Food Research 7 (Suppl. 1) : 80 - 90 (2023)
Journal homepage: https://www.myfoodresearch.com

FOOD RESEARCH

FULL PAPER

Amylase, protease, and lipase-producing microbes of local origin as potential starter cultures for low-salt *moromi* fermentation

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Article history:
Received: 5 February 2022
Received in revised form: 25 March 2022
Accepted: 4 October 2022
Available Online: 27 April 2023

Keywords:
Starter cultures,
Low-salt soy sauce,
Fermentation,
Aspergillus,
Bacillus

DOI:
https://doi.org/10.26656/fr.2017.7(S1).22

Abstract

High salt concentration (18%-22% NaCl) is essential for halophilic microbes to grow and develop aroma during soy sauce fermentation. Due to health concerns, recent efforts have focused on reducing salt during fermentation by introducing novel yeast species. However, the natural fermentation of soy sauce involves a more complex microbial community essential for achieving optimal sensory qualities. This study aimed to select potential bacterial and fungal species from traditional soy sauce factories in Indonesia for use in low-salt fermentation. Isolates that showed positive enzymatic activities at 20%, as well as 5% NaCl, were subjected to growth profile characterisation in tryptic soy broth (TSB) with different salt concentrations (0%, 5%, and 20%) and pH (4.0, 5.0, and 6.0). A total of 47 bacterial and 39 fungal isolates were obtained. Fourteen bacterial and 12 fungal isolates demonstrated positive enzymatic activities at 20% and 5% NaCl. The majority of bacterial strains were identified as *Bacillus subtilis*, while most of the fungal isolates belonged to the *Aspergillus* genus (*Aspergillus oryzae* and *Aspergillus tamarii*). The majority of *Bacillus* isolates performed rapid growth in 5% NaCl and relatively low pH (5.0), indicating their potential application in low-salt soy sauce production.

1. Introduction

Soy sauce is a traditional fermented condiment that is widely used in many Asian cuisines. Although the production practice varies among countries and regions, three main ingredients are used in soy sauce production, including soybeans, wheat, and brine. These ingredients are fermented through a two-step process, called *koji* and *moromi*, also known as solid-state and brine fermentation, respectively (Lioe *et al*., 2004; Wah *et al*., 2013; Feng *et al*., 2015; Song *et al*., 2015;). In *moromi*, brine contains high NaCl concentration ranging from 18% to 22%, which is important to prevent the growth of spoilage and pathogenic microbes while encouraging the growth of halophilic microbes essential for flavour and aroma development. However, high sodium consumption is associated with an increased risk of high blood pressure, and therefore cardiovascular diseases (World Health Organization, 2018), which led to the NaCl reduction initiatives of food products. Furthermore, as consumer awareness of healthier lifestyles continues to grow, the demand for low-sodium food products increases. Therefore, it gives considerable opportunities for the soy sauce industry to diversify its product to a healthier low-salt soy sauce.

Reducing NaCl content in soy sauce remains a challenge since it can negatively impact soy sauce's sensory quality and safety (Song *et al*., 2015). Some soy sauce desalting methods have been previously studied and patented, including nanofiltration (Luo *et al*., 2009), ion exchange (Motoki *et al*., 1977), reverse osmosis (Otomi *et al*., 1992), freezing (Watanabe *et al*., 1996) and extraction (Matsuyoshi *et al*., 1998). Although some of these methods are claimed to have no adverse effect on the soy sauce flavour, they seem to be nonviable for commercial purposes due to high operating costs. More recent studies have focused on reducing the NaCl content during fermentation by introducing appropriate starter cultures. Song *et al*. (2015) have demonstrated the feasibility of performing low-salt *moromi* fermentation using two yeast species isolated from Korean traditional soy sauce, namely *Torulaspora delbrueckii* JBCC-623 and *Pichia guilliermondii* JBCC-848. These yeast strains were able to preserve the sensory properties of low-salt soy sauce without compromising its safety. Moreover, another strain of *P. guilliermondii* found in Thai soy sauce was shown to enhance the production of aroma compounds in soy sauce when co-cultured with common soy sauce starter cultures, *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* (Singracha *et al*., 2017). However, those studies only focused on yeast isolates, while *moromi* fermentation is driven by a complex

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eISSN: 2550-2166
Published by Rynnye Lyan Resources

microbial community, which is also made up of various halotolerant bacterial and mould species (Han *et al*., 2020; Ito and Matsuyama, 2021; Liu *et al*., 2021; Qi *et al*., 2021). These microorganisms produce extracellular enzymes that break down starch, protein, and lipid contained in the raw materials into precursors of flavour and aroma compounds. Since the essential microbes perform best in a high-salt environment, finding new microbial isolates that maintain their growth and enzymatic activity in a low-salt environment is paramount in retaining or improving the sensory quality of low-salt soy sauce. This study aims to isolate amylase, protease, and lipase-producing microbes potential for future applications as low-salt soy sauce starter cultures.

2. Materials and methods

2.1 Sample collection

Koji and *moromi* samples were collected from three local traditional soy sauce factories in Java, Indonesia. The age of *koji* samples varied from 3 to 7 days, while the age of *moromi* was ranging from 7 to 30 days. Samples were collected in sterile 50 mL falcon tubes and stored in -20ºC freezer until analysis.

