Characterizations of Milkfish Sauce on Amino Acid Content with Variations in Addition of Salt and Pineapple Extract Concentration

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Abstract. Fish sauce is synonymous with salty taste and distinctive fish aroma. The good quality of fish sauce is supported by the amino acid content, proximate analysis and the assessment of the organoleptic preferences of the product by the panelists. This research aimed to determine the effect of increasing the concentration of pineapple extract on the organoleptic characteristics of fish sauce and the most abundant amino acid content in fish sauce and the best quality of fish sauce treatments. The research is based on randomized design of 4 days fermentation with salt : pineapple extract concentration ratio of 20%:5%, 20%:10%, 15%:15% and 25%:20% respectively. Research analysis such as amino acid content and proximate analysis were evaluated by the ANOVA test and DMRT test, while organoleptic test was evaluated by Kruskal Wallis test. The higher of the concentration of pineapple extract, the lower of amino acid content of the fish and the taste getting sourer. The best treatment for milkfish sauce was found in the treatment with salt concentration : pineapple extract = 15%:15% indicated by the highest amino acid content of glutamate, lysine and aspartate compared to other treatments, the highest protein content and water content and the best organoleptic assessment.

1. Introduction
Fish sauce is synonymous with salty taste and distinctive fish aroma. It is a clear brown liquid and salting taste [1]. Fish sauce contains all essential amino acid and many vitamins and minerals. It is composed of various nitrogen-containing compounds, including amino acid with an extraordinarily high content of lysine [2].

Traditional fish sauce is produced by fermenting fish mixed with salt at the ratio of fish to salt of 2:1 or 3:1 and fermenting at the temperature range of 35-40°C up to 12-18 months or longer to complete [3]. Fermentation is the process of breaking down complex protein compounds which are converted into simpler compounds with the help of enzymes derived from the body of fish or natural bacteria under controlled conditions. It is one of the technique used in preserving perishable fish due to its high salt concentration [4].

The traditional fermentation process is normally continued for a long time and using the high salt concentration. Modern fish sauce is produced in levels of salt 10%-20% by weight. It’s also produced of salt considered excessive, 25-40% by weight which are acceptable in South East Asia [5]. Jeotgal, a fermented fish sauce of Korea, is made from sea animals (whole fish, fish roe, internal organs of fish and shellfish) and high salt 20-30% (w/w) [6]. Most microorganisms except halophiles will not survive due to very high salt concentration in fish sauce (20-25%). However, bacterial metabolites such as free
fatty acids, amines, and various nitrogen-containing compounds are major contributors to fish sauce flavour [7]. The high levels of salt, actually reduced enzyme activity and potential nutritional value of these sauce. The more salt content added will increase the shelf life but decrease the taste of fish sauce. To speed up the fermentation process and reduce salt levels, fish sauce is fermented using enzymes. The addition of bromelain enzymes, papain enzymes, ficin, viscera, koji, and lactic acid bacteria has been shown to shorten the fermentation time [8].

Enzymes that can be used in making fish sauce include papain and bromelain enzymes [9]. Pineapple contains a proteolytic enzyme, namely bromelain which is a protease enzyme that is able to break down protein, therefore it increase dissolved protein content. Making fish sauce using a combination of enzymatic and fermentation has several advantages, such as it requires a short time, high protein value and has a taste that is liked by consumers [10].

Several studies on the manufacture of fish sauce with pineapple extract have been carried out previously. [11] observed the microbiological quality of tuna fish sauce with the addition of pineapple extract. [10] examined the manufacture of fish sauce with raw materials of petek fish and the addition of pineapple juice as a source of bromelain enzymes. Research on the addition of bromelain enzyme with a concentration of 9% can function as a catalyst that helps accelerate the hydrolysis process in fish sauce fermentation has been carried out by [12]. Based on previous research, it is necessary to conduct research on the quality of fish sauce by enzymatic fermentation with the comparison of salt levels and bromelain enzymes from pineapple extract seen from the amino acid content, proximate analysis and the panelists' preference level.

