

## Isolation and identification of halophilic microorganisms in soy sauce

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### Abstract

Microorganisms play an important role in the fermentation of soy sauce as they produce flavoring compounds which contribute to the desired taste and quality of soy sauce. The aim of this study was to isolate and identify halophilic microorganisms that are involved in the first stage (Koji) and second stage (Moromi) of soy sauce fermentation. In this study, soy sauce samples were collected from a local company located in Johor Bahru. The microorganisms were identified using Analytical Profile Identification (API) system and 16s ribosomal RNA (bacteria)/Internal Transcribed Spacer region (fungi and yeast) sequencing. In the koji fermentation, one fungus was isolated and identified as *Aspergillus oryzae*. During the moromi fermentation, one lactic acid bacteria and two yeasts were identified, including *Tetragenococcus halophilus*, *Candida versatilis* and *Candida etchellsii*. These halophilic microorganisms can be used as starter culture in moromi stage to shorten the fermentation period.

## 1. Introduction

Soy sauce is a traditional fermented food that has been widely used as a seasoning agent with a salty taste in Asian and European countries. Traditionally, soy sauce is made through natural fermentation with combination of soybean, wheat flour and salt. These combinations will produce an aromatic flavor, hence make it suitable for seasoning and coloring agent in cooking due to its salty and umami taste. In Malaysia, there are many types of soy sauces available but the most popular ones are “Kicap Masin” and “Kicap Lemak Manis”, which are equivalent to the light and dark soy sauce, respectively. Soy sauce in Malaysia is usually made traditionally and slightly different from soy sauce in Japan and China.

The process of making soy sauce involved two steps: Koji fermentation and Moromi fermentation. Koji fermentation process includes the combinations of soybean, wheat flour, and inoculation of spore (*Aspergillus oryzae*). During Koji fermentation, the mold produces proteolytic and amylolytic enzymes through enzymatic reaction which later hydrolyze the soybeans into simpler forms. Proteolytic enzymes break down proteins from soybean into peptides and amino acids. Meanwhile, amylase hydrolyzes starch from wheat flour into simpler sugars. The hydrolyzed substrate will be utilized by the natural microorganisms including yeasts

and lactic acid bacteria, and this is reflected by the decrease in pH value during moromi fermentation. The breakdown products will be converted into volatile compounds that are responsible for the development of aroma and flavor of the soy sauce.

*Tetragenococcus halophilus* is lactic acid bacteria which is involved in moromi fermentation and produces lactic acid. *Tetragenococcus halophilus* undergo fermentation to produce lactic acid and various secondary metabolites. The most common aroma compounds in soy sauce are acetic acid, formic acid, methyl acetate, benzaldehyde, ethyl-2-hydroxypropanoate, 4-hydroxy-3-methoxybenzaldehyde and 2-hydroxy-3-methyl-2-cyclopenten-1-one, which are produced by *Tetragenococcus halophilus* (Lee *et al.*, 2013). Halophilic yeasts are usually added into the fermentation process to increase the quality and enhance the flavour of soy sauce. Common aromatic halophilic yeasts are *Zygosaccharomyces rouxii*, *Candida versatilis* and *Candida etchellsii*. *Candida* sp. produces 4-ethyl-guaiacyl phenol and 4-ethyl phenol, which contribute to clove and smoked flavours. The wheat-based ingredient is hydrolysed into ferulic acid and coumaric acid. Then, it is converted into 4-ethyl-guaiacyl phenol and 4-ethyl phenol via biotransformation process. Under high-salt condition, *Candida* sp. may biotransform maltose into ethanol. In contrast, *Zygosaccharomyces rouxii* has no capability to undergo biotransformations (Feng, 2012).

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*Zygosaccharomyces rouxii* which are involved in the formation of ethanol, alcohol, isobutyl alcohol, isoamyl alcohol, 2-phenylethanol (Van Der Sluis *et al.*, 2001; Jansen *et al.*, 2003), 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone (HEMF) (Sasaki, 1996), and 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF) (Hauck *et al.*, 2003). These compounds contribute the distinct flavour in the final product during moromi stage.

The purpose of this study was to isolate the potential halophilic microorganisms that can be further implemented as starter cultures in moromi fermentation and contribute to flavor and aroma in soy sauce.

## 2. Materials and methods

### 2.1 Soy sauce samples

Few soy sauce samples were collected from a local company in Johor Bahru under aseptic condition. The samples were stored in icebox at -4°C and transported back to laboratory aseptically.

