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Application of lactic acid bacteria and yeasts as starter cultures for reduced-salt soy sauce (moromi) fermentation

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ABSTRACT

The use of starter cultures (*Tetragenococcus halophilus* TS71, *Zygosaccharomyces rouxii* A22, and *Meyerozyma (Pichia) guilliermondii* EM1Y52) in reduced-salt moromi fermentation was investigated. Reduced-salt moromi fermentation (12%NaCl) with starter cultures was monitored for changes of microbiological and biochemical properties including volatile flavor compounds (VFCs) and biogenic amines (BAs) during three months. The inoculation was done after one-month fermentation with two combinations: TS71 and A22 (L2); and TS71, A22 and EM1Y52 (L3). Controls were 12% salt (CL) and 18% salt traditional (CT) without inoculation. Total bacteria counts significantly increased ($P < 0.05$) in CL. Total lactic acid bacteria and total yeasts in the reduced-salt moromi with inoculation (L2 and L3) were significantly higher than both controls. Key VFCs including ethanol, 2-methyl-1-propanol, 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF) and 3-hydroxy-2-methyl-4H-pyran-4-one (maltol) were detected in L2 and L3 at higher levels than the traditional CT. Lower amount of BAs was accumulated in the reduced-salt moromi fermentation, particularly with lactic acid bacterium (L3) than other fermentations. Hence, the reduced-salt moromi fermentation with using starter cultures was therefore feasible for the soy sauce production without undesirable impact on formation of VFCs and safety levels of BAs.

1. Introduction

Soy sauce is one of fermented soybean products which is consumed as a seasoning or a condiment due to its umami and salty tastes, and unique aroma (Van Der Sluis, Tramper, & Wijffels, 2001). Soy sauce has been used in oriental countries since antiquity and now used worldwide (Kataoka, 2005; Yokotsuka, 1986). Soybeans, wheat, and salt brine are three main raw materials of soy sauce fermentation (Fukushima, 2004; Wah, Walaisri, Assavanig, Niamsiri, & Lertsiri, 2013). In traditional Thai soy sauce production, koji is firstly prepared by growing the koji mold (i.e. *Aspergillus oryzae*) on mixture of steamed soybeans and wheat flour or rice flour. After that, the matured koji is then mixed with salt brine containing 18–22% (w/v) of NaCl at ratio 1:3 (w/v) to obtain moromi mash which is further fermented for at least 3 months. The well-aged moromi is filtered, formulated according to a recipe, and heated to precipitate protein and pasteurize, prior to bottling. Up to now, application of LAB and yeast starter cultures has not been used in Thai soy sauce industry yet (Lertsiri, Wanakhachornkrai, Assavanig, Chaiseri, & Suwonsichon, 2011; Mongkolwai, Assavanig, Amnajsongsiri, Flegel, & Bhumiratana, 1997). As a condiment, high daily consumption of soy sauce can lead to excessive intake of NaCl causing risk of hypertension and cardiovascular disease as well as renal dysfunction (Kremer, Mojet, & Shimojo, 2009). Currently, global average daily intake of sodium is 3.95 g which is nearly double higher than recommended 2 g by the World Health Organization (WHO). While daily allowance of sodium intake is 2 g (equivalent to 5 g salts) (WHO, 2012), long-term overconsumption can lead to the onset of chronic disease (Luo, Ding, Chen, & Wan, 2009). According to high concentration of sodium chloride in soy sauce, this condiment has relatively strong contribution to the dietary intake of sodium salt. Consequently, the reduction of salt content in soy sauce becomes one of approaches for healthy food industries.

There are various methods for reduced-salt soy sauce

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production that have been studied including using minimum brine solution concentration that is necessary for avoiding contamination; using alcohol with water; or eliminating salt by means of electrodialysis, a membrane treatment (Luo et al., 2009), ion exchange (Japan Patent No. 52,120,197, 1977), reverse osmosis (Japan Patent No. 4,016,162, 1992), freezing (Watanabe, Tesaki, & Arai, 1996) and extraction (Japan Patent No. 10,295,320, 1998). In addition, potassium chloride and gamma-aminobutyric acid are added to a reduced common salt soy sauce, so as to obtain a low common salt soy sauce (Yamakoshi et al., 2010). Reduction of salt by those physical approaches may give adverse effects on flavor and taste such as bitterness (Luo et al., 2009). On the other hand, if the moromi fermentation takes place under low salt concentration, control of microflora growth becomes necessary. In Japanese soy sauce, a salt-tolerant lactic acid bacterium (LAB), so-called *Tetragenococcus halophilus*, grows at the early stage and produces lactic acid. After pH drop in the moromi, salt-tolerant yeasts such as *Zygosaccharomyces rouxii* grow and produce compounds related to flavor development during the fermentation (Fukushima, 2004). The use of suitable microbial starter cultures in both koji making and moromi fermentation can lead to consistent quality of soy sauce with production of desirable flavor compounds (Wah et al., 2013). Previously, we have reported that *Pichia guilliermondii* found in Thai soy sauce moromi fermentation produces phenolic compounds which are important volatile flavor compounds (VFCs). The strain of *P. guilliermondii* also enhances production of VFCs (*i.e.* higher alcohols) by *Z. rouxii* when co-cultured during moromi fermentation (Wah et al., 2013). To demonstrate the approach of using combination of such starter cultures in reduced-salt concentration to 12%, moromi fermentations were conducted to understand growth of LAB and activities of *Z. rouxii* with and without co-culture of *P. guilliermondii*. It is well known that salt and other solutes in food products could inhibit microbial growth by lowering the water activity (a_w) values and cause high osmotic pressure to the bacterial cells. Most pathogenic bacteria do not grow below 0.94 a_w in which equivalent to the a_w of 10% NaCl solution (Lund, Baird-Parker &Gould, 2000; Taormine, 2010; Strelkauskas, Edwards, Fahnert, Pryor, & Strelkauskas, 2016) Therefore, in this study, reduced NaCl concentration to 12% in moromi fermentation could provide safe reduced-salt soy sauce product. Hence, the trial salt-reduced fermentation was conducted and changes of microbial, physicochemical and biochemical properties were monitored in pilot scale of 3-month fermentation with industrial practice. The knowledge obtained would be beneficial for production reduced-salt soy sauce and development of rapid fermentation process.

