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Effect of Enzyme on Physicochemical and Sensory Characteristics of Black Soy Sauce

Lyda Yuok[1], Luka Ly[1], Marinich Net[2], Parakulsuksatid Pramuk[3], Reasmey Tan[1,2*]

[1] *Research and Innovation Center, Institute of Technology of Cambodia, Russian Federation Blvd., P.O. Box 86, Phnom Penh, Cambodia*

[2] *Faculty of Food and Chemical Engineering, Institute of Technology of Cambodia, Russian Federation Blvd., P.O. Box 86, Phnom Penh, Cambodia*

[3] *Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok, Thailand*

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Abstract: *Soy sauce is a brown liquid and widely used as a seasoning in East Asia, and its popularity in the Western part of the world is growing due to its unique taste and aroma. The black soy sauce is used to flavor dishes, but more importantly, it is used to darken the color of sauces, fried rice and noodles. In Cambodia, there are only local light soy sauces and there is no black soy sauce produced locally. Therefore, the purpose of this study was to develop black soy sauce by using different methods during moromi fermentation. There are two stages of fermentation including koji fermentation and moromi fermentation. To evaluate the quality of each sample of soy sauce, the main physicochemical parameters were analyzed such as moisture content, α-amylase, protease activity and pH during 72h koji fermentation. During 24-week moromi fermentation, 3 different methods were used: normal black soy sauce without addition of enzyme (M1), addition of natural enzyme from pineaple (M2), and addition of papain commercial enzyme (M3). In addition, the pH, salt content, total acidity, amino acid nitrogen, and reducing sugars were measured during moromi fermentation. After filtering, the molasse was then added in order to make black soy sauce. The results showed that the moisture content was decreased from 51% to 28% while pH, α-amylase, and protease activity were increased during 72h koji fermentation. During 24-week moromi fermentation, there were no significant differences in pH, TA, and salinity. For pH, it was higher in M2 with pH of 4.62. However, there were significant differences in amino acid nitrogen (AAN), and reducing sugars (RS) when 3 different methods were used. RS was remarked high in M1 with 1.68 ±0.026g/100ml. However, each parameter for both koji and moromi samples was within the range of the standard quality of soy sauce. According to the result of sensory evaluation,* among 3 conditions*, M1, M2 and M3 got the overall score of 6.26, 6.31 and 6.55, respectively. Therefore, M3 with addition of natural enzyme was preferred by panelists.*

Keywords: Black soy sauce; Enzyme; Koji fermentation; Moromi fermentation; Physicochemical analysis

1. INTRODUCTION

Soy sauce, a dark-colored seasoning, is added to enhance the sensory properties of foods. Soy sauce can be consumed as a condiment or added during the preparation of food [1]. Soy sauce, a traditional liquid condiment, is a widely consumed fermented product renowned for its unique flavor and umami taste. It serves as the primary seasoning in Japan, China, Korea, and other Asian countries. Throughout its production, soy sauce undergoes two main stages: Koji and moromi fermentation. Koji involves solid-state fermentation of soybeans and wheat using mold spores, typically *Aspergillus oryzae* or *Aspergillus sojae*, while moromi entails immersing the resulting koji in a brine solution and fermenting it for several months to up to four years. Lactic Acid Bacteria (LAB) contribute to the complex microbial community, generating key volatile compounds, amino acids, peptides, and sugars that define soy sauce's characteristics. LAB, particularly prevalent in the moromi stage, plays a crucial role in fermentation, acting as a natural preservative, flavor enhancer, aroma booster, and texture improver, thereby extending the soy sauce's shelf life and enhancing its nutritional value. In modern production, a combination of LAB (*Tetragenococcus halophilus*), yeast (*Zygosaccharomyces rouxii*), and Candida species ensures consistent product quality [2]. In Cambodia, some local soy sauces were made by traditional fermentation method.

* Corresponding author: Reasmey Tan
E-mail: rtan@itc.edu.kh; Tel: +855-12-602-202

Incorporating *A. oryzae* or *A. sojae*, vital for fermentation and recently utilized as koji starter cultures, is crucial for ensuring high-quality output, particularly on a larger scale [3]. In addition, there were only local light soy sauces, no local dark soy sauce available yet. Therefore, this study aimed to develop dark soy sauce without and with addition of commercial enzyme and natural enzyme during mormi fermentation. The changes of physicochemical characteristics during koji fermentation and moromi fermentation were measured, and sensory evaluation was also conducted for final products.

