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Investigation of the Physicochemical Properties and its Correlation during Koji-Moromi Fermentation Stage of Production Soy Sauce Naturally Brewed in Central Java, Indonesia

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***Abstract*—Soy sauce, a widely consumed condiment, undergoes intricate physicochemical transformations during its natural fermentation process. This research delved into the analysis of physical and chemical properties in naturally brewed soy sauce, aiming to elucidate the impact of fermentation stages (Koii, earlier moromi, final moromi, soy sauce product) and sample variations on these properties. Parameters such as pH, total acid, total soluble solids, and water activity (a_w) were assessed. Additionally, chemical attributes were analyzed, including total nitrogen, crude protein, formol nitrogen, and sodium chloride (NaCl) content. The correlation between physical and chemical properties during the fermentation stages was determined using the principal component analysis (PCA) method. The fermentation process significantly affected soy sauce's physical and chemical properties. Key factors include total dissolved solids, water activity, total acids, salt, total nitrogen, crude protein, and formol nitrogen, which display distinct patterns. These fluctuations were primarily driven by microbial activity and enzymatic hydrolysis, and total nitrogen, crude protein, and formol nitrogen play pivotal roles in characterizing soy sauce quality. Remarkably, total acid content and pH values exhibited significant variations that deviate from expected correlations due to weak acids. Samples C and D were deemed the most suitable out of the five tested, aligning better with quality standards for soy sauce. This study sheds light on the complex physicochemical changes during soy sauce fermentation, emphasizing the impact of raw materials and microbial processes on its properties.**

***Keywords*— Koji; Moromi; physicochemical; soy-sauce.**

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I. INTRODUCTION

Soy sauce is typically used as a table seasoning or condiment when preparing certain Indonesian or Chinese dishes. In particular, the most common form of soy sauce in Indonesia is dark sweet soy sauce, also known as "kecap manis". Kecap manis, or Indonesian sweet soy sauce, is a processed soybean with the addition of brown sugar up to 40% (w/v), resulting in a sweet, dark color, and typical caramel aroma due to further heat processing [1]. Chinese soy sauce, on the other hand, is made from soybeans, brine, grain, and mold, such as *Aspergillus sojae* or *A. oryzae*, and sodium chloride to prevent contamination. [2]. Furthermore, Chinese-type soy sauce mostly uses more soybeans than wheat, with a ratio of 80:20 and 70:30 [3].

Traditional soy sauce fermentation requires two steps, namely koji fermentation (solid and dry condition) and moromi fermentation (brine and wet condition) [4], [5]. In the koji stage, koji mold (*Aspergillus oryzae* or *A. sojae*) and roasted wheat are inoculated into steam-cooked soybeans. The koji processing produces a culture placed in a closed tank, added 20% salt brine, and mixed to produce moromi in mash shape before being sun-dried for 3 to 4 months. At this stage, a hydrolysis process contains enzymes such as proteases, amylases, and other enzymes, and the hydrolysis product contributes to the final flavor of the product [6]. The second step is Moromi, which contains *Pediococcus halophilus*, yeasts, *Zygosaccharomyces rouxii*, and lactic acid bacteria. Simple sugars produced in koji fermentation are hydrolyzed through a metabolism process by *Pediococcus halophilus* into acetic acid and lactic acid, which causes the pH of moromi to drop below 5.5. *Z. rouxii* of yeast produces alcohol which acts as a precursor for the final aroma of soy sauce [7]. The Moromi stage begins by adding 18% brine in the fermentation process for three months; this stage is important in the protein hydrolysis process, producing amino nitrogen that acts as a flavor precursor and a good quality soy sauce [8].

Refining is the final step in soy sauce fermentation. The separation process is performed by pressing the cake of raw soy sauce (solidly suspended) consistently through the layers of filters [9]. This stage produced a dark-colored liquid, pasteurized, added with palm sugar, and packaged in bottles for easier consumption. Sophisticated chemical and biological processes occur throughout the soy sauce manufacturing process. Soybeans, water, and salt are the primary raw materials used in soy sauce processing [10]. In the process of koji fermentation or solid-state fermentation (SSF), molds are known as the essential components that produce protease and amylase enzymes [11]. These enzymes help soy protein to be converted into peptides and amino acids that influence the quality of soy sauce, such as its sensational aroma and unique taste, such as umami. The distinct umami taste of soy sauce requires nitrogenous compounds (45%) and must contain free amino acids, specifically glutamic acid, which is an essential component of soy sauce [12], [13]. Different components, such as raw materials and production methods, lead to differences in the quality of soy sauce because each hydrolysis will produce different compounds [14].

Good quality soy sauce generally comprises total nitrogen (w/v) 1.0-1.65%, reducing sugar 2-5%, organic acids 1-2%, ethanol 2.0-2.5%, and sodium chloride (w/v) 17-19% [6]. Furthermore, there is an Indonesian National Standard, namely SNI 3543:2013, that regulates the standard quality of soy sauce, such as the protein content that must exceed 1% (b/b) [15]. Unfortunately, to date, limited research is being conducted on the quality of local soy sauce with traditional fermentation processes, particularly focusing on each fermentation stage from the koji phase to the final moromi.