2.2 Microbial isolation

Microbial isolation from *koji* and *moromi* samples was performed by homogenizing 1 g of each sample in 9 mL of phosphate-buffered saline (PBS) (Sigma-Aldrich, Saint Louise, USA). The homogenized mixture was then subjected to serial dilution, followed by spread plating onto three types of agar media supplemented with 5%, 10%, and 20% NaCl (Merck, Darmstadt, Germany). Total mesophilic aerobic bacteria (TMAB) were isolated using nutrient agar (NA) (Merck, Darmstadt, Germany) supplemented with cycloheximide (10 µg/mL) (Sigma-Aldrich, Saint Louise, USA). Lactic acid bacteria (LAB) were isolated using de Man, Rogosa, and Sharpe (MRS) agar (Merck, Darmstadt, Germany), added with cycloheximide (10 µg/mL). Fungi (yeast and mould) were isolated using potato dextrose agar (PDA) (HiMedia, Mumbai, India) supplemented with chloramphenicol (50 µg/mL) (HiMedia, Mumbai, India). NA was incubated for 48 hrs at 30°C, MRS agar was incubated for 72 hrs at 37°C, and PDA was incubated for 96 hrs at 25°C. Colonies with distinct morphologies were picked and purified several times using a four-way streak method on the same agar medium. The purified colonies were maintained in 30% glycerol solution and stored at -80°C.

2.3 Screening for amylase, protease, and lipase activity

The ability of bacterial and fungal isolates to produce amylase, protease, and lipase was tested at 5% and 20% NaCl concentration in three replicates. Amylase activity was tested using starch agar medium, containing 1% soluble starch (Merck, Darmstadt, Germany), 5 g/L peptone (Becton Dickinson, Le Pont de Claix, France), 3 g/L yeast extract (HiMedia, Mumbai, India), and 15 g/L agar (Deben Diagnostics, Suffolk, UK). For bacteria, the agar plates were incubated at 37ºC for 24 hrs, while for fungal isolates, the agar plates were incubated at 25ºC for 96 hrs. Positive colonies were selected based on the formation of a clear zone after an iodine solution was poured onto the agar.

Protease activities were observed by culturing the isolates on skim milk agar. The skim milk agar used contained 1% skim milk powder (Interfood, Jakarta, Indonesia), 5 g/L peptone, and 15 g/L agar. The skim milk and peptone-agar mixture were autoclaved separately at 115ºC for 10 mins and 121ºC for 15 mins, respectively, and left to cool down. Then, they were mixed using a homogenizer (Wise-Tis, UK) and poured into Petri dishes. The agar plates were incubated for 24 hrs at 37ºC for bacteria, and 96 hrs at 25ºC for fungi. Positive results were indicated by the formation of a clear zone around the colony.

The screening was done on tributyrin agar for lipase activities. The agar contained 1% liquid tributyrin (HiMedia, Mumbai, India), 5 g/L peptone, 3 g/L yeast extract, and 15 g/L. Bacterial isolates were incubated for 24 hrs at 37ºC, while fungal isolates were incubated for 96 hrs at 25ºC. Positive results were shown by clear zones surrounding the colonies. Each experiment was conducted in triplicate (N = 3).

2.4 Growth profile of isolates at different NaCl concentrations and pH

The isolates showing positive results for amylase, protease, and lipase at 20% and 5% NaCl were selected for growth assay at different NaCl concentrations (0%, 5%, and 20% NaCl) and pH (pH 4.0, 5.0, and 6.0). The selected isolates were first activated in tryptic soy broth (TSB) (Merck, Darmstadt, Germany) media, supplemented with 5% of NaCl, and the pH was adjusted to 5.0. The study of growth profile was done in triplicate (N = 3) in a 96-well plate with 200 µL of working volume. For the growth profile characterisation at different NaCl concentrations, the initial pH of TSB was adjusted to 5.0, while the NaCl concentration was varied to 0%, 5%, and 20%. For the growth study at different pH, the NaCl concentration used was 5%, and the pH was varied to 4.0, 5.0, and 6.0, using HCl or NaOH. Each isolate was inoculated to the medium to a final optical density (OD) of ~0.1. The isolates were then incubated statically at 30ºC, and the growth was assessed by measuring the OD at λ 600 nm periodically using a

https://doi.org/10.26656/fr.2017.7(S1).22

plate reader (NanoQuant Plate™ 200, Tecan, Switzerland).

2.5 DNA extraction

Bacterial DNA was extracted by breaking the cells using a thermal cycler (SimpliAmp™, Thermo Fisher Scientific, Waltham, MA, USA) at 94ºC for 10 mins (Romo *et al*., 2007). For fungi, DNA extraction was done using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA), according to manufacturer instruction. First, the fungal isolates were cultured in 10 mL of yeast-peptone broth at 25ºC for seven days and two days, respectively, in a shaker incubator. Then, the cultures were centrifuged at 16,000×*g* for 2 mins. The pellets were then washed twice using 293 µL of 50 mM EDTA followed by resuspension in 300 µL of nuclei lysis solution and 100 µL of protein precipitation solution using vortex for 20 s. After that, the supernatant was transferred to 1.5 mL microtubes containing 300 µL of isopropanol and centrifuged at 16,000×*g* for 2 mins. The supernatant was collected and mixed with 70% ethanol, followed by another round of centrifugation at 16,000×*g* for 2 mins. Then, the supernatant was removed, and the pellet was left to dry for 30 mins. The pellet was rehydrated using 50 µL of DNA rehydration solution and 1.5 µL of RNAse solution. After brief centrifugation, the samples were incubated at 37ºC for 15 mins and stored at 4ºC before further use.