### 2.2 Isolation of halophiles from soy sauce samples

The soy sauce sample was diluted via serial dilution method. Soy sauce sample (1 mL) was diluted into 9 mL of 0.85% saline and homogenized using a vortex. The diluted sample (100 µL) was transferred onto Potato Dextrose agar, MRS agar (supplemented with 1.5% CaCO<sub>3</sub>, 12% NaCl, 4 µg/mL sodium azide and 0.1% cycloheximide) and Malt Extract Agar (supplemented with 5% NaCl and 50 mg/mL chloramphenicol) to isolate fungal, lactic acid bacteria and yeast, respectively. Salt concentration on MRS agar and MEA agar varied from 8% to 19% NaCl. MRS agar was incubated anaerobically at 37°C for 3-5 days. Malt Extract and Potato Dextrose agar were incubated at 30°C for 3-5 days. The isolation work was carried out in the laminar-flow chamber under sterile and aseptic conditions. The growing colonies were observed daily to check for bacterial and fungal growth.

### 2.3 Morphological identification

Gram staining was performed for the halophilic lactic acid bacteria and yeasts to identify their shape and arrangement of the cells. Crystal violet staining reagent was applied to the heat-fixed smear for 60 s and washed the slide with water. Then, the slide was flooded with Iodine for 60 s and washed the slide with water. Decolorizing agent was applied to the smear for 10-15 s and safranin was flooded for 30-60 s. The slide was washed gently and blotted dry with absorbent paper. Result of stained bacterial smear was observed under light microscope (100x) using immersion oil.

### 2.4 Catalase test

Catalase test was done to identify the presence of catalase, an enzyme that was able to hydrolyze hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) into oxygen and water with a visible effervescence. 18 to 24 hrs bacterial colony was smeared on a clean glass slide and dropped with 3% hydrogen peroxide solution. Visible effervescence indicated catalase-positive organism (Reiner, 2010).

### 2.5 Genomic DNA extraction

Genomic DNA of halophilic lactic acid bacteria and yeasts were extracted using heat treatment. Two colonies from an overnight culture were selected. The colonies were suspended in 1 mL of sterile ddH<sub>2</sub>O and boiled at 100°C for 10 mins in water bath. The suspension was centrifuged at 1000 rpm for 5 mins to collect the supernatant and discard the pellet (Dashti *et al.*, 2009).

### 2.6 PCR

Halophilic lactic acid bacteria and yeasts were identified using species barcoding full length 16s rRNA and ITS region, respectively. Bacterial 16s rRNA gene was amplified via PCR with universal primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-ACG GYT ACC TTG TTA CGA CTT-3'). ITS region of the halophilic yeasts was targeted using both ITS1 (50-TCCGTAGGTGAACCTGCGG-3') and ITS4 (50-TCCCTCCGCTTATTGATATG-3') primers. The targeted DNA was amplified in the total volume of 50 µL containing 25 µL of 2x PCRBIO HS Taq Mix, 5 µL of template DNA, 2 µL of 0.4µM reverse primer, 2 µL of 0.4µM forward primer, and 16 µL of sterile ddH<sub>2</sub>O. DNA amplification through PCR begun with 5 mins of predenaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 60 s, and elongation at 72°C for 5 mins. The PCR product was detected through 1.5% agarose gel electrophoresis at voltage 100V for 15-20 mins and band was observed under UV light. The amplified target DNA was cut and placed in 1.5 Eppendorf tube, then followed by sequencing. The result was analysed with sequence homology via NCBI Blast database. The phylogenetic tree was constructed with MEGA version 5.0 (Jiang *et al.*, 2019).

## 3. Results and discussion

### 3.1 Isolation and identification of halophiles

Halophilic lactic acid bacteria and yeasts were grown on MRS media and MEA media, respectively, and counted within 30-300 colony forming unit (CFU)/mL. Lactic acid bacteria grew on MRS with salinity of 8%, 10%, 12% and 14% NaCl and there were significant

differences between them. Based on Figure 1, the highest lactic acid bacteria count was at 8% NaCl ( $3.6 \times 10^3$  CFU/mL) while the lowest count was at 10% NaCl ( $2.4 \times 10^3$  CFU/mL). The lactic acid bacteria count decreased as from 8% NaCl to 10% NaCl, but the count increased slightly at 12% NaCl. Apart from lactic acid, *Micrococcus*, *Bacillus*, *Streptococcus* and other related bacteria are also present in the soy sauce mash (Ho et al., 1984; Roberts et al., 2000; Tanasupawat et al., 2002). Their existence contributes to high bacterial count at 8% salt concentration. As the salt concentration increases to 10%, most of the mesophilic bacteria could not tolerate with such salinity, hence incapable to survive. Halophilic lactic acid bacteria dominate the entire moromi fermentation when the salt concentration increased to 12%. There was significant difference between yeasts count on different salt concentrations in MEA agar. The yeast count gradually decreased as the salt concentration increased. Figure 1 shows the highest yeast count was at 8% NaCl ( $2.1 \times 10^6$  CFU/mL) while the lowest at 19% NaCl ( $3.67 \times 10^4$  CFU/mL). When salt concentration increased, the chance of survival for halophilic yeasts decreased. Based on Figure 2, there is significant difference between growth of lactic acid bacteria and yeasts at salinity 8% NaCl. As compared with the bacterial ( $3.6 \times 10^3$  CFU/mL) and yeasts ( $2.1 \times 10^6$  CFU/mL) count on salinity 8% NaCl, yeasts have a higher count than lactic acid bacteria. This is due to the domination of yeasts population during moromi fermentation and the finding was parallel with the theory explained by Van Der Sluis (2001). During the earlier stage of moromi fermentation, lactic acid bacteria grew exponentially and produced lactic acid. As lactic acid produced by bacteria accumulated in the moromi, the pH dropped below 5.0 and the condition was ambient enough for the yeasts to grow. The halophilic yeasts utilized the glucose and other compounds to produce desirable aroma and volatile compounds.