Therefore, this research aimed to allow a common understanding of the physicochemical properties of sweet soy sauce, such as pH, a_w, crude protein, total acid, ammonium salt, total nitrogen, and total soluble solids. Furthermore, this research attempted to provide additional data on its correlation during the koji-moromi fermentation stage of soy sauce production naturally brewed in Central Java, Indonesia.

II. Materials and Methods

A. Soy Sauce Samples

Twenty samples were collected from five different brands of soy sauce produced by small microenterprises in Central Java using traditional methods. The areas in Central Java covered Slawi-Tegal, Pati, Kudus, and Yogyakarta. The formula was made by fermenting good quality soybeans without the addition of wheat flour, with variable ratios of soybeans and salt brine solution based on the manufacturer's instructions. Twenty samples were coded as FA, FB, FC, FD, and FE. Each sample consisted of four stages of fermentation, namely: koji (coded with FX1 as a dry soybean fermented sample, fully coated with fungi), initial moromi (coded with FX2 as a salt liquid koji fermentation which in the early time of fermentation within a month), final moromi (coded with FX3 as a salt-liquid koji fermentation which takes some months duration of fermentation), and ready-to-eat soy sauce products (coded with FX4 as a product that was ready to be consumed). The samples were kept refrigerated and out of direct sunlight until they were analyzed.

B. Physicochemical Analysis

1) Total Acidity: Total acidity was determined by the titration method using total titratable acid [16]. The soy sauce diluent (10 mL) was combined with deionized water (30 mL) and then titrated to a pH value of 8.2 using NaOH (0.05 M). The total acidity was calculated based on the amount of NaOH needed to change the color of the solution during the titration process. The formaldehyde solution was then added in 10 mL increments. The next step was the mixture titrated with NaOH (0.05 M) to pH 9.2. The total acidity content was shown according to the volume of used NaOH to raise the pH value from 8.2 to 9.2. Distilled water served as the control group.

2) pH, a_W, and Sodium Chloride Analysis: The pH was measured using a digital pH meter with type Sartorius PB10, Goettingen, Germany. Sodium chloride concentration was determined using Mohr's method [17] by volumetric titration with $AgNO_3$. The homogenization process was performed by diluting a sample (2 g) in distilled water (18 mL) before being titrated with $AgNO_3$ (0.1 M) with 10% (wt/vol) of K_2CrO_4 solution as an indicator. The water activity (a_w) was measured by an electric hygrometer (Novasina lab swift) at room temperature. Around 5 g of homogenate and slurry samples were placed in a disposable cup, until the slurry thoroughly covered the bottom of the cup, filling it to no more than half of the disposable cup. A hygrometer with an accuracy of 0.003 was used to directly measure the value of a_w.

3) Total Nitrogen, Crude Protein, and Formol Nitrogen: The Kjeldahl technique AOAC No. 979.09 in 2001.1 [18] was used to assess total nitrogen concentration. A boiling tube was filled with 10 mL of supernatant, followed by a mixture of 3 g potassium sulfate and cupric sulfate. After that, sulfuric acid (10 mL) was added to the solution, and mixed. The digested step of the solution was performed in a graphite digester and heated at 260°C for 105 minutes followed by another 120 minutes at 420°C. The digestion liquid was distilled, and titration was performed with a standard hydrochloric acid solution (0.1 M). Total nitrogen was calculated using the volume of used hydrochloric acid. Based on SNI-2354-2006 [19], a conversion factor of 5.75 was used to determine crude protein content for koji and moromi samples, while a conversion factor of 6.25 [1] was used to compute the crude protein level for soy sauce ready to consume. Formol nitrogen (FN) was determined following the [20]. The supernatant (50 mL) was neutralized with NaOH (0.1 N) until it reached a pH value of 8.5. Formaldehyde was added subsequently (20 ml, pH 8.5), and then the composite was titrated using NaOH (0.1 N) up to the final pH value of 8.5.

4) Data Analysis: The experiments were carried out in triplicate and analyzed by Two-way ANOVA and followed by Tukey analysis at a significance level of $p < 0.05$ using IBM SPSS Statistics 26. The data shown in the figures, along with the standard deviation as error bars, were explained descriptively. PCA (Principal Component Analysis) Origin

2022 software was utilized to analyze the association between all physical and chemical properties with fermentation stages.

III. Results and Discussion

A. Analysis of Physical Properties in Naturally Brewed Soy Sauce

Based on the results shown in Figures 1a. and 1b., it was observed that the variations in the samples and the fermentation stage significantly influenced the total acid and pH of the soy sauce. Typically, a high total acid content in a solution was associated with a low pH. Total acid content referred to the concentration of all acid compounds present in a solution, while pH measured the acidity or alkalinity of a solution on a scale from 0 to 14. Samples A and B exhibited the highest total acid levels, while sample D had the lowest pH (acid). In general, the final moromi phase displayed the highest total acid, and soybean sauce displayed the lowest pH (acid) values, ranging from 10.16% to 26.28% and from 4.53 to 7.73, respectively.

a.

b.