2.6 PCR amplification

DNA samples obtained from the previous step were then subjected to partial gene amplification using a thermal cycler (SimpliAmpTM, Thermo Fisher Scientific, USA). For bacterial isolate identification, the *16S rRNA* gene was amplified using 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). For fungal isolates, universal primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG -3') and NL4 (5'- GGTCCGTGTTTCAAGACGG-3') were used to amplify the D1/D2 domain of *26S rRNA* gene. Each PCR reaction contained 10 µL 5× GoTaq, 4 µL of 25 mM $MgCl_2$, 1 µL dNTPs, 1 µL of 10 µM primer (forward and reverse), 0.25 mL Taq polymerase, 5 µL template and 27.75 µL nuclease-free water. The following PCR conditions for primer pair 27F and 1492R were used: initial denaturation at 94ºC for 5 mins, 40 cycles of denaturation at 95ºC for 30 s, annealing at 60ºC for 1 min, extension at 72ºC for 45 s, and a final cycle at 72ºC for 10 mins (Jang *et al*., 2011). For primer pair NL1 – NL4, the following PCR conditions were set: initial denaturation at 95ºC for 2 mins, 35 cycles of denaturation at 95ºC for 30 s, annealing at 57ºC for 1 min, extension at 72ºC for 60 s, and a final cycle at 72ºC for 10 mins (Hajar *et al*., 2012).

2.7 Amplified DNA analysis using gel electrophoresis

The amplified DNA from the previous step was analysed using gel electrophoresis. Agarose gel was prepared by solubilising 2.4 g of agarose in 120 mL of 1× TAE buffer using a microwave. The agar solution was cooled down to 60ºC, mixed with 3 µL of EtBr, and left to solidify in a casting tray. The agarose gel was then placed in a tank containing 1× TAE buffer. DNA ladder (5 µL, 1 kb) was also run along with the samples at 120 V, 400 mA for 60 mins (Murakami *et al*., 2009). Gel visualisation was done using SynGene Gel Documentation System with UV light. Then, the DNA samples were sent to Macrogen Inc., South Korea, for further analysis. The DNA sequence was then analysed using BLAST to compare them with sequences in the GenBank database.

3. Results and discussion

In this study, halophilic microbes important to the fermentation of soy sauce were isolated from three traditional factories and screened based on their enzymatic activities, including amylase, protease, and lipase. Starch, protein, and lipid hydrolysis during soy sauce fermentation are crucial for generating small molecules that contribute to sensory qualities and nutritional value of the final product (Su *et al*., 2005; Cui *et al*., 2014). In this study, a total of 47 bacterial (Table 1) and 39 fungal (mould and yeast) (Table 2) isolates with distinctive morphology were obtained. Although all the isolates were halotolerant, 30% of bacterial and 13% of fungal isolates did not show any of amylase, protease, or lipase activity at either 5% or 20% NaCl. The results indicated that these isolates might play a role in metabolizing substances other than starch, protein, and lipid during *moromi* fermentation.

A number of bacterial and fungal isolates that displayed enzymatic activities at 5% NaCl lost their activities when cultured at 20% NaCl (Figure 1). At 5% NaCl, most of the bacterial isolates showed positive results for amylase (57%), followed by protease (45%) and lipase (40%). In contrast, the numbers dropped to 32%, 4%, and 4%, respectively, when the isolates were cultured at 20% NaCl. Unlike bacteria, at 5% NaCl, the majority of fungal isolates showed positive results for lipase (72%), followed by protease (46%), and amylase (38%) (Figure 1). When cultured at 20% NaCl, only 5% of fungal isolates exhibited lipase activity, while 31% and 28% of isolates showed amylase and lipase activity, respectively. Since high salt concentration is inhibitory to the growth of most microbes, their enzymatic

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Table 1. Qualitative assay of amylase, protease, and lipase produced by bacterial isolates at 5% and 20% NaCl concentration