2. Materials and Methods
2.1. Materials and Tools
The raw material used in this study was milkfish weighing 300 grams obtained from Kalanganyar Market, Sedati, Sidoarjo, East Java. The pineapple fruit and the salt used were obtained from Kalanganyar Market, Sedati Sidoarjo, East Java. The testing materials used include milkfish (300 grams); salt: pineapple extract (bromelain extract) = 20%:5%, 20%:10%, 15%:15% and 25%:20%, while the equipment used includes a coolbox (28x18x20), cutting board, analytical balance, incubator, oven, stove, pan, filter, sample bottle, erlenmeyer, hedonic organoleptic test equipment including ballpoint pen, paper, aqua glass, and bowl.

2.2. Research Method
2.2.1. Pineapple crude extract manufacture
Pineapple fruit was cut into small pieces and weighed as much as 1000 g, then blended, squeezed and filtered with a filter cloth to obtain a clear liquid (crude extract of bromelain). The crude extract was filtered again with Whatman No.1 filter paper, then the clear filtrate is taken to be used in making fish sauce [13].

2.2.2. Fish Sauce Fermented
Fish sauce fermentation was carried out using the method of [14]. The milkfish used comes from a traditional market in Sedati District, Sidoarjo, during transportation the fish is placed in a coolbox containing ice cubes to maintain the freshness of the fish. Fresh milkfish are killed and weeded. The fish is then soaked in the orange solution for 5 minutes. Then, the milkfish is boiled for 15 minutes to make it easier to separate the meat and fish bones. After boiling, the milkfish is blended until smooth and then added salt and coarse pineapple extract into each bottle in a ratio (20%:5% or 4:1; 20%:10% or 2:1; 15%:15% or 1:1; and 25%:20% or 5:4). Milkfish solution was incubated for 4 days at room temperature (20-25°C). After being incubated for 4 days, the milkfish solution was filtered, pasteurized for 2 hours (heating on low heat at 70°C), and added seasonings in the form of bay leaves, lemongrass leaves and brown sugar. Next, the cooked fish sauce is filtered again to obtain a separate soy sauce with spices. The fish sauce was then packaged in sterilized bottles for further testing.
Hydrolysis on the 4th day carried out proximate testing, organoleptic hedonic testing (appearance, aroma, taste and texture) and amino acid testing. Proximate and amino acid tests were carried out at Saraswanti Indo Genetech, Surabaya, East Java, while organoleptic hedonic tests were carried out at the Fisheries Product Quality Testing Laboratory, Sidoarjo Polytechnic of Maritime and Fisheries.

2.2.3. Data Analysis
Proximate and amino acid test results data were analyzed by Anova and DMRT further test. While the data from the Hedonic Organoleptic test will be analyzed using Non-Parametric Analysis with the Kruskal Wallis Test and the Mann Whitney Advanced Test.

3. Result and Discussion
3.1. Hedonic Organoleptic Test
Milkfish meat that has been weeded, washed and blended with a mixture of salt and pineapple extract then put into a plastic bottle and hydrolyzed at room temperature (22-25°C) for 4 days. On day 4, the filtrate was taken and filtered with 2 layers of filter cloth to separate the filtrate and residue to obtain clear fish sauce. The filtrate was pasteurized at 70°C for 2 hours to kill bacteria from outside that could damage the quality of fish sauce. The pasteurized fish sauce was then presented to the panelists for hedonic organoleptic testing. The results of the hedonic organoleptic test can be seen in Table 1.