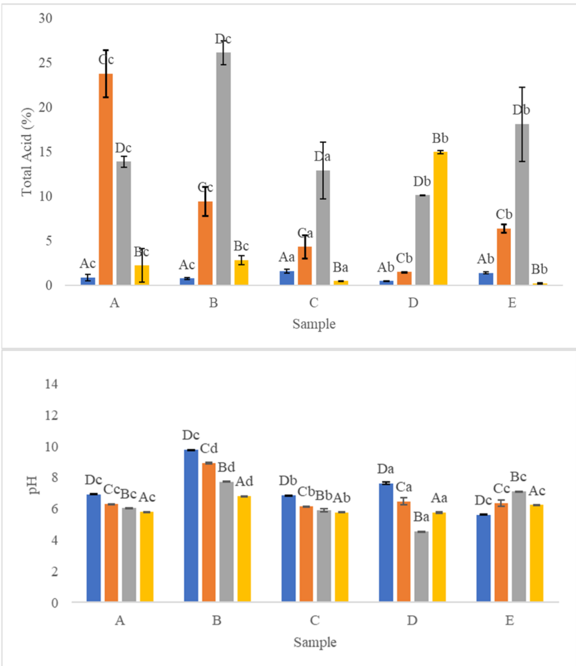


Fig. 1 The dynamic changes of total acids and pH at koji (blue), earlier moromi (orange), final moromi (grey) and soybean sauce (yellow) stages of fermentation processing of soy sauce products in Central Java. Values are mean ± standard deviation. Different letters above the bars indicate statistically significant differences at $P < 0.05$ between stages (upper case) and between samples (lower case).

In general, an increase in the total acid content of soy sauce resulted in a decrease in the pH value. However, in this study, a high total acid content did not necessarily lead to a low pH value. pH represents the concentration or level of hydrogen ions in a solution [21]. According to Blanco and Blanco [22], weak acids could have lower hydrogen ion concentrations than strong acids, even if they had the same acid concentration. Therefore, the fact that the pH of soy sauce in this study did not significantly decrease despite the increase in acid concentration was likely because the acids formed were weak acids, thus preventing a drastic decrease in the pH of the solution. The total acid number during soy sauce fermentation supports the physical properties of soy sauce. The koji stage generally produced the lowest total acid number, as shown in Fig. 1. The total acid number, in general, continued to rise until it peaked during the moromi fermentation. The increase in total acid content in fermented soy sauce led to a decrease in pH value [23]Some of this research's results differed from those of previous studies, possibly due to the quality of the raw materials used to make the soy sauce. According to Liang *et al.* [24], soy sauce quality is strongly affected by the use of raw materials, such as whole soybeans or defatted soybeans, and the activity of microorganisms.

The decrease in pH value potentially occurred due to the fermentation activity of yeast and salt-tolerant lactic acid bacteria, such as *Tetragenococcus halophilus*, which optimally produce lactic acid [25]. When the pH value begins to drop to around 4.0 to 5.0, according to Devanthi and Gkatzionis [4], it leads to the diminishing of the bacterial population because the fermentation system is no longer suitable for LAB (lactic acid bacteria) formation. Thus, yeast continues the fermentation process. This aligns with to Wang *et al.* [26]'s assertion that LAB growth, especially halophilic LAB like *Tetragenococcus halophilus*, is hindered when the pH value falls below 5.0. As the fermentation period progresses, supplies of simple sugars from proteolytic activity start decreasing, further reducing yeast proliferation, and leading to a decrease in the system's acidity.