Isolate code	Source	5% NaCl			20% NaCl		
		Amylase	Protease	Lipase	Amylase	Protease	Lipase
12.1.KK.NA	*koji*	-	-	-	-	-	-
12.1.KK.NB	*koji*	-	-	-	-	-	-
12.1.KM1.NA	*moromi*	+	+	+	+	-	-
12.1.KM1.NB	*moromi*	-	+	+	-	-	-
12.1.KM1.NC	*moromi*	+	+	+	+	-	-
12.1.KM1.ND	*moromi*	+	+	+	+	-	-
12.1.KM1.NE	*moromi*	+	+	+	+	-	-
12.1.KM1.NF	*moromi*	+	+	+	+	-	-
12.1.KM2.NA	*moromi*	+	-	+	+	-	-
12.1.KM2.NB	*moromi*	+	+	+	-	-	-
12.1.KM2.NC	*moromi*	+	+	+	-	-	-
12.1.KM2.ND	*moromi*	+	+	+	+	-	-
12.1.KM2.NE	*moromi*	-	-	-	-	-	-
12.1.KM2.NF	*moromi*	-	-	+	-	-	-
12.1.KM2.NG	*moromi*	-	-	-	-	-	-
12.1.KM2.NH	*moromi*	-	+	-	-	-	-
12.1.KM2.NJ	*moromi*	+	+	+	+	-	-
12.1.KK.MA	*koji*	-	-	-	-	-	-
12.1.KK.MB	*koji*	-	-	-	-	-	-
12.1.KK.MC	*koji*	-	+	-	-	-	-
12.1.KM1.MA	*moromi*	-	-	-	-	-	-
12.1.KM1.MB	*moromi*	-	-	-	-	-	-
12.1.KM2.MA	*moromi*	-	-	-	-	-	-
12.1.KM2.MB	*moromi*	-	-	-	-	-	-
12.2.KK.N10N-4.B	*koji*	-	-	-	-	-	-
12.KK.N10N-4.C	*koji*	-	-	-	-	-	-
12.2.KK.N10N-2.A	*koji*	-	-	-	-	-	-
12.2.M1.N10N-2.A	*moromi*	+	+	+	+	+	+
12.2.M2.N10N-2.A	*moromi*	+	+	+	+	-	-
12.2.M1.N5N-4.A	*moromi*	+	+	+	+	-	-
12.2.M1.N5N-4.B	*moromi*	+	+	+	+	-	-
12.2.M2.N10N-2.B	*moromi*	+	+	+	+	+	+
12.2.M2.N5N-4.A	*moromi*	+	+	-	+	-	-
12.2.M2.N5N-4.B	*moromi*	+	-	-	+	-	-
12.2.KK.N5N-4.A	*koji*	-	+	-	-	-	-
12.3.KMD.N5N.A	*moromi*	+	-	-	-	-	-
12.3.KMD.N5N.B	*moromi*	+	-	-	-	-	-
12.3.KK.N5N.B	*koji*	-	-	-	-	-	-
12.3.KMF.N10N.B	*moromi*	-	+	+	-	-	-
12.3.KMD.N5N.C	*moromi*	+	-	-	-	-	-
12.3.KMD.N10N.B	*moromi*	+	-	-	-	-	-
12.3.KMD.N10N.C	*moromi*	+	-	-	-	-	-
12.3.KMD.N10N.A	*moromi*	+	-	-	-	-	-
12.3.KMF.N10N.A	*moromi*	+	+	+	-	-	-
12.KK.N5N.A	*koji*	+	-	-	-	-	-
12.3.KK.M10N.A	*koji*	+	-	-	-	-	-
12.3.KK.M10N.B	*koji*	+	-	-	-	-	-

https://doi.org/10.26656/fr.2017.7(S1).22

Table 2. Qualitative assay of amylase, protease, and lipase produced by fungal (mould and yeast) isolates at 5% and 20% NaCl concentration

Isolate code	Source	5% NaCl			20% NaCl		
		Amylase	Protease	Lipase	Amylase	Protease	Lipase
12.1.KK.PA	*koji*	-	-	+	-	-	-
12.1.KK.PB	*koji*	-	-	+	-	-	-
12.1.KK.PC	*koji*	-	-	-	-	-	-
12.1KK.PD	*koji*	-	-	+	-	-	-
12.1.KK.PE	*koji*	-	-	-	-	-	-
12.1.KK.PF	*koji*	-	-	+	-	-	-
12.1.KK.PG	*koji*	-	+	-	-	+	-
12.1.KK.PH	*koji*	+	+	+	-	+	-
12.1.KK.PI	*koji*	+	+	+	+	+	-
12.1.KK.PJ	*koji*	+	+	+	+	+	-
12.1.KM1.PA	*moromi*	+	+	+	+	+	-
12.1.KM1.PB	*moromi*	+	-	+	+	-	-
12.1.KM1.PC	*moromi*	+	+	+	+	+	-
12.1.KM1.PD	*moromi*	-	+	-	-	-	-
12.1.KM2.PA	*moromi*	-	-	-	-	-	-
12.1.KM2.PB	*moromi*	-	+	-	-	-	-
12.1.KM2.PC	*moromi*	-	+	-	-	-	-
12.2.M1.P5-2.A	*moromi*	+	+	-	+	+	-
12.2.M1.P5-2.C	*moromi*	-	-	+	-	-	+
12.2.M1.P5-3.C	*moromi*	+	+	+	+	+	-
12.2.M2.P5-3.A	*moromi*	-	-	+	+	-	-
12.2.M2.P5-3.B	*moromi*	+	+	+	+	+	-
12.2.KK.P5-4.A	*koji*	+	+	+	+	+	-
12.2.KK.P5-4.B	*koji*	+	+	+	+	+	+
12.2.KK.P5-4.C	*koji*	+	-	+	+	-	-
12.3.4KMD.P5N.C	*moromi*	-	+	+	-	-	-
12.3.4KMD.P5N.B	*moromi*	-	-	-	-	-	-
12.3.2KMF.P10N.B	*moromi*	+	-	+	-	-	-
12.3.KK.P5N.A	*koji*	+	+	+	-	-	-
12.3.KK.P5N.C	*koji*	+	+	+	-	-	-
12.3.KMD.P10N.C	*moromi*	-	-	+	-	-	-
12.3.3KMD.P10N.B	*moromi*	-	-	+	-	-	-
12.3.KMD.P10N.B	*moromi*	-	-	+	-	-	-
12.3.2KMF.P10N.A	*moromi*	-	-	-	-	-	-
12.3.KK.P5N.B	*koji*	-	+	+	-	-	-
12.3.3KMD.P10N.A	*moromi*	-	-	+	-	-	-
12.3.KK.P10N.B	*koji*	-	-	+	-	-	-
12.3.KK.P10N.C	*koji*	-	-	+	-	-	-
12.3.KK.P10N.A	*koji*	-	-	-	-	-	-