| Parameter | Code of Samples | 854 | 542 | 428 | 288 |
|-----------|-----------------|-----|-----|-----|-----|
| Colour    |                 | 3.53±0.52\(^{a1)}\) | 5.47±0.52\(^{c}\) | 6.73±0.46\(^{d}\) | 2.67±0.49\(^{a}\) |
| Texture   |                 | 3.67±0.49\(^{a1)}\) | 5.47±0.74\(^{b}\) | 6.47±0.83\(^{c}\) | 3.40±0.74\(^{a}\) |
| Aroma     |                 | 2.27±0.46\(^{a1)}\) | 3.53±0.52\(^{b}\) | 6.67±0.62\(^{d}\) | 5.47±0.74\(^{c}\) |
| Flavor    |                 | 2.33±0.46\(^{a1)}\) | 3.60±0.51\(^{b}\) | 6.87±0.35\(^{d}\) | 5.33±0.49\(^{c}\) |

\(^{a1)}\) Different codes on the same line indicate a significant difference in the treatment group

Table 1 shows that the fish sauce color parameters ranged from 2.67±0.49 (slightly dislike) to 6.73±0.46 (like very much). The color of fish sauce with the addition of salt and different pineapple extracts showed a significant difference in the level of acceptance of the panelists between the treatment groups. The color of fish sauce that was still acceptable to the panelists was in the treatment group with addition of salt and pineapple extract 20%:10% (somewhat like) and 15%:15% (like very much). Fish sauce with the addition of 15% salt and 15% pineapple extract has specifications of brown color, clear and clean, a specific smell of fish sauce, and a salty taste. The higher the pineapple extract added, the faster the substrate hydrolysis process into simpler compounds by the activity of proteolytic enzymes in pineapple and bacteria found in the digestive tract, gills and fish skin surface. The addition of pineapple extract in large quantities will produce flavor and aroma-forming components, such as alcohol, ether, organic acids and certain peptides. The higher concentration of pineapple extract can digest fish muscle tissue in a short time and cause the color of fish sauce to become more brown due to the reaction between reducing sugars and amino groups of protein [11]. The higher the salt content added to the manufacture of fish sauce, the slower the browning reaction [15]. The color of fish sauce between treatment groups can be seen in Figure 1.
Figure 1. The Colour of Milkfish Sauce for Each Treatment Groups

Image caption from left to right:
- a. Group A (fish sauce with added salt: pineapple extract = 20%:5%)
- b. Group B (fish sauce with added salt: pineapple extract = 20%:10%)
- c. Group C (fish sauce with added salt: pineapple extract = 15%:15%)
- d. Group D (fish sauce with added salt: pineapple extract = 25%:20%)

The fish sauce texture that was still acceptable to the panelists was found in the treatment group with addition of salt and pineapple extract 20%:10% (somewhat like) and 15%:15% (like). The most preferred fish sauce texture by the panelists was the addition of salt: pineapple extract = 15%:15% (1:1). This is because the bromelain contained in pineapple can shorten the time and accelerate the digestion of fish muscle tissue in a short time so that the perfect liquid texture is obtained [9].

Panelists’ assessment of the aroma of fish sauce ranged from 2.27±0.46 (dislike) in the sample with the addition of salt and pineapple extract (20%:5%) to 6.67±0.62 (like very much) in the sample with the addition of salt and pineapple extract (15%:15%). Fish sauce that was still acceptable to the panelists was found in the addition of salt and fish sauce 15%:15% and 25%:20%. Fish contain aggressive endogenous enzymes, e.g. in viscera, such as proteases and nucleases that digest proteins and nucleic acids, respectively. The more pineapple extract added will accelerate the hydrolysis of protein in fish muscles into amino acids, resulting in a distinctive aroma such as alcohol, ether, organic acids and certain peptides. The alcohol compounds formed in fish sauce during fermentation are mostly aliphatic alcohols and aromatic alcohols which are thought to be the result of hexose fermentation and a small portion of amino acid and fat degradation [16]. The most important contributor to umami in fish sauces is free glutamate and aspartate. The combination of umami compounds and salt works synergistically to enhance the saltiness of fish sauce. The less salty fish sauces typically lead to more flavourful products, since the salt tends to limit enzymatic activity [9].