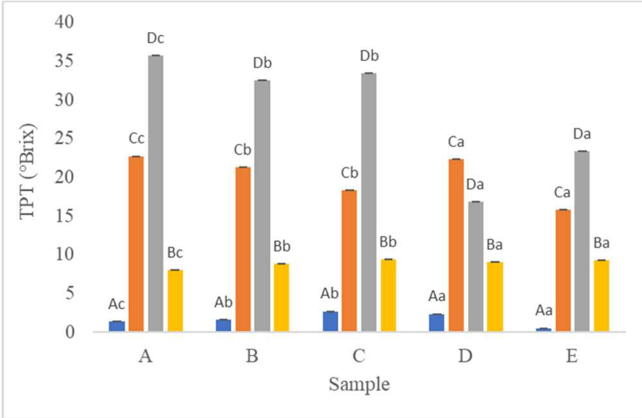
The pH value of soy sauce samples, overall, was at its highest during the koji stage fermentation, indicating that at this stage, it was not too acidic. This phenomenon is in accordance with to Devanthi and Gkatzionis [4] that states fungi activities during the koji stage increase the pH of koji fermentation from 6.5 to around 7.3 due to heat production. The fungi from the koji microbial community, particularly *A. oryzae*, produces a variety of extracellular proteins during fermentation [27]. The koji stage fermentation process is subsequently followed by the moromi fermentation process, which is carried out by halophilic lactic acid bacteria due to the high salt condition. The pH value was currently falling as well, showing a higher level of acidity as the fermentation process advances from start to finish. Halophilic lactic acid bacteria metabolize simple sugars into lactic acid, gradually lowering the pH value even to 4.0-5.0 at the final moromi stage [6]. The product's highest total acid value can be linked to the cell destruction (autolysis) mechanism of microbial cells and the formation of FFA (free fatty acids) [7]. Previous studies [3] claimed that the extended fermentation period afforded sufficient time for enzymes to destroy starches, proteins, and lipids in soy sauce into saccharides, volatiles, peptides, and free amino acids. Besides FFA, amino acids, and peptides containing carbonylic side chains, organic acids were also formed during fermentation, contributing to improving the organoleptic taste (flavor and aroma) of soy sauce while affecting the pH value and acidity characteristics and inhibiting the growth of spoiling bacteria [13]. After the moromi stage is complete, the pH will continue to drop into the last stage of the moromi stage into soybean sauce [28]. As per the Indonesian National Standard (SNI) 3543-01-2013 [15], soy sauce is required to adhere to a pH level standard falling within the range of 3.5 to 6. The findings revealed that the pH values of the soy sauce sample in the study varied between 5.75 and 6.81. Consequently, it can be deduced that all the samples, except for sample B and E, complied with the pH standard. The organic acids that occur during the fermentation stages of soy sauce are mainly L-malic acid, L-lactic acid, acetic acid, citric acid, succinic acid, and fumaric acid [29]. Fungi from koji step fermentation, such as *Aspergillus sojae* mainly produce fumaric acids and malic acids, meanwhile, *Aspergillus oryzae* produces citric acids [30]. Yeast *Z. rouxii*, during the moromi stage, produces a large amount of malic acids and succinic acids, while *C. versatilis* produces a high amount of acetic acids [7]. Halophilic lactic acid bacteria from the moromi stage were dominated by *Tetragenococcus halophilus*, which produce lactic acids and acetic acids [31].

The result of total dissolved solids content shows the total amount of the soluble fraction of food components in the product that was formed during processing [32]. The total dissolved solids value from soy sauce making could be measured based on total organic acids, sugars, reducing sugars, salts, proteins, and amino acids [33]. The higher total dissolved solids obtained shows that the hydrolysis process of substrates with various microbes during fermentation occurs at a great acceleration [34]. Several enzymes can perform hydrolysis activity to reduce complex compounds into their derivatives that contribute to total dissolved solids, including protease, amylase, and others, produced by microbes, started during the koji fermentation. Solid protein bonds are catalyzed by the protease enzyme so that soluble proteins and amino acids can occur [8], [35]. Furthermore, the amylase enzyme, which transforms polysaccharides into simple sugars, contributes to total dissolved solids [36].

Total soluble solids referred to the concentration of all dissolved solids in a liquid, typically expressed in terms of percentage or degree Brix (°Bx). The results of the analysis of total dissolved solids in the five soy sauce samples tested in this study are shown in Figure 2. The final moromi phase exhibited the highest total dissolved solids compared to the other samples, with a range of 16.8 to 35.67 °Bx. In contrast, the koji phase only showed a minor dissolution of solids, ranging from 0.5 to 2.67 °Bx. Sample A possessed the highest average total dissolved solids, with a value ranging from 1.37 to 35.67 °Bx.

Based on Fig. 2, total dissolved solids mainly reached its highest during the final moromi stage. This shows that the total dissolved solids increased massively from the koji to the moromi stage. The increase of total dissolved solids during the final moromi stage happened because soluble sugars and macromolecules in the fermentation mash were decomposed during the koji stage because of yeasts and other microorganisms' activity that softened the fermented soybean [37]. Soluble sugars are a result of the activity performed by the amylase enzyme on wheat starch and soybean components during moromi fermentation (brine step) [38].

According to Zhu *et al*. [39], the degradation rate created by proteases and amylases produced by microbes during the moromi stage is far greater than its forming activity of volatile substances, thus explaining the increase of total dissolved solids. The addition of salt will also alter the osmotic pressure between the salt solution and soybean, causing the salt to bind to the substrate [40]. According to to Zahidah and Lo [41], the increase of total dissolved solids can be affected by the hygroscopic activity of salts. Meanwhile, during the soy sauce stage, total dissolved solids were rapidly decreased. This might be caused by the fermentation process of brine in the moromi stage, which takes longer to prevent microbe development; as a result, TDS production will begin to fall [40].



