aromatic vinegar (Xu et al., 2011), Korean fermented soybean paste (Kim et al., 2009), and Japanese rice black-vinegar (Haruta et al., 2006). Combinatory use of culture-dependent quantitative identification and culture-independent direct analysis would therefore render comparable and complementary results for analysis of microbial community in naturally fermented foods (An et al., 2010; Masco, Huys, De Brandt, Temmerman, & Swings, 2005).

Among various culture-dependent rapid identification technologies, BOX-PCR fingerprint technology, based on the diversity of short tandem repeats sequences (BOX elements) in the genome, has been widely used for molecular typing of strains due to its good reproducibility and discrimination power, simple procedure, and low cost (Olive & Bean, 1999; Zhu et al., 2006). This study aimed to gain insights into the bacterial diversity of a popular Japanese Enzyme® product by combinational use of the culture-dependent BOX-PCR fingerprint technique and the culture-independent 16S rDNA PCR-DGGE technique. The bacterial diversity in the Enzyme® product was quantitatively evaluated and its health-promoting function was proposed.

2. Materials and methods

2.1. *Japanese Enzyme® sample*

The Japanese Enzyme® (in powder) product was provided by Polo Biology Corporation (Beijing, China). The Bottled products were stored at 4 °C.

2.2. *Plate counting assay and culture condition*

Two gram powder samples were respectively added to a 150 ml Erlenmeyer flask containing 50 ml sterile Solute A (1% NaCl, 1% Tryptone, 0.05% Tween-80) and small glass beads. The suspensions were incubated in a rocking bed at 150 rpm, 37 °C for 20 min to disperse cells completely. Each suspension was serially diluted by using Solution B (1% NaCl, 1% Tryptone). Appropriate dilutions were spread (in triplicate) onto MRS agar plates (Oxoid). The MRS agar plates were incubated anaerobically at 37 °C for 3 days using BACTRON II (SHEL LAB). The plates with 100–300 colonies were used for further analysis. All colonies (100–300) grown on MRS agar plates were individually inoculated into a 2.0 ml centrifugal tube containing 1.8 ml of MRS broth. The cultures were incubated in BACTRON II anaerobically at 37 °C for 2 days. While 0.8 ml of culture of each colony was stored at −80 °C with addition of 0.2 ml of 50% glycerol. The residual cultures were used for DNA extraction and BOX-PCR analysis.

2.3. *DNA extraction of cultures*

A previously reported hexadecyl trimethyl ammonium bromide (CTAB) method (Zhu et al., 2006) was used for DNA