Panelists’ assessment of the taste of fish sauce ranged from 2.33±0.49 (dislike) to 6.87±0.35 (like very much). The lowest value was obtained in fish sauce with the addition of salt: pineapple extract = 20%:5%, while the highest value was found in fish sauce with the addition of salt: pineapple extract = 15%:15%. The taste of fish sauce that was still acceptable to the panelists was found in the addition of salt and pineapple extract 25%:20% and 15%:15%. The greater the concentration of the addition of pineapple extract, the faster the breakdown of protein in fish into several components such as peptides, peptones, and amino acids that interact to create a distinctive taste [8]. However, the higher the salt concentration will inhibit the process of hydrolysis of proteins into amino acids, peptides and other compounds that create a distinctive savory taste. In addition, the addition of high amounts of salt will enter the substrate and reduce the water content of the fish sauce, so the taste of fish sauce becomes more salty and flavourless [15].

3.2. Proximate Analysis

The milkfish sauces was composed of water content, crude ash, protein, lipid and carbohydrate as shown in Figure 2 and Table 2. The highest value of crude protein content was approximately 3.38±0.19% (w/w) using the Kjeldahl method. The higher the pineapple extract added, the greater the protein content.
which is hydrolyzed into soluble amino acids. In addition, the bromelain enzyme contained in pineapple extract will hydrolyze more connective tissue, causing the meat structure to be more tender and the hydrolyzed protein easily dissolved. Bromelain enzyme acts as a protease enzyme that can break down proteins in collagen and muscle fibers. Proteolysis of collagen and muscle fibers can cause the shear force of collagen and decreasing muscle fibers, so the density of the meat is reduced. Myofibril proteolysis produces protein fragments with shorter peptide chains. The more proteolysis in myofibrils, the greater the amount of dissolved protein. The higher volume of pineapple added, the greater the concentration of enzymes contained in it, while the speed of hydrolysis will increase as the enzyme concentration increases [17].

Water content represented the greatest proximate component of the milkfish sauce, which could have been derived from the hydrolysis of protein from fish muscle into amino acids and peptides which are then broken down into smaller components so as to break down the substrate into water. The highest water content value was found in the fish sauce treatment group with the addition of salt and pineapple extract by 15%:15%. This is because the ratio between pineapple extract and salt is balanced (1:1). The lowest water content value was found in the fish sauce group with the addition of salt and pineapple extract by 25%:20%. The salt added in making fish sauce is humectant and bactericidal. The greater the salt content, the lower the water activity (aW) in food because salt has the ability to bind water of substrate [18].

The results of the ash content test in milkfish sauce can be seen in Table 2. The ash content of all fish sauce treatment groups was <0.02% and was not significantly different between treatment group [16].

| Parameter                  | Milkfish Sauce (Salt : Pineapple Extract) (%) |
|----------------------------|-----------------------------------------------|
| Water Content              | 20%:5% 20%:10% 15%:15% 25%:20%                |
| Ash Content                | 58.73±2.57<sup>a</sup> 63.33±1.68<sup>b</sup> 70.87±1.01<sup>c</sup> 58.46±2.68<sup>a</sup> |
| Protein Content            | 15.52±0.37<sup>a</sup> 13.46±0.37<sup>b</sup> 11.78±0.41<sup>a</sup> 16.81±0.28<sup>d</sup> |
| Carbohydrate Content       | 3.2±0.23<sup>c</sup> 3.07±0.05<sup>b</sup> 3.38±0.19<sup>c</sup> 2.78±0.17<sup>a</sup> |

1) Different codes on the same line indicate a significant difference in the treatment group.

The decrease of water content is caused by the hydration of salt ions which attract water molecular ions in a food ingredient. Salt will penetrate into the body of the fish. The salt that enters the fish's body will replace the free water in the fish's body (hygroscopic). In addition, salt can attract water from microorganism cells so that plasmolysis occurs and inhibits the work of proteolytic enzymes in pineapple extract to hydrolyze carbohydrates, proteins, fats in producing simple molecules [16].