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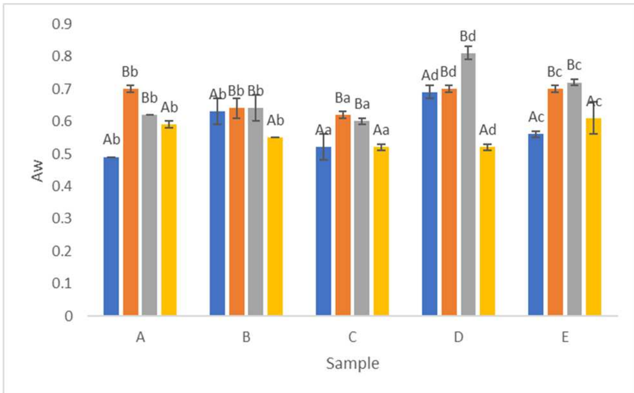


(b)

Fig. 2 The dynamic changes of total soluble and a_w solid at koji (blue), earlier moromi (orange), final moromi (grey), and soybean sauce (yellow) stages of fermentation processing of soy sauce products in Central Java. Values are mean ± standard deviation. Different letters above the bars indicate statistically significant differences at $P < 0.05$ between stages (upper case) and between samples (lower case).

Controlling water activity was essential for ensuring the microbiological safety, shelf life, quality, and overall characteristics of soy sauce. Based on the results obtained and shown in Figure 2, it was determined that the a_W value in the koji and soybean phases was lower than in the pre and final moromi phases. The a_W value in the koji phase ranged from 0.49 to 0.69, whereas in the soy phase, it ranged from 0.52 to 0.61. In sequence, the samples with the lowest to highest a_W values were C, A, B, D, and E.

Water activity (a_w) analysis needed to be done because it is a major factor in preventing and suppressing microbial growth so that it won't exceed the permitted limit, becoming the primary parameter of food stability, observing the microbial response to finally determine the type of microorganisms existing in specific food [42].The research on a_w value of fermented sauce production has been done to prevent any spoilage potential caused by microorganisms by emphasizing the decrease of water activity during the fermentation process [43].

The a_w value, as of Fig 2., shows that it remained within acceptable limits during the koji stage and did not exceed 0.8. This is consistent with Dwipa *et al*. [44], who states that mold can only thrive in an a_w environment that does not exceed 0.8, implying that the koji fermentation process is complete. The reduction of a_w during 46 hours of koji fermentation, mainly by *A. oryzae*, prevented spoilage or contamination by unwanted microorganisms with a specific temperature control between 30 and 43°C [27]. Koji fermentation molds, especially from the genus *Aspergillus* sp., are classified as primary colonizers fungi that can grow well in a water activity below 0.8 [45]. Enzymatic metabolism of koji fermentation molds caused the increase of total reduced soluble sugars. Reduced soluble sugars such as sucrose, glucose, and fructose can maintain the low water activity of below 0.8 because of their osmotic activity that can bind water molecules [46].

The a_w value increased initially, then decreased throughout the moromi stage. These phenomena might be explained by the absorption of water caused by the addition of salt by the principle of the osmosis process. The presence of salt and the increase of total solutes may inhibit the proliferation of microorganisms by affecting the osmotic pressure exerted on the bacterial cytoplasmic membrane [47]. The a_w value in the final product of soy sauce fell. Other than the salt osmosis activity during the previous stage, this occurrence can be related to adding palm sugar when the final soy sauces were made. Palm sugar, according to Dewi *et al*. [48] consists of two monosaccharide sugars, namely glucose and fructose, which increase the total reduced soluble sugars and lower water activity because of their osmotic activity. The decrease of water activity at the final product leads to an environment that is less favorable to microbial growth, especially spoilage bacteria, which cannot survive at a_w below 0.85 [49].

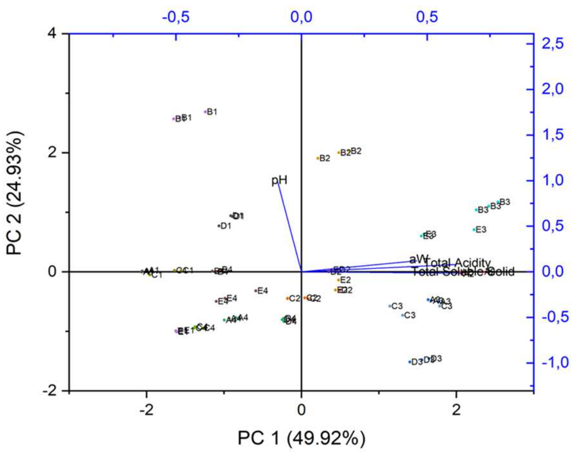


Fig. 3 The correlation relationship between physical properties and the fermentation stages using Principal Component Analysis (PCA). (A) Koji, (B) Earlier Moromi, (C) Final Moromi, (D) Soybean Sauce.

The Origin program was used to conduct a correlational association analysis between physical properties and fermentation stages. Figure 3 indicated that, in the presented PCA plots, the principal components sequentially explained 74.85% (PC1 49.92%; PC2 24.93%) of the total variance in the data. Based on the biplot obtained, it was found that during the soy sauce production process, the total acid value, dissolved solids, and a_W had a relatively strong positive correlation, and all three variables had relatively weak correlations with the pH value because they formed an angle that was nearly 90 degrees. Regarding variability for each variable, the pH and aW values had less variability than the total acid and total dissolved solids because they had shorter vector lengths. The biplot interpretation regarding each variable's variability is consistent with the results shown in Figures 1 and 2.