2. METHODOLOGY

2.1 Koji starter preparation

The commercial mold (*A. oryzae)* preserved in glycerol at –80°C was cultured on PDA (Millipore, Germany). To prepare the medium, 3.9g of PDA powder was dissolved in 100mL of deionized water and autoclaved at 121°C for 15 minutes. Subsequently, the *A. oryzae* strain were inoculated onto PDA plates and incubated in an incubator (Memmert, Germany) at 30°C for 5 days. For koji starter preparation, approximately 10g of rice were cleaned and soaked in water for 4 hours. Subsequently, the soaked rice was transferred into an Erlenmeyer flask and autoclaved at 121°C for 20 minutes to eliminate undesired microorganisms. *A. oryzae* spores, previously cultured on PDA media, were then inoculated into the autoclaved rice with 1:10 ratio, which was then covered with cotton and aluminum foil. The inoculated rice was left to incubate at ambient temperature for 7 days to develop into a suitable koji starter.

2.2 Mold spore counting

To prepare spore suspension, the spores from koji starter were firstly collected using the diluted tween 80 solution. 0.01 mL of 10^{-4} dilution sample was then taken and injected into counting chamber of a hemocytometer. The spores were enumerated under a microscope (40×) by counting four corner squares and the center square [3]. The spore count was determined using below equation.

$$\text{Number of spores} = \frac{N_1}{N_2} \times 10^4 \times \text{df} \qquad \text{(Eq. 1)}$$

where N_1 = number of spores in squares, N_2 = number of squares counted, and df = dilution factor.

2.3 Koji fermentation

For Koji production, the soybeans were soaked for 6 to 8 hours, and autoclaved at 121°C for 30 minutes. Subsequently, the soybeans were mixed with wheat flour at a 1:1 ratio (w/w). Approximately 0.2% of *A. oryzae* (commercial strain) mixture was spread evenly on a pastry tray with a thickness of 3 to 5 cm, and fermented at 30°C for 72 hours It is essential to periodically stir the contents [4].

2.4 Moromi fermentation

After 72 h koji fermentation, 20% brine solution was added. Moromi fermentation was carried out in 5L glass jars. During the first week of fermentation, the moromi was stirred daily, and thereafter, every two weeks. At week 8, natural enzyme from (pineapple) and papain commercial enzyme were added to M2 and M3, and 3 different conditions were conducted: without addition of enzyme (M1), with addition of nature enzyme (M2), and with addition of commercial enzyme (M3) at concentration of 0.3% per kg of raw material. Throughout fermentation, koji-derived proteolytic enzymes break down soybean and wheat proteins into amino acids and low molecular weight peptides and starch was converted to simple sugars [4,6]. For making black soy sauce, sugarcane molasse and sugar were added with the ratio of 2:1:8.

2.5 Physicochemical analysis

2.5.1 Moisture content

The moisture content of the koji sample was determined at 0 h, 24 h, 48 h, and 72 h following the AOAC (1999) guidelines. Initially, 3 g of the koji sample was weighed in dried aluminum dish and dried at 105°C for 24 h. The dried koji sample was then placed in a desiccator to cool down and reweighed. The moisture content was then calculated using the following formula.

$$\text{Moisture content} = \frac{Ms-(Mo-M1)}{Ms} \times 100 \qquad \text{(Eq. 2)}$$

where M_S = mass of sample (g), M_1 = mass of aluminum cup (g), and M_0 = mass of dried sample with aluminum cup (g).

2.5.2 pH

The pH values of koji samples at 0 h, 24 h, 48 h, and 72 h were measured, and of moromi samples from week 0 to week 24 were also determined using a pH meter (Laqua, pH 1200, Japan). The calibration was performed using pH buffers of 7, 4, and 10. Subsequently, approximately 5 mL of the sample was analyzed and waited until the reading stabilized.

2.5.3 Salt content

The salt content of moromi samples from week 0 to week 24 was measured using a salt meter (model ES-421; ATAGO). Prior to the experiments, all samples were diluted and calibration with distilled water was conducted. Each sample was then placed into

an electric salt meter, and click start to measure the salt content. The results were recorded on the screen, and the dilution factor was computed after determining that the unit of salt was g/100mL.