activities are also expected to be inhibited (Su and Lee, 2001). This phenomenon could result in the extension of the time period required for the enzymatic hydrolysis, consequently, the overall maturation of high salt soy sauce (van der Sluis *et al*., 2001; Yang *et al*., 2017). Therefore, lowering salt concentration during *moromi* fermentation could shorten the fermentation period, as the amylolytic, proteolytic, and lipolytic activity of the microbes are higher.

The isolates that displayed enzymatic activities at 20% NaCl indicated their prominent roles in *moromi* fermentation. Most of them also showed enzymatic activities at 5% NaCl, except for fungal isolate 12.2.M2.P5-3.A. Since these isolates showed enzymatic activities at both NaCl concentrations, they were considered to be the potential candidates for low-salt *moromi* starter cultures. From 39 fungal isolates, there were 11 isolates for amylase positive, 11 isolates for protease positive, and 2 isolates for lipase positive (Table 2). Meanwhile, from the 47 bacterial isolates, there were 15 isolates for amylase positive, 2 isolates for protease positive, and 2 isolates for lipase (Table 1).

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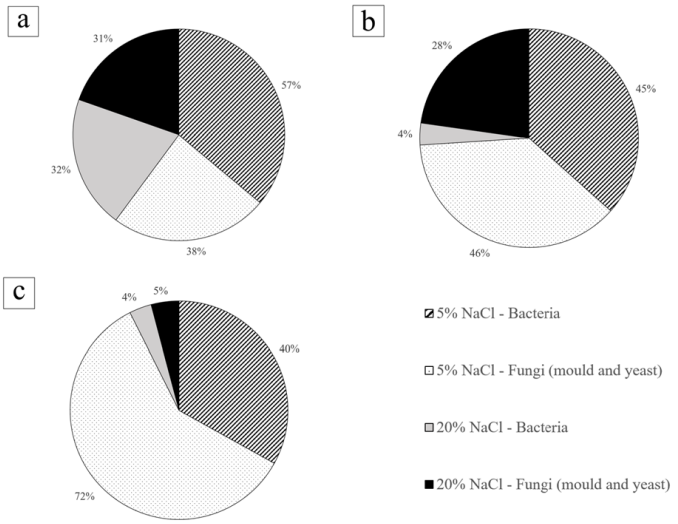

Figure 1. The number of bacterial and fungal (mould and yeast) isolates showing positive results for amylase (a), protease (b), and lipase (c) at 5% and 20% NaCl concentrations after culturing on starch, skim milk, and tributyrin agar, respectively.

Moromi fermentation process is mainly driven by bacteria and yeast (Yan *et al*., 2013; Singracha *et al*., 2017). In this study, after establishing the enzymatic activities of the isolates at different NaCl concentrations, the isolates showing positive enzymatic activities at both NaCl concentrations were then selected for growth profile characterisation. The characterisation only focused on bacterial isolates as none of the yeast isolates showed positive enzymatic activities. In general, the growth of isolates was suppressed in the presence of NaCl, and the inhibition was proportional to the NaCl concentration (Figure 2). None of the isolates reached OD above 0.2 when cultured in 20% NaCl, while in 0% and 5% NaCl, the maximum OD values reached were 1.66 and 1.25, respectively. Interestingly, some bacterial isolates (12.1.KM1.N10ND, 12.1.KM2.N10NJ, 12.1.KM1.N10NF, 12.2.KM1.N10N-2.A, 12.1.KM1.N5NA, and 12.1.KM1.N5NC) maintained their growth when the NaCl concentration was increased from 0% to 5% NaCl. The rapid growth of the bacterial isolates in 5% NaCl indicated their potential to perform faster starch, protein, and lipid hydrolysis during low-salt *moromi* fermentation and therefore, may accelerate the *moromi* maturation period. Moreover, the rapid growth also demonstrates the bacteria's competitiveness against unwanted microorganisms that are more likely to be present when the salt concentration is lowered.