Based on the samples' proximity to the variable vectors, sample B in the koji phase had a higher pH value than the other samples. The Final moromi phase had higher a_W, total acid, and total dissolved solids values compared to the other phases because it was aligned with the vectors of the three variables. On the other hand, the earlier moromi and soybean phases tended to have lower values of total acid, a_W, total dissolved solids, and pH compared to the other phases.

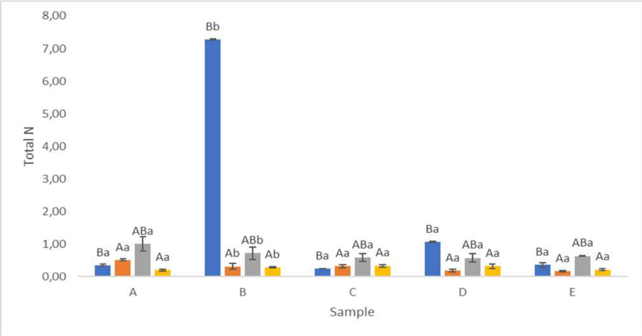
B. Analysis of Chemical Properties in Naturally Brewed Soy Sauce

The chemical properties that were analyzed included the formol nitrogen value, total nitrogen value, crude protein value, and NaCl. Formol nitrogen refers to non-protein nitrogen compounds present in a sample, total nitrogen represents the total sum of all nitrogen compounds in a sample, both protein and non-protein, while crude protein provides an estimate of protein content based on the total nitrogen content.

The results of the analysis are shown in Figures 4a and 4b. The results obtained in terms of crude protein and total nitrogen content had a similar pattern, where crude protein and total nitrogen experienced fluctuations, with the highest amounts obtained in the koji phase. The values of crude protein and total nitrogen in the final moromi phase, on the other hand, were not significantly different from those in the koji phase. In both parameters, Sample B had the highest average crude protein and total nitrogen content compared to the other samples.

Total nitrogen, crude protein, and formol nitrogen values are among the chemical properties. Total nitrogen resembles the composition of simple peptides (45%) and amino acids (50%-75%) in the sample; the higher the content, the greater the quality of the soy sauce [8]. The result showed a lower total nitrogen value compared to previous research. According to Kuang *et al*. [50], the amount of total nitrogen could represent the utilization of raw materials and directly affect the development of soy sauce's flavor. According to Zhao *et al*. [51], the total nitrogen content of Chinese-type soy sauce was 1.35-1.57 g/100mL. However, the total nitrogen content of salty soy sauce was 0.32-1.14 g/100 mL and sweet soy sauce was 0.14-0.25 g/100mL [22].

As specified in Fig. 4, the total nitrogen value showed a significant positive correlation with the crude protein value. This may be connected to the definition and basic formula of crude protein calculation. According to [52], crude protein reflects the total content of nitrogen from a protein degradation process, which is calculated using the Kjeldahl conversion factor. Moreover, Urbat *et al*. [53] stated that the conversion factor of Kjeldahl's method is based on the assumption that proteins have a nitrogen content of 16%.



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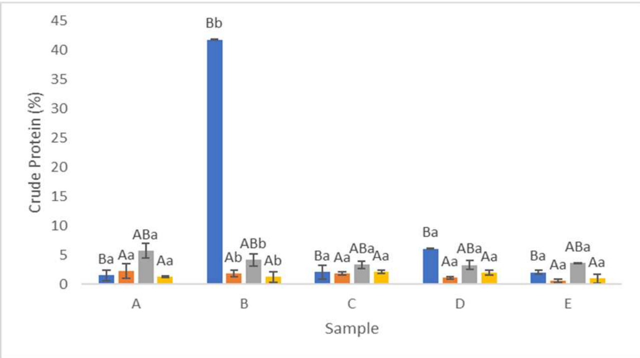


(b)

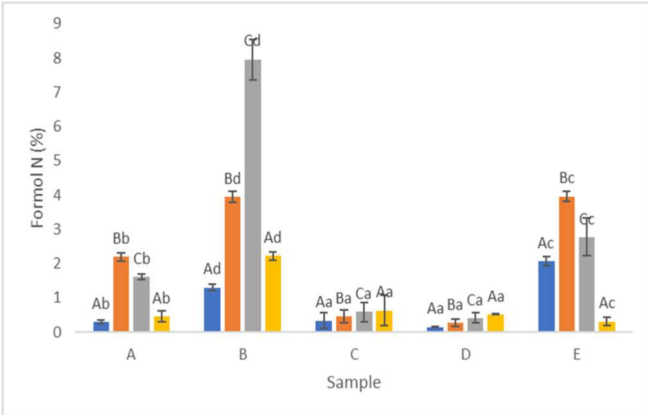
Fig. 4 The dynamic changes of total N and crude protein at koji (blue), earlier moromi (orange), final moromi (grey), and soybean sauce (yellow) stages of fermentation processing of soy sauce products in Central Java. Values are mean ± standard deviation. Different letters above the bars indicate statistically significant differences at P < 0.05 between stages (upper case) and between samples (lower case).