2.5.4 Alpha-amylase activity

The activity of α-amylase was assessed using the procedure outlined by [5]. In this method, 3 mL of a 0.2% starch solution were pre-heated at 60°C for 10 min. Then, 3 mL of either a pH 6.9 buffer or a diluted koji enzyme were added and incubated for another 10 min. To halt the enzyme activity, 1.5 mL of 1 M HCl was introduced into the reaction. 3 mL of the reaction mixture were then transferred to a new tube, mixed with 3 mL of distilled water, and 0.1 mL of iodine solution was added to bind with the starch, producing a blue color. The absorbance was then measured using a UV-VIS spectrophotometer (Shimadzu, Japan) at a wavelength of 580 nm. The α-amylase activity was determined as the following equation below.

$$\text{Alpha-amylase activity (Unit/g)} = \frac{m_{control} \times m_{assay}}{\text{time} \times V} \times \text{df} \quad \text{(Eq. 3)}$$

where m (control) and m (assay) = mass obtained from calibration curve of 0.2% soluble starch using absorbance value, time = incubation time of assay (10 min), and V = volume of sample (extracted enzyme) used (mL).

2.5.5 Protease activity

The protease activity assay was conducted following the procedure outlined by [5]. Initially, a 2% casein solution was prepared by dissolving casein in sodium phosphate buffer at pH 7.0. Subsequently, 5 mL of this casein solution were transferred to a test tube and pre-heated at 40°C for 10 minutes. Next, 1 mL of the enzyme sample was added to the test tube and incubated for an additional 10 minutes to allow for enzymatic reaction. To terminate the reaction, 5 mL of 0.11 M trichloroacetic acid (TCA) was added and left for 30 minutes. For the blank sample, 1 mL of the enzyme sample was added to a test tube containing 5 mL of TCA and kept it for 10 minutes. Following this, 5 mL of casein were added to the test tube and incubated for 30 minutes. Afterward, the mixture from both the sample tested and the blank was filtered through Whatman No. 1 filter paper (China), and 1 mL of the filtrate was mixed with 5 mL of 0.5 M Na_2CO_3. Then, 1 mL of Folin-Ciocalteu's phenol reagent (diluted with water with the ratio of 1:3) was added, and the solution was left for 30 minutes. The absorbance of the resulting solution was measured at 660 nm using a UV-VIS spectrophotometer (Shimadzu Corp., 06002, Japan). The protease activity was determined as the following equation below.

$$\text{Protease activity (Unit/g)} = \frac{C \times V_{assay}}{V_{sample} \times t_{assay}} \times \text{df} \quad \text{(Eq. 4)}$$

where m (control) and m (assay) = mass obtained from calibration curve of soluble starch 0.2% using absorbance value, time referred to the incubation time of assay (10 min), and V = volume of sample (extracted enzyme) used (mL).

2.5.6 Total acidity

The total acidity (TA) was determined using a titration method as described by [6]. The sample was initially diluted 20-fold by adding 19 mL of distilled water to 1 mL of the sample, followed by mixing with a vortex mixer. The 20 mL of the diluted sample were then combined with 60 mL of distilled water and titrated with a 0.05 mol/L NaOH solution until the pH reached 8.2. The volume of NaOH used was recorded as the sample measurement. Additionally, 80 mL of distilled water were titrated with a 0.05 mol/L NaOH solution to pH 8.2, and the volume of NaOH required was recorded as the blank measurement. The TA content was calculated based on the volume of NaOH consumed, using the formula provided below.

$$\text{Total acidity (\%)} = \frac{N_{NaOH} \times V_{NaOH} \times 0.09}{V_{sample}} \times 100 \quad \text{(Eq. 5)}$$

where C (NaOH) = concentration of NaOH used (0.05 mole/L), V (NaOH) = volume of NaOH used in sample test (mL), and V (sample) = initial volume of sample used (1 mL).

2.5.7 Amino acid nitrogen

The determination of AN followed the protocol of The Chinese National Standard (GB/T 5009.39-2003). The Moromi sample was initially diluted 20 times by adding 38 mL of distilled water to 2 mL of the sample, and the mixture was thoroughly blended using a vortex mixer. The 20 mL of the diluted sample were then combined with 60 mL of distilled water and titrated with a 0.05 mol/L NaOH solution until the pH reached 8.2. Subsequently, 10 mL of formaldehyde solution (37%–40%) was added, and the solution was further titrated with 0.05 mol/L NaOH until the pH reached 9.2. The volume of NaOH used was recorded as the sample test. For the blank sample, 80 mL of distilled water were titrated with a 0.05 mol/L NaOH solution to pH 8.2, followed by the addition of 10 mL of formaldehyde solution (37%–40%) and continued titration to pH 9.2. The volume of NaOH consumed was recorded. The AN content was then calculated using the formula provided below.

$$\text{AN(g/100ml)} = \frac{C_{NaOH} \times |V_{test} - V_{blank}|_{NaOH} \times 0.014}{V_{sample}} \times 100 \quad \text{(Eq. 6)}$$

where C_{NaOH} = concentration of NaOH (M), V(test) = volume of NaOH consumed for sample (mL), V (blank) = volume of NaOH consumed for the blank (mL), and V (sample) = initial volume of sample used (mL).