2. Materials and methods

2.1. Microorganisms and preparation of inoculums

All starter culture strains of halotolerant lactic acid bacteria (LAB) and yeasts previously isolated during moromi fermentation of Thai soy sauce (*T. halophilus* TS71, *Z. rouxii* A22, *Meyerozyma (Pichia) guilliermondii* EM1Y52) were obtained from the culture collection of the Department of Biotechnology, Faculty of Science, Mahidol University. These LAB and yeast strains have been investigated for commercial application in food on their acid production (Sombat, 2007) and activity to produce important characteristic flavor compounds in Thai soy sauce (Wah et al., 2013) respectively. To prepare inoculum, a single colony of each strain was transferred to a proper liquid medium and statically incubated at 37 °C for TS71 or at 30 °C with 150 rpm shaking for the yeasts to obtain one unit of optical density at 600 nm (OD_{600}) (Wah et al., 2013). The cells were harvested by centrifugation at 5000 x*g*, 4 °C for 20 min. The cell pellets were then suspended to the same volume of moromi mash for further use as 5% inoculum at a concentration of ~10^6 cells/ml.

2.2. Soy sauce fermentation

The fermentation was conducted in the SS soy sauce factory in Samutprakan province and based on industrial practice of Thai soy sauce production. Koji was prepared by mixing of cooked soybeans, wheat flour and rice flour at ratio of 7:1:1 (w/w), and then inoculated with 0.1–0.2% of commercial starter *A. oryzae* and incubated at 30–32 °C for 2 days. The koji was added with brine (12% or 18%; w/v NaCl) at ratio of 2:5 (w/v), to obtain moromi mash. There were three treatments conducted at 12% (w/v) NaCl; CL: non-starter cultures; L2: co-cultures of TS71 and A22; and L3: co-cultures TS71, A22 and EM1Y52. Each treatment and control fermentation were conducted in 50-L batch in duplicate. Each starter culture was inoculated after one month of fermentation at the equal ratio. The fermentation was prolonged statically for 3 months at ambient temperature (~30 °C). These treatments and control experiment were run in comparison with industrial scale fermentation of traditional soy sauce in 2000-L fiberglass tank (CT). About 500 ml of moromi samples were taken after stirring every month (0M, 1M, 2M and 3M). Each sample was aseptically separated for microbiological analyses. The remaining was centrifuged at 5000 x*g* for 20 min to obtain the supernatant and kept at −20 °C until further analyses. At the end of 3-month moromi fermentation, all samples (1 L) were filtered by cheesecloth and then centrifuged at 5000 x*g* for 20 min. The supernatant obtained were filled in 500-ml Duran bottles and heated in water bath at 80 °C for 15 min and analyzed microbial count, physicochemical and biochemical properties.

2.3. Microbiological analyses

Moromi sample was 10-fold serially diluted in 0.5 M phosphate buffer pH 7.2. Aliquot of 100 μl was spread on agar plates of TSA (Tryptic soy agar with 10% NaCl), MRS (supplemented with 10% NaCl and 50 mg/ml cycloheximide) and YPD (supplemented with 5% NaCl and 50 mg/ml chloramphenicol) in triplicate for determining total bacteria, lactic acid bacteria and yeasts, respectively. Total bacteria and LAB were anaerobically incubated at 37 °C for 1–2 days and 2–3 days, respectively, while yeasts were aerobically incubated at 30 °C for 2–3 days. Colonies were counted from plates with 30–300 colonies appeared and reported as log CFU/ml (Park et al., 2015).

2.4. Physicochemical and biochemical properties analyses

pH of each sample was measured by pH meter at 25 °C (IQ Scientific Instrument, Inc., Carlsbaad, CA, USA). Salt concentration was determined by Volhard's method (AOAC, 2000). Total titratable acidity (%TA) was analyzed by titration of 1 ml of each sample and 10 ml of distilled water with standard 0.1 N NaOH, using phenolphthalein as an indicator and expressed as % lactic acid (AOAC, 1984). Glucose content was assayed with a commercial glucose oxidase kit (Sigma Chemical Co., St. Louis, MO). Each was performed in triplicate.

2.5. Determination of volatile flavor compounds (VFCs)

VFCs analyses were done based on the work of Wah et al. (2013). Briefly, 5 ml of moromi sample saturated with NaCl was added with 2-methyl-3-heptanone as an internal standard in a 20 ml headspace vial sealed with a PTFE septum in an aluminum cap. After preheated at 40 °C for 10 min in oil bath, VFCs in the headspace were collected with solid phase microextraction fiber Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) (Supelco,