Generally, the overall value of crude protein can be utilized as an indicator that will show the quality of soy sauce. Commercial soy sauce typically contains a protein concentration of 3 to 4%. Soy sauce must have a minimum protein level (Nx6.25) of 1%, according to the Indonesian National Standard (SNI) 3543-01-2013 [15]The result showed that the crude protein value of soy sauce in the research ranged between 0.93% and 2.09%. Therefore, it can be concluded that the samples met the protein standard except for sample E.

This study obtained the highest formol nitrogen values in the soy sauce samples during the pre and final moromi phases, with values ranging from 0.28 to 7.96%. However, during the soy sauce phase, the formol nitrogen values decreased again, and on average, they were lower than those in the koji phase. The formol nitrogen values differed significantly among each sample, with the highest values starting from samples B, E, A, and C, and the lowest being sample D.

In general, the pattern of the graph formed by the NaCl content during the soy sauce production process began with an increase from the koji phase towards the pre-moromi phase. It reached its peak during the final moromi phase, which was followed by a decrease in NaCl content during the soy sauce phase. The highest NaCl content commonly occurred during the moromi phase because adding salt during this stage was critical in ensuring proper fermentation, flavor development, and preservation in soy sauce production. The highest NaCl content was possessed by sample B (0.7-32.46%), while the lowest NaCl content was observed in sample E (1.47-13.65%).

Based on the results shown in Fig. 5, the total nitrogen and crude protein increased during the fermentation process from koji to moromi but decreased in the final product. The escalation of total nitrogen and crude protein value may be attributed to the presence of dilution and the diverse concentrations of salt, leading to a rise in the total amount of nitrogen [54]. Moreover, according to Gao *et al*. [55], the final moromi fermentation enhanced the total nitrogen concentration due to the combination and contribution of various free amino acids (FAAs) and peptides to the total nitrogen content. It is attributed to proteases and peptidases' enzymatic activity, which catalyzes the hydrolysis of protein substrates into peptide fragments, amino acids, and ammonia.



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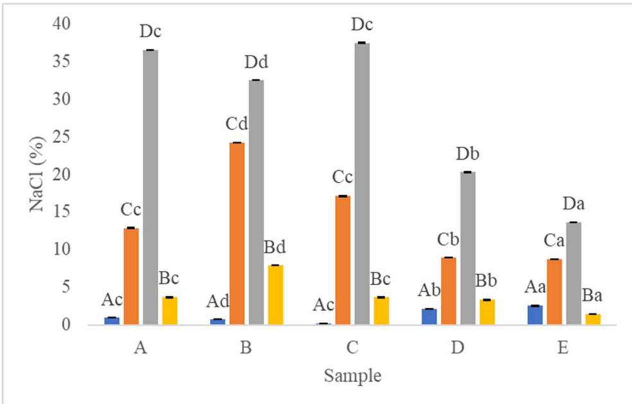

(b)

Fig. 5 The dynamic changes of formol N and NaCl at koji (blue), earlier moromi (orange), final moromi (grey), and soybean sauce (yellow) stages of fermentation processing of soy sauce products in Central Java. Values are mean ± standard deviation. Different letters above the bars indicate statistically significant differences at P < 0.05 between stages (upper case) and between samples (lower case).

Therefore, the total nitrogen increased rapidly at the beginning of the fermentation and subsequently decreased in the final stage because a high salt concentration denatures the proteases and glucoamylases. The decrease in total nitrogen value may indicate a lower level of digestible protein during the fermentation process. According to Abdurrauf and Aceh [40], higher amounts of nitrogen implicate a greater level of protein hydrolysis into amino acids and simple peptides. The presence of palm sugar and different types of sweetening ingredients that have been observed also showed a high potential in decreasing the proportionate number of nitrogenous compounds [22].

Formol nitrogen explicitly reflects the content of small peptides and amino acids that were produced during the protein hydrolysis process. Moreover, formol nitrogen is an essential indicator of soy sauce quality. In accordance, Gao *et al*. [56] stated that formol nitrogen represents the content of free amino acids (FAAs), which plays a vital role as a taste contributor in soy sauce. According to Ciou *et al*. [57], based on the reaction of formol nitrogen and amino acid that will liberate one H^+ ion from the amino group and be determined through the titration using sodium hydroxide solution, formol nitrogen content can be utilized to measure the level of total free amino acids in soy sauce.

As specified by the result, the value of formol nitrogen, in general, showed an escalation from the koji to the moromi phase. This may be related to the hydrolysis process of high molecular weight materials, which means proteins, to release amino acids [58]. In accordance, a similar increase at the beginning of the fermentation process was reported by a previous study on fish sauce [59] that showed an escalation of formol nitrogen caused by protein hydrolysis, with the influence of some enzymes, such as endogenous and koji proteinases. Finally, the value of formol nitrogen started to decline after the final moromi state and reached its lower value at the refining state. According to Zhu *et al*. [39] The decline of formol nitrogen at the end of the soy sauce fermentation process was caused by microorganisms utilizing formol nitrogen as energy, the decrease of enzymes in brine solution, and a lower rate of protein degradation into amino acids than the rate of amino acid conversion into volatile compounds.