2.5.8 Reducing sugars

The determination of reducing sugars (RS) was determined using the 3,5-dinitrosalicylic acid (DNS) method, as described by [7], with slight modifications. Initially, the DNS solution was prepared by combining solution A and solution B. Solution A was made by mixing 1 g of DNS reagent with 20 mL of 2N NaOH, while solution B was prepared by dissolving 30 g of potassium sodium tartrate tetrahydrate in 50 mL of distilled water. These solutions were then combined and diluted with distilled water to a final volume of 100 mL, resulting in the DNS solution. Subsequently, 0.5 mL of the appropriately diluted sample solution (diluted 50 times) was placed in a test tube, and 0.5 mL of distilled water was added. Next, 2 mL of the prepared DNS solution were added, and the mixture was heated in a boiling water bath for 5 minutes. After heating, the solution was quickly cooled to room temperature, and 7 mL of distilled water were added. The absorbance was measured at 540 nm using UV-VIS spectrophotometer (Shimadzu, Japan). The standard glucose solution of 0.1% was used to make a calibrated curve.

2.5.9 Sensory evaluation

A hedonic test according to [8] was conducted by 50 untrained panelists for black soy sauce without addition of enzyme (M1), with addition of nature enzyme (M2), and with addition of commercial enzyme (M3). The panelists were untrained students. The samples were put in cup with different codes such as M1, M2, and M3. The nine-point hedonic scale is a bipolar scale with like and dislike on each end and a neutral point in the middle.

2.5.10 Statistical analysis

The data were analyzed by One-way Analysis of Variance (ANOVA) to determine whether there is any statistical difference level (p <0.05), using SPSS (25.0). All results from these experiments were presented as means of duplicate samples ± the standard deviation and the standard error.

3. RESULTS AND DISCUSSION

3.1 Physicochemical changes during koji fermentation

3.1.1 Moisture content

Fig. 1. shows the result of moisture content of samples during koji fermentation. The moisture contents were not significant difference (p>0.05) during fermentation among all samples. According to the result, the moisture content of all samples at 0h was about 51%. However, the moisture content decreased to approximately 28% after 72h fermentation. According to [9], the moisture content decreased to 22% after 72h. The result showed that the moisture content decreased over the time of fermentation due to the utilization of water and water evaporation by heat during *A. oryzae* growth [5]. The amount of moisture loss depends on the type of mold used, the temperature and humidity of the fermentation environment, and the length of fermentation time. The moisture content may decrease slightly after 24h because of the free molds start to grow and produce enzymes that break down the starches and proteins in the soybeans and wheat [6].

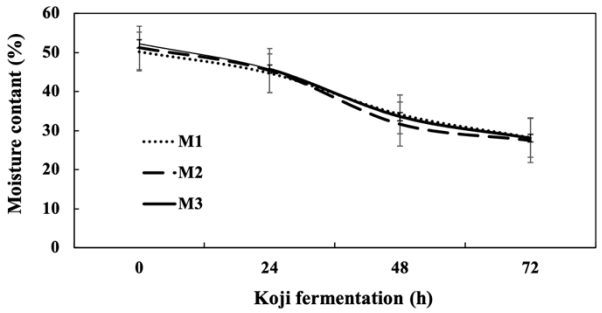

Fig. 1. Moisture content during koji fermentation

3.1.2 pH value

The result of pH change during fermentation is shown in Fig. 2. The result of pH of M1, M2, and M3 at the 0h, 48h and 72h were not significant difference (p>0.05), but there was significant difference (p>0.05) among samples during 24h of fermentation. The initial pH at 0h was approximately 6.22, while at the 72h it was increased to about 6.32. According to [12], the initial pH of koji with *A. oryzae* was 6.32, then the pH decreased to 6.12 after 24h of fermentation [2], the enzymatic activity of fungi increased the pH of koji from around 6.5 to 7.3, accompanied by heat production. However, it increased to 6.97 at the end of the fermentation period. The pH of koji at 72h was increased because of pH of fermented matter and due to microbial metabolic activities, especially various extracellular protein production[6]. Generally, *A. oryzae* has optimal growth at a temperature of 32–36°C (±1°C) with pH ranging between 5.0 and 6.0, and it can germinate at a pH of 2.0 to 8.0 [7].