Another strategy that could be done to prevent the growth of undesirable microbes is by lowering the pH of *moromi* since the growth of most microbes is inhibited at low pH. However, pH 4.0 was found to be unsuitable for the isolates to grow, as none of the isolates reached OD above 0.08 (Figure 3). Most of bacterial isolates (12.1.KM2.N5NA, 12.1.KM1.N10ND, 12.1.KM2.N10NJ, 12.1.KM1.N10NF, 12.2.KM2.N5N-4.B, 12.2.KM1.N5N5-4.B, 12.2.KM1.N10N-2.A, 12.1.KM1.N5NC, 12.2.KM2.N5N-2.A, and 12.2. KM2. N10N-2.B) grew best at pH 6.0 and maintained their growth at pH 5.0. Bacterial isolate 12.2.KM2.N10N-2.A was the only isolate that showed a decrease in growth when cultured in pH 5.0, as its maximum OD decreased from 1.13 to 0.27. Interestingly, some bacterial isolates (12.2.KM1.N5N-4.A, 12.1.KM2.N5ND, and 12.1.KM1.N5NA) exhibited higher growth in pH 5.0 than pH 6.0. In natural fermentation, these isolates probably grow in the later stage of *moromi* fermentation when the pH is becoming acidic due to the growth of lactic acid bacteria (Syifaa *et al*., 2016).

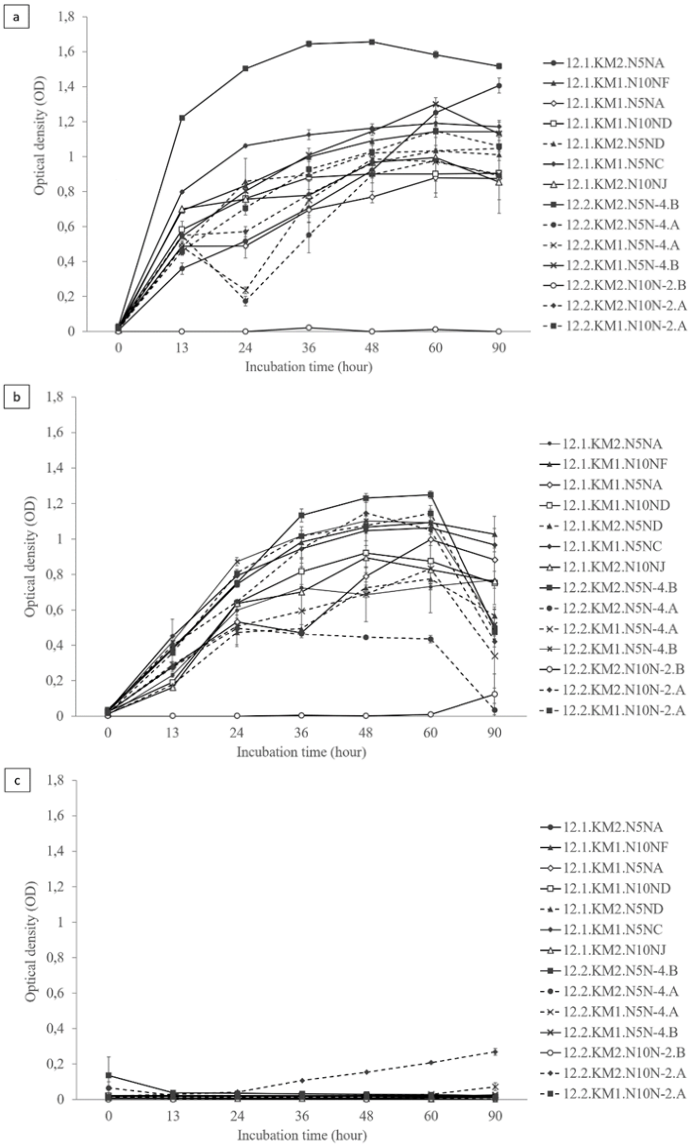

Figure 2. The optical density (OD) of the selected bacterial isolates in tryptic soy broth (TSB) medium (pH 6.5) containing 0% NaCl (a), 5% NaCl (b), and 20% NaCl (c), over 96 hrs at 30ºC.

The bacterial and fungal isolates showing positive enzymatic activities at 20% NaCl as well as 5% NaCl were selected for identification through DNA sequencing. The majority of selected bacterial isolates belonged to the genus *Bacillus* (Table 3). Six isolates (12.1.KM1.N5NA, 12.1.KM1.N5NC,

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12.1.KM1.N10ND, 12.2.KM1.N5N-4.B, 12.2.KM2.N5N-4.A, and 12.1.KM1.N10NF) were identified as *Bacillus subtilis*, 2 isolates (12.2.KM2.N10N-2.A and 12.1.KM2.N5NA) were identified as *Bacillus licheniformis*, and 2 isolates (12.1.KM2.N5ND and 12.1.KM2.N10NJ) were identified as *Bacillus* sp. (Table 3).