Lastly, salt played a significant role during the manufacturing process of soy sauce products. Salt regulates microbiological safety and organoleptic properties, including texture and aroma. Statement from Devanthi and Gkatzionis [4] shows that a high concentration of salt in brine will subdue the growth of spoilage and pathogenic microorganisms and provide favorable conditions for halotolerant microorganisms involved in the formation of soy sauce's flavor. Moreover, Hao *et al*. [60] stated that sodium chloride, combined continuously and synergistically with other characteristic umami tastes (glutamic acid), will improve the taste of soy sauce. The concentration of sodium chloride found in this research was substantially lower than that found in other investigations. The sodium chloride content in sweet soy sauce in the literature was within the range of values 3.30-6.27 g/100mL [22]. Overall, the result shows that the sodium chloride concentration escalated from the koji to the moromi phase and reached its peak at the final moromi state. According to Li *et al*. [37], the addition of sodium chloride in high concentration, along with the evaporation of water, which happens when the soy sauce product is left unsealed in a sun-exposed environment, may be connected to the escalation of sodium chloride concentration level. Simultaneously, the salt will stay intact, resulting in a highly concentrated solution. Finally, the sodium chloride will decrease at the refining state, resulting in a lower concentration level of soy sauce. Blanco and Blanco [22] stated that the lower concentration of sodium chloride was probably caused by different types of soy sauce processing, specifically due to the excessive dilution of moromi after the fermentation.

Figure 6 explains the correlation between chemical characteristics and the soy sauce fermentation stages. The available biplot data explained 85.93% of the total data variance, comprising 50.30% for PC1 and 35.63% for PC2.

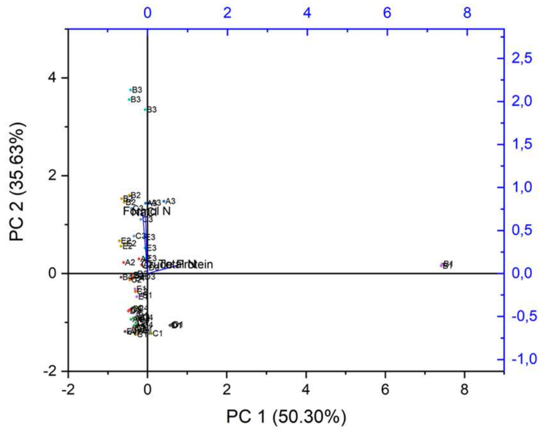

Fig. 6 The correlation relationship between chemical properties and the fermentation stages using Principal Component Analysis (PCA). (A) Koji, (B) Earlier Moromi, (C) Final Moromi, (D) Soybean Sauce.

The biplot graph obtained shows that during the soy sauce production process, Crude Protein and Total Protein exhibited a significant positive correlation. However, they did not correlate strongly with NaCl and Formol nitrogen, both of which displayed a substantial positive correlation. Regarding variability for each variable, Crude Protein, and Total Nitrogen exhibited less variability compared to formol nitrogen and NaCl because they had shorter vector lengths.

By examining the proximity of the sample positions to the vectors of each variable on the biplot graph, it is apparent that sample B in the koji phase had the highest values for Crude Protein and Total Nitrogen compared to the other samples. On average, the values of Crude Protein and Total Protein tended to be lower. Still, generally, in the final moromi phase, their values slightly increased because they were closer to the vectors than the samples in other phases. Sample B in the final moromi phase had the highest levels of formol nitrogen values and NaCl compared to the other samples.

By Figure 6, the koji and moromi phases were characterized by low values of formol nitrogen and NaCl because their positions were opposite and distant from the vectors. Conversely, the final and earlier moromi phases tended to exhibit higher values of formol nitrogen and NaCl.

IV. Conclusion

The fermentation stage (Koji, earlier moromi, final moromi, soybean sauce) significantly impacted the physical and chemical properties of soy sauce. During the soy sauce fermentation process, various properties, including total dissolved solids, water activity, total acids, salt, total nitrogen, crude protein, and formol nitrogen, exhibited similar curves, increasing until the moromi phase, and then decreasing in the final phase of soy sauce production. These fluctuations in chemical properties were primarily driven by microbial activity and enzymatic hydrolysis. Notably, total nitrogen, crude protein, and formal nitrogen values played pivotal roles in characterizing soy sauce quality. Interestingly, the total acid content and pH values showed significant variations throughout fermentation, deviating from the expected correlation between acid content and pH due to weak acids.

Out of the five tested samples, samples C and D were considered the best soy sauce due to their physicochemical properties aligning better with the standards for good soy sauce. Overall, this research provided insights into the intricate physicochemical transformations occurring during soy sauce fermentation, highlighting the influence of raw materials and microbial processes on its properties. Future studies may explore other compounds contained in soy sauce, which fermentation stages and impact soy sauce quality could influence.

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