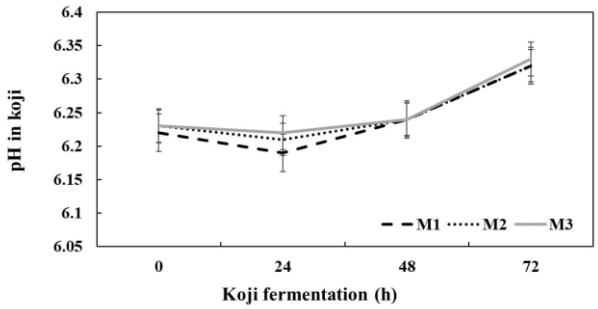

Fig. 2. pH during koji fermentation

3.1.3 Alpha-amylase activity

At 0h, alpha-amylase activities of M1, M2, and M3 were 0.078±0.020Unit/g, 0.056±0.014Unit/g, 0.042±0.014Unit/g, respectively. After 24h, they were 4.452±0.68Unit/g, 4.316±0.63Unit/g, 4.082±0.41Unit/g, respectively. The result of alpha-amylase activities at 0h and 24h were not significant difference (p>0.05). However, during 48h and 72h fermentation, alpha-amylase activity of each sample was higher increased and significant difference to about 51.030±5.08Unit/g, 43.773±11.59 Unit/g, and 44.944±9.96Unit/g, respectively. According to the study of [8], the increasing of enzyme activity was related to the growth of *A. oryzae*. The asexual cycle or spore forming is related to the secondary metabolite production, such as enzyme and organic acid. Amylase activity was rapidly increased after 24 h and highest activity at 72 h of cultivation period [8]. Alpha-amylase activity is used to break down starches and proteins in soybeans and other grains into smaller monosaccharides and amino acids [9].

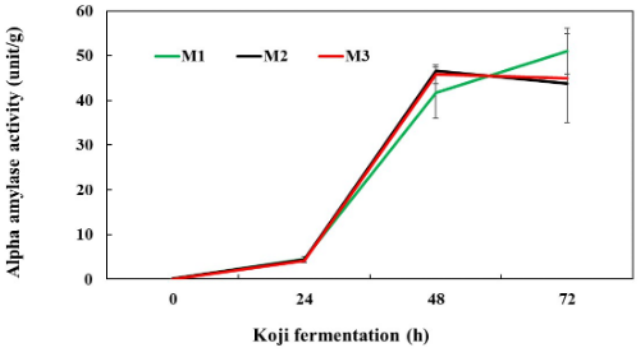

Fig. 3. Alpha amylase during koji fermentation

3.1.4 Protease activity

One of the most crucial enzyme activities in koji fermentation is protease activity, an enzyme responsible for breaking down proteins into smaller molecules, such as amino acids [13]. According to Fig. 4, at 0h the protease activities of M1, M2, and M3 were 3,929±4.083Unit/g, 1.571±6.49Unit/g, 11,768±5.40Unit/g, respectively. According to the study, the alkaline protease activity was detected only a very small amount during the first 24h. At 48h koji fermentation, the protease activity was significantly increased to 165.00±44,91Unit/g, 106.607±27.45Unit/g and 76.607±5.40Unit/g, respectively. The significant difference may due to molds that just started to grow and produce small amount of protease activity while *A. oryzae* in koji leaded to increase in protease activity at 24 h, and 48 h. According to the result of [8], the protease activities of koji increased rapidly after 24h of fermentation and highest activity at 48 h of fermentation and it might be caused by increasing of pH of koji. The protease activities of M1, M2, and M3 were significant difference at 72h with 777,464±18.91Unit/g, 452.179±21.16Unit/g and 428.214±12.49Unit/g, respectively. The variability in protease activity may be influenced by the presence of free mold, which can produce other enzymes that inhibit protease function [11].

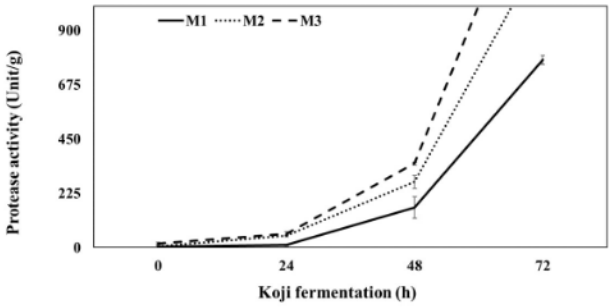

Fig. 4. Protease activity during koji fermentation

3.2 Physicochemical changes during moromi fermentation

3.2.1 pH value

The pH value of moromi is important for flavor and quality of soy sauce. Fig. 5. shows the change in pH values for each sample during 24-week moromi fermentation. According to Fig. 5, the pH values of M1, M2, and M3 were approximately 6.16 at week 0. The halotolerant lactic acid bacteria produce lactic acid from the glucose released from starch and lower the pH of the mix from about 6.6 to 7.0 to <5.0 [10]. LAB propagates rapidly at the beginning of the moromi fermentation, and the pH gradually decreases due to lactic acid fermentation and other metabolic products [11]. Furthermore, a decrease in pH can also result from the gradual accumulation of free fatty acids, amino acids, and peptides with carbonylic side chains. These compounds are generated through the hydrolysis of raw materials and the autolysis of microbial cells [17]. The pH was then stabilized until