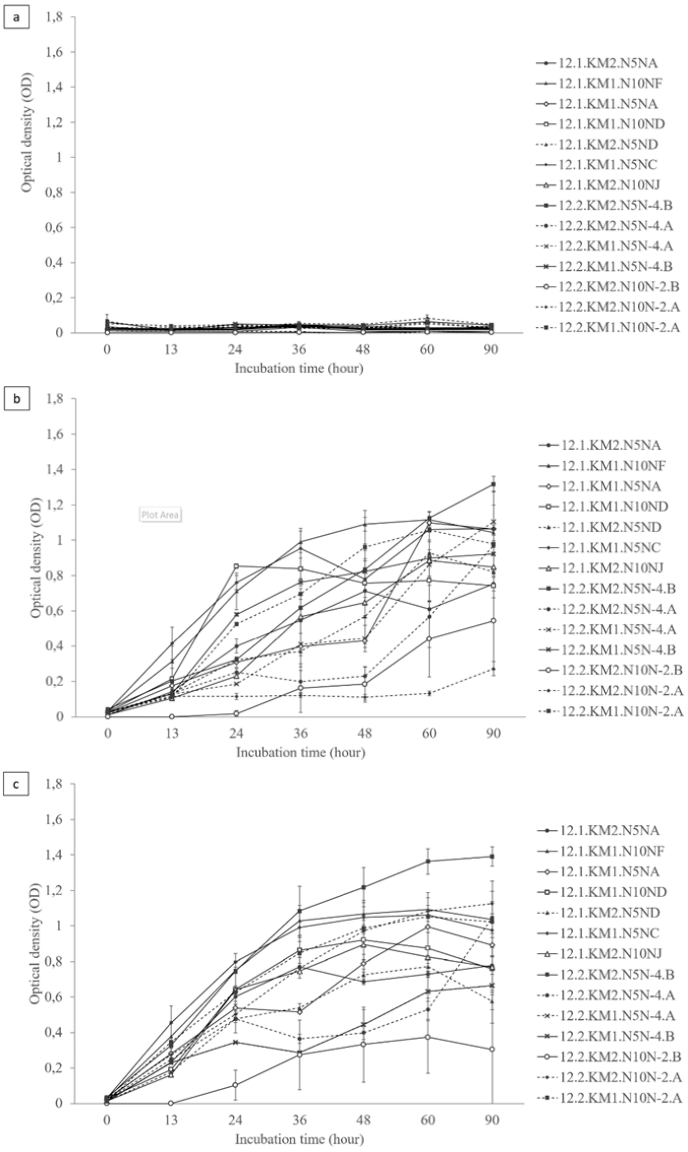

Figure 3. The optical density (OD) of the selected bacterial isolates in tryptic soy broth (TSB) medium (5% NaCl) at pH 4.0 (a), pH 5.0 (b), and pH 6.0 (c) over 96 hrs at 30ºC.

Bacillus subtilis is an endospore-forming, Gram-positive, and rod-shaped bacteria, which is able to grow in 5%-10% NaCl (Kim *et al*., 2011). *B. subtilis* has been reported as one of the dominant species frequently found not only during *koji*, but also *moromi* step of soy sauce fermentation, due to its salt tolerance (Yan *et al*., 2013; Wei *et al*., 2013; Song *et al*., 2015; Yang *et al*., 2017). Its prevalence has also been reported in other soybean-based fermented foods, such as *doenjang* (Nam *et al.,* 2012), buckwheat *sokseongjang* (Eom *et al*., 2014), *cheonggukjang* (Nam *et al.,* 2012), *douchi* (Chen *et al*., 2011), and *tempeh* (Barus *et al*., 2017). *B. subtilis* is known as an excellent amylase and protease producer, which makes it an attractive starter culture for improving the fermentation process and enhancing the umami taste of soy sauce (Sakaguchi, 1959; Kijima and Suzuki, 2007; Det-Udom *et al*., 2019; Jiang *et al*., 2019). Furthermore, *B. subtilis* has been reported to enhance the formation of several volatile compounds responsible for soy sauce characteristics aromas, such as guaiacol, 4-vinyl guaiacol, and other phenolic substances (Jiang *et al*., 2019). A recent study conducted by (Lee *et al.,* 2017) has demonstrated some probiotic properties of *B. subtilis* isolated from Korean soy sauce. The presence of this bacteria in soy sauce was claimed to be safe as they showed antimicrobial activity against pathogenic microbes, did not produce biogenic amines, and was non-haemolytic. Its potential use as starter culture in low salt fermentation has been previously demonstrated in low-salt *doenjang* (Korean soybean paste) fermentation, which contained 6.5–7.5% NaCl (Jeon *et al*., 2016).

Another *Bacillus* found in this study was *Bacillus*

Table 3. Bacteria isolates showing positive results for amylase, protease, and lipase at both 20% NaCl and 5% NaCl identified using 16S rRNA gene sequencing

Isolate code	16S rRNA gene sequence of the closest match observed in BLAST analysis		
	Species	GenBank Accession No.	Sequence similarity
12.1.KM1.N5NA	*Bacillus subtilis*	MN744690	100%
12.1.KM1.N5NC	*Bacillus subtilis*	MN604374	100%
12.1.KM1.N10ND	*Bacillus subtilis*	MN604374.1	100%
12.2.KM1.N5N-4.B	*Bacillus subtilis*	CP025941.1	100%
12.2.KM2.N5N-4.A	*Bacillus subtilis*	MN960275.1	100%
12.1.KM1.N10NF	*Bacillus subtilis*	MN604374	98,60%
12.2.KM2.N10N-2.A	*Bacillus licheniformis*	MN515089.1	100%
12.1.KM2.N5NA	*Bacillus licheniformis*	MN756663.1	100%
12.1.KM2.N5ND	*Bacillus* sp.	CP045926.1	100%
12.1.KM2.N10NJ	*Bacillus* sp.	MN759435.1	100%
12.2.KM1.N10N-2.A	n.d	n.d	n.d
12.2.KM1.N5N-4.A	n.d	n.d	n.d
12.2.KM2.N10N-2.B	n.d	n.d	n.d
12.2.KM2.N5N-4.B	n.d	n.d	n.d

n.d: not detected.