Ash content is one of the important parameters used to indicate the presence of minerals contained in a food ingredient. Foodstuffs consist of 96% organic matter and water, while the rest are mineral elements which are inorganic substances (ash content). In the combustion process, organic materials are burned but inorganic substances are not, which is why they are called ash [19]. The results of the ash content of milkfish sauce with variations in the addition of salt concentration and pineapple extract showed a significant difference in each treatment group. The highest ash content value was 16.81±0.28% (w/w) in the fish sauce group with the addition of salt and pineapple extract = 25%:20%, while the lowest ash content value was 11.78±0.41% (w/w) in the fish sauce group with the addition of salt and pineapple extract = 15%:15%. This is inversely proportional to the water content. The higher the water content of a material, the lower the ash content of the material. The higher the addition of salt to fish sauce, the higher the mineral content, so the ash content is also higher [20].

The results of the fat content test in milkfish sauce can be seen in Figure 2. The fat content of all fish sauce treatment groups was <0.02% and was not significantly different between treatment groups. During the hydrolysis process, the fat in fish sauce decreased due to fat degradation into fatty acids. During fermentation, a lot of organic acids are produced so that it will coagulate proteins. The protein bonds that make up the molecular configuration are damaged due to protein coagulation/denaturation, so the fat bound to the protein (lipoprotein) is released and leaves the tissue. The hydrolysis reaction makes the peptide bonds in the protein broken, so the protein is degraded into simple parts in the form...
of amino acid and carboxyl components and the bound fat will come out [21]. The lower the fat content in fish sauce, the longer the shelf life of the fish sauce.

The results of the analysis of the carbohydrate content of milkfish soy sauce with different treatment ratios of salt concentration and pineapple extract affect the carbohydrate content of the product. This is indicated by the significant difference between the treatment groups. The treatment of adding salt and pineapple extract by 20%:5% was not significantly different from the addition of salt and pineapple extract by 25%:20%, but the two treatment groups were significantly different from the fish sauce treatment group with the addition of salt and pineapple extract 20%:10 % and 15%:15%. The higher the salt content added in making fish sauce, the higher the carbohydrate content in the fish sauce. This is because the presence of NaCl solution can inhibit the work of proteolytic enzymes derived from pineapple extract and from the body of fish to hydrolyze carbohydrates into glucose, thus maintaining high amounts of carbohydrates [16]. The work of salt in inhibiting the hydrolysis of carbohydrates on the substrate decreased along with the increase in the concentration of the bromelain enzyme in pineapple extract. The low carbohydrate content in fish sauce is also influenced by the high content of amino acids in the product [22]. The amino acid content of glycine helps the process of glycogen metabolism into glucose and regulates glucose levels in the fish body, so that high glycine content will reduce carbohydrate levels in the product [23]. Carbohydrate levels in milkfish sauce in all treatment groups were still higher than commercial fish sauce (ABC) with a value of 3.6% [24]. This is due to the effect of adding brown sugar during the cooking process of milkfish sauce. This is in accordance with the results of research by [22] which stated that the carbohydrate content of tuna fish sauce was high because of the caramelization process of brown sugar which was added when cooking tuna fish sauce.