the fermentation was finished. Based on the Institute of Standard of Cambodia, the pH value in soy sauce should be around 4.2 to 4.6. On the final day of moromi fermentation (week 24), the values were found 5.08±0.005%, 5.07±0.005%, and 5.15±0.005%, respectively. M2 had the highest pH value because of fermentation condition at higher pH promoted more extensive Maillard reaction activity. This facilitated the conversion of reducing sugars to their aldehydic forms, which in turn reacted more efficiently with amino acids, leading to more Maillard products. The Maillard reaction tends to be more pronounced under higher pH conditions [12].

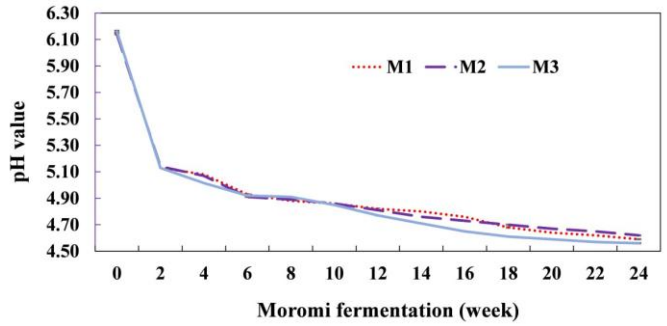

Fig. 5. pH during moromi fermentation

3.2.2 Total acidity

Fig. 6. illustrates the variation in total acidity throughout the moromi fermentation process, from week 0 to week 24. As shown in Fig. 6, the initial total acidity of the moromi was 0.05±0.00g/100ml, 0.05±0.00g/100ml, and 0.05±0.00g/100ml, of M1, M2, and M3, respectively. From week 0 to week 2, total acidity rose significantly, about 0.99±0.00g/100ml, 0.95±0.00g/100ml, and 0.90±0.00g/100ml, of M1, M2 and M3, respectively. This increase in total acidity is attributed to the rise in organic acids, such as lactic acid, fumaric acid, and succinic acid, which are produced by microorganisms that metabolize sugars in meju [13]. The rapid increase of total acidity during fermentation mainly due to the production of organic acid, mostly lactic acid and acetic acid from bacteria fermentation. After week 2, total acidity continued to increase slowly until week 24 of fermentation. This phenomenon has been identified as yeast continued to grow and had activity. In moromi, *Z. rouxii* yeast was dominant which produced the alcohol and several organic acid compounds added to the flavor of soy sauce. *Z. rouxii* is known to ferment glucose and other sugars into alcohol under high salt concentration during fermentation [14]. At week 24, the total acidity expressed as the fluctuates graphic with the significant difference of 2.21 ± 0.00, 2.41 ± 0.03, and 2.43 ± 0.06 g/100ml, of M1, M2, and M3, respectively. The yeast continues to grow during fermentation depending on the availability of air and the pH level of the mash, which falls within an optimal range for yeast growth. In this scenario, the yeast generates ethanol and other organic compounds, leading to a rapid increase in total acidity [10].