https://doi.org/10.26656/fr.2017.7(S1).22

licheniformis, which is also commonly found in soy sauce (Wei *et al*., 2013; Yan *et al*., 2013; Song *et al*., 2015) and other soybean-based fermented foods, such as *doenjang* (Nam *et al.*, 2012), *cheonggukjang* (Nam *et al.,* 2012), and *tempeh* (Barus *et al*., 2017). It has also been reported as one of the most dominant microbes during Chinese-sesame flavoured liquor and plays an important role in the formation of several aroma compounds, such as 2,3-butanediol, isobutyric acid, guaiacol, and 4-vinyl guaiacol (Wu *et al*., 2014). It is known for its excellent proteolytic activity, which can tolerate high salinity of up to 30% NaCl (Toyokawa *et al*., 2010). Apart from its proteolytic activity, *B. licheniformis* also demonstrates an antimicrobial activity during the fermentation process (Su-Yeon *et al*., 2017).

The majority of selected fungal isolates belonged to the genus *Aspergillus*, including three *Aspergillus flavus* (12.1.KK.P10NJ, 12.1.KM1.P5NC, and 12.2.M1.P5-3.C), one *Aspergillus tamarii* (12.2.M2.P5-3.B), and two *Aspergillus* sp. isolates (12.1.KK.P10NI and 12.2.M1.P5-2.A) (Table 4). Isolate 12.1.KM1.P5NB and 12.2.M1.P5-2.C were identified as *Lictheimia ramose* and *Absidia* sp., respectively. *Aspergillus* species, including *A. oryzae* and *A. tamarii*, have been widely used in *koji* fermentation due to their importance in starch, protein, and lipid hydrolysis. However, their growth and enzymatic activities are usually terminated during *moromi* fermentation, due to their inability to tolerate salinity stress (Su and Lee, 2001; Su *et al*., 2005). For this reason, the selection of *koji* moulds that produce protease, amylase, and lipase with superior saline tolerance may contribute to the acceleration of *moromi* fermentation time, but this requires further study (Su and Lee, 2001).

4. Conclusion

This study confirmed that several bacterial and fungal species play a vital role in protein, lipid, and carbohydrate hydrolysis during high-salt soy sauce fermentation can also have similar enzymatic activities as well as enhanced growth potential at low-salt conditions. Knowledge of the growth and enzymatic properties of individual microbial species is paramount to successfully designing a defined mixed starter culture of soy sauce fermentation. Since soybean mainly contains proteins (40-50%), lipids (20-30%), and carbohydrates (26-30%), starter cultures with excellent protease, lipase, and amylase production capacity in saline conditions are of high interest. The predominant isolates, *Aspergillus* sp. and *B. subtillis*, found in this study, have the potential to be used as a starter culture for low-salt soy sauce production due to their protease, lipase, and amylase activities. Having these characteristics might not only compensate for the biochemical changes caused by salt reduction but also shorten the fermentation period, which normally takes several months in high salt conditions. However, further studies are needed to evaluate the performance of each starter culture candidate in the natural environment of low-salt *moromi*.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We would like to thank Lee Ga Young, Fahreza Gifari Avicena, Purwita Putri Ariana, Priscilla Jessica, Muhammad Novianto Bayu Saputro for the technical assistance. Also, we are grateful to Dr. Hani El Kadri for helpful advice in writing.

Table 4. Fungal isolates showing positive results for amylase, protease, and lipase at both 20% NaCl and 5% NaCl identified using ITS region sequencing

Isolate code	16S rRNA gene sequence of the closest match observed in BLAST analysis		
	Species	GenBank Accession No.	Sequence similarity
12.1.KK.P10NJ	*Aspergillus flavus*	CP047255.1	100%
12.1.KM1.P5NC	*Aspergillus flavus*	CP047255.1	99.83%
12.2.M1.P5-3.C	*Aspergillus flavus*	MH271094.1	100%
12.2.M2.P5-3.B	*Aspergillus tamarii*	MK638758.1	99.66%
12.1.KK.P10NI	*Aspergillus* sp.	MH876377.1	99%
12.2.M1.P5-2.A	*Aspergillus* sp.	MN648727.1	100%
12.1.KM1.P5NB	*Lictheimia ramosa*	KJ737434.1	99.85%
12.2.M1.P5-2.C	*Absidia* sp.	AY944892.1	98.66%
12.2.KK.P5-4.C	n.d.	n.d.	n.d.
12.2.KK.P5-4.B	n.d.	n.d.	n.d.
12.2.KK.P5-4.A	n.d.	n.d.	n.d.
12.2.M1.P5-2.A	n.d.	n.d.	n.d.

n.d: not detected.

https://doi.org/10.26656/fr.2017.7(S1).22

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