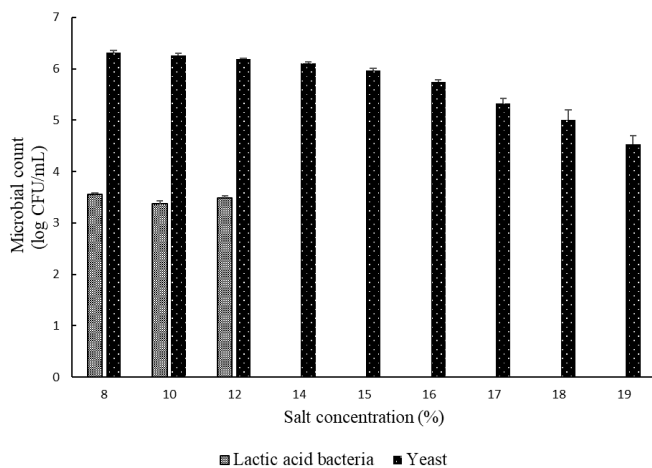


Figure 1. Halophilic microbial count at salt concentration 8% to 19% NaCl

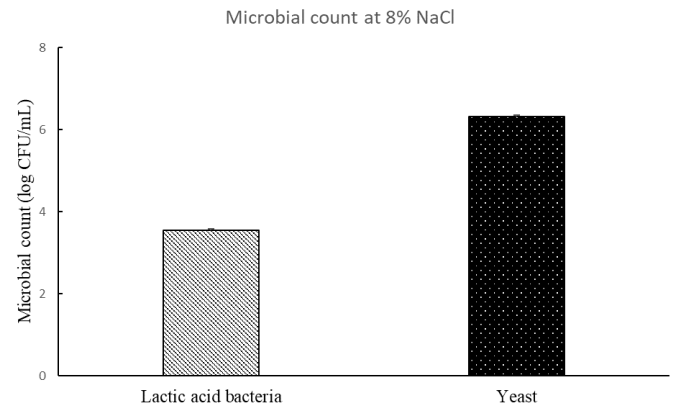


Figure 2. Microbial count at 8% salt concentration

The halophilic lactic acid bacteria were observed under light microscope as Gram-positive, coccus and clustered arrangement. The morphology was shown as small, circular, flat and entire colony. Catalase test showed negative results in lactic acid bacteria because no effervescence was formed. Hence, it is an anaerobic bacteria. Meanwhile, halophilic yeasts were stained and observed as oval shape and budding under the light microscope.

The extracted genomic DNA was amplified and viewed on 1.5% agarose gel under UV light. Clear bands were shown. The isolated strains were further identified through comparing genomic sequence with NCBI Blast database. Two species of halophilic lactic acid bacteria were identified as *Tetragenococcus halophilus* with similarity 100% and fungal was identified as *Aspergillus oryzae* with similarity 100%. On the other hand, five strains of halophilic yeast were identified as *Candida versatilis* and *Candida etchellsii* with both similarity of 100%. *Tetragenococcus halophilus*, *Candida versatilis* and *Candida etchellsii* were widely found in moromi fermentation and implemented as starter cultures in production of high-salt soy sauce with salinity from 18% to 24% NaCl in Chinese soy sauce (Wanakhachornkrai and Lertsiri, 2003).

#### 4. Conclusion

Halophilic microorganisms were isolated and identified as *Aspergillus oryzae*, *Tetragenococcus halophilus*, *Candida etchellsii* and *Candida versatilis*. *Aspergillus oryzae* grew during Koji fermentation and produced enzymes that were further utilized by halophiles in moromi fermentation. Meanwhile, *Tetragenococcus halophilus*, *Candida etchellsii* and *Candida versatilis* survived during moromi fermentation and produced aromatic flavouring compounds which contributed to taste and quality of soy sauce. These halophilic microorganisms will be used as starter culture in koji and moromi to shorten the fermentation period and produce excellent quality of soy sauce.

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