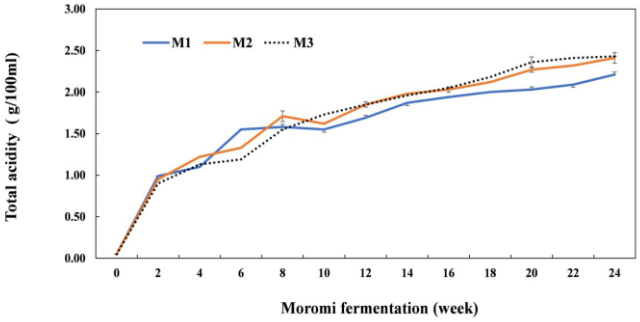

Fig. 6. Total acidity during moromi fermentation

3.2.3 Amino acid nitrogen

Amino acid nitrogen (AAN) plays an important role in the quality of soy sauce. It is a measure of the amount of free amino acids in soy sauce, which are responsible for its flavor and aroma. Fig. 7 shows about the changing of amino acid nitrogen during 24-week moromi fermentation. The amino acid nitrogen values of M1, M2, and M3 were approximately 0.13/100ml, at the initial of moromi fermentation. The total nitrogen content of the soy sauce was consistent at 0.27 to 0.70% during the fermentation period [13]. The amino acid nitrogen of three samples were not significant differences (p>0.05) at week 0. According to the graph, three samples increased gradually between weeks 0 and week 2. After that, from week 2 fermentation amino acid nitrogen continued to increase slowly until week 24 to 0.89±0.002g/100ml, 1.01±0.01g/100ml, and 1.030±0.003g/100ml, of M1, M2, and M3, respectively. However, the changes in amino nitrogen between the two samples from week 2 to week 24 showed a significant difference (p<0.05), with the amino acid nitrogen levels being lower in the moromi without addition of enzyme compared to the moromi with addition of enzyme. The higher amino nitrogen content in moromi with addition of enzyme is attributed from the increased activity of proteases and amylases at the higher controlled temperature of 37°C, which enhanced the breakdown of proteins and starch in the soybean and wheat moromi, resulting in the production of various small nitrogen compounds and sugars [15]. Amino acid nitrogen shows the nitrogen content of free amino acids in soy sauce which directly related to the umami flavor of soy sauce. According to institute of standard of Cambodia, the amino nitrogen in soy sauce must be equal or bigger than 0.26 g/100ml. The amino acid nitrogen of M1 was lower than that with additional enzyme may due to the low amount of enzyme that helped to break down the protein in the soy sauce mash [16].

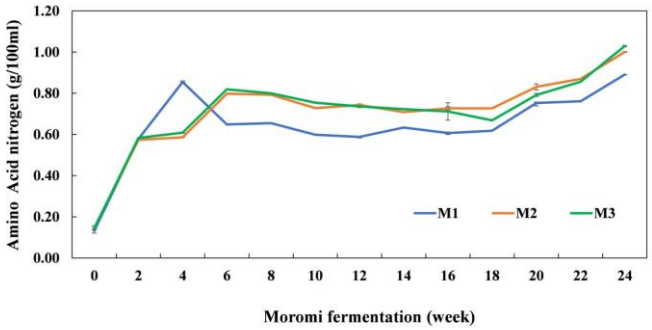

Fig. 7. Amino acid nitrogen during moromi fermentation

3.2.4 Reducing sugars

Fig. 8 shows about the changing of reducing sugar during 24-week moromi fermentation. The reducing sugars were present at low level at week 0 with a value of 0.32±0.009g/100ml, 0,29±0.003g/100ml and 0,42±0.091g/100ml, of M1, M2 and M3, respectively. In week 2, they were highly increased to 2.92±0.023g/100ml,3.31±0.025g/100ml, and 2.56±0.024g/100ml, of M1, M2 and M3, respectively. *A. oryzae* is the main factor to break down the raw materials such as protein, carbohydrates, and lipids [13]. This is reflected, in yeast cells, which increased from the initial log phase to several log phases during the first month, followed by a decline in log levels during the third month after inoculation. The fluctuations in reducing sugar of the three samples during soy sauce fermentation are likely due to a combination of microbial dynamics, raw material variability, enzyme activity, and environmental factors like temperature and pH [17]. The sugars, including maltose and glucose, were hydrolyzed in the presence of yeast through enzymatic action. Enzymes produced by the microorganisms play a key role in breaking down starches into sugars. If enzyme activity varies over time or is inhibited (example changes in temperature or pH), the breakdown of starches into reducing sugars may fluctuate, impacting sugar levels in the fermentation. This interaction leads to the Maillard reaction, a complex process that produces a variety of compounds, including aroma substances, UV-absorbing intermediates, and dark-brown polymeric compounds like melanoidins [18].

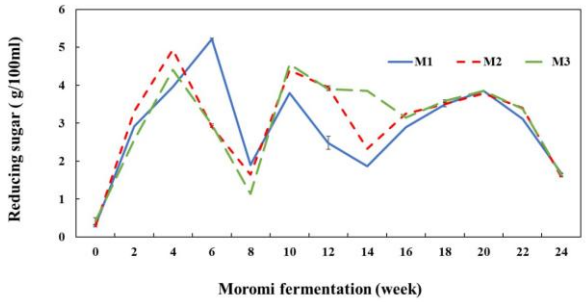

Fig. 8. Reducing sugars during moromi fermentation

3.2.5 Salt content

Fig. 9 illustrates the variation in salt content during moromi fermentation. The salt concentrations at week 0 were approximately 17.40%, respectively. They continued to decrease to approximately 15.63% during 24-week moromi fermentation. This decrease in salt concentration was due to precipitation during the fermentation process [13]. The rise in saltwater concentration elevated osmotic pressure, which slowed down the dissolution of proteins and amino acids. Additionally, high saltwater concentrations significantly inhibited protease activity, leading to a reduction in the amount of nitrogen-containing amino acids. Osmosis of koji which is made from steamed soybeans and wheat causing the salt content of the moromi to decrease. Lactic acid bacteria and yeasts will consume some of the water in the moromi, which will also lead to a decrease in the salt content [19].

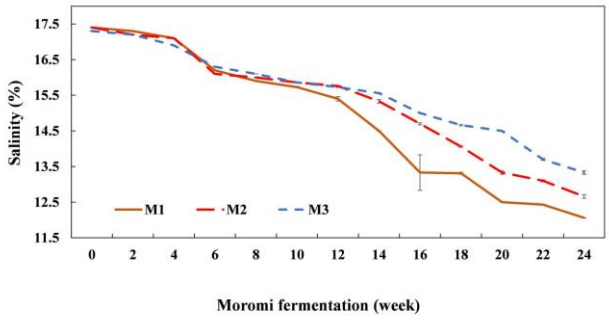

Fig. 9. Salinity during moromi fermentation

3.3. Sensory evaluation of different black soy sauces

According to Fig. 10, the characteristics of sensory evaluation by indicating color, odor, texture, aroma, sourness, sweetness, and bitterness in each condition of soy sauce are shown for 3 different conditions. The overall scores in each condition (M1, M2, and M3) were 6.26 ± 0.92, 6.31 ± 0.92, and 6.55 ± 0.90, respectively. The results also showed that odor, sourness, aroma, saltiness, texture, were not significantly different. However, other indicators such as bitterness, color, sweetness were significantly different (p<0.05) among the three conditions. Over 24 weeks of fermentation period, the samples were acceptable for consumer-preferred. Among these 3 conditions, M3 sample was accepted and scored higher than M1, and M2. The result of sensory evaluation shows points of colour, aroma, sweetness, sourness, and overall acceptableness of black soy sauce. The participants rated M3 higher than the M1 and M2 as it had a more complex umami flavour, as well as a darker colour, aroma, and sweetness.

Therefore, the addition of enzymes contributed to the increase of attributes during sensory evaluation.

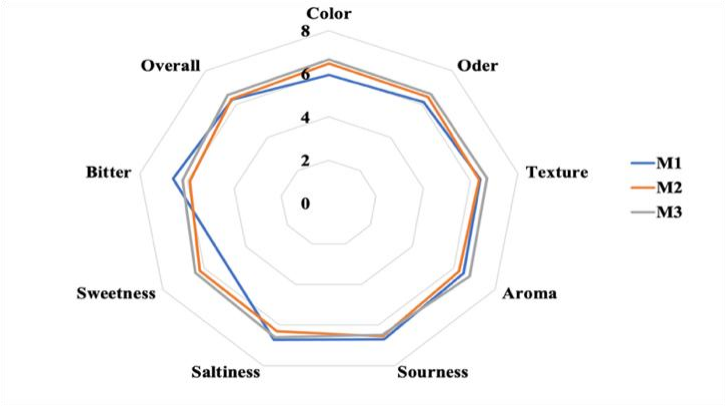

Fig. 10. Sensory evaluation of black soy sauces

4. CONCLUSION

In conclusion, the moisture content was decreased while the pH, alpha-amylase activity and protease activity were increased during 72h koji fermentation. Total acidity, amino acid nitrogen, and reducing sugar, of soy sauce samples with and without addition of enzyme were within the range of the standard quality of soy sauce. The quality of soy sauce with the addition of enzyme is better due to the fact that enzyme plays a vital role in the fermentation to improve the quality of soy sauce. According to the result of sensory evaluation, M3 overall score demonstrated that the panelists preferred and got higher score rating than the other 2 conditions. Over 24 weeks of fermentation period, the black soy sauce samples were acceptable for consumer-preferred. Besides, each parameter of these 3 final products during moromi fermentation was acceptable and under the standard of Cambodia, and the National Food Safety Standard of the People's Republic of China. The results showed that the enzyme contributed to improving the quality of soy sauce. As recommendations, other parameters such as volatile compounds, bioactive compounds, and antioxidants should be investigated during moromi fermentation in further studies.

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