![Figure 2. Proximate Analysis of Milkfish Sauce Treatment Groups](image)
3.3. Amino Acid Contents

Free amino acid contents were identified in all of the treatments of milkfish sauce (Figure 3). The dominant free amino acids contained in milkfish sauce include glutamic amino acids, lysine amino acids and aspartic amino acids. Lysine amino acid has a side chain in the form of a polar or positively charged R group, while the glutamic and aspartic amino acids have a negatively charged side chain R group. All fermented sauces contain high levels of free amino acids, particularly Glu and Asp are known to elicit umami taste [9]. The highest amino acid content in milkfish sauce is the amino acid glutamate. Glutamate is responsible for the distinctive umami taste of fish sauce [25]. The free amino acid contents of glutamate accounted approximately for 3491.17±0.01 to 4312.42±0.01 mg/kg, respectively. The highest value of amino acid content was found in the fish sauce group with the addition of salt and pineapple extract by 15%; 15%. This also applies to the content of the lysine and aspartate amino acids. The results of the study are in accordance with the research of [26] that the highest amino acid content in tuna fish sauce (Thunnus albacores) fermented with the enzyme papain and salt is amino acid glutamate at 1.82% (w/w) which followed by phenylalanine (1.31% w/w) and histidine (1.27% w/w). It was also found in some research on both soy sauce and fish sauce. Fish sauce was found to have the highest glutamate acid number followed by leucine and valine with the number of 128.50 mg/100 mL, 92.70 mg/100 mL, and 78.20 mg/100 mL correspondingly [27]. Other research on soy sauce also reports that glutamate acid showed the highest number of concentration followed by aspartate acid and isoleucine with the value of 2.2-2.9%; 1.05-1.46%; and 1.04-1.28% in that order [28]. The fermentation process will break down amino acids from raw materials through enzyme activity into glutamic acid, glycine and aspartate. Glutamic acid contributes to the savory/umami taste of fish sauce. The higher the activity of proteolytic enzymes during the fermentation process, the higher the amino acids will be. Fish sauce with the addition of a lot of salt will inhibit the activity of proteolytic enzymes, so that the resulting savory taste will be decrease [18]. Salt functions as a fermentation controller, is bactericidal and inhibits the work of proteolytic enzymes (bromelain enzymes) in hydrolyzing carbohydrates, proteins and fats, so that the ability to produce simple molecules and volatile compounds is inhibited [16].

Aspartate is a non-essential amino acid content that functions to help detoxify the liver, improve the immune system, inhibit tumor cell growth, help convert carbohydrates into cell energy [19]. Aspartate in the fish body plays an important role in stimulating the gustatory organs (tasting organs) and olfactory organs (olfactory organs) [23]. Aspartate amino acid is a polar amino acid that is charged, acidic with a side chain containing a carboxyl group and an aliphatic chemical structure. The higher the aspartate content in the product, the better the taste of the product [9]. The highest aspartic amino acid content in the salt addition treatment: pineapple extract = 15%: 15% with a value of 2784.89±0.47 mg/kg, while the lowest amino acid content in the salt addition treatment group: pineapple extract = 15%: 15% at 2720, 90 mg/kg or 272.09 mg/100 mL, while the lowest lysine amino acid content was in the salt addition treatment: pineapple extract = 25% : 20% of 2028.85 mg/kg or 202.89 mg/L. The brown colour of fish sauce resulted in a Maillard reaction between protein and sugar reduction indicates lysine blocking. The decreasing of lysine in fish sauce with the adding of salt : pineapple extract = 25% : 20% caused by E-amino lysine deamination is due to Maillard reaction formed melanoidin a brown colour indication. Thus foodstuff contains lysine, its colour tends to be brown colour [26].
4. Conclusion
The results showed that the best milkfish sauce was found in the addition of salt : pineapple extract = 15% : 15% which was characterized by the highest hedonic organoleptic value by the panelists in terms of colour, texture, aroma and taste with a range of 6.47-6.87 values; the highest water content with a value of 70.87±1.01, the highest protein content with a value of 3.38±0.19; and the highest amino acid content compared to other treatment groups. The highest amino acid content found in milkfish sauce is the glutamate amino acid, followed by the aspartate and the lysine amino acid. The higher the pineapple extract added in the manufacture of fish sauce, the faster the hydrolysis process which results in the colour of the fish sauce getting brown with higher water content and increasing amino acid content. Increased salt content will slow down the hydrolysis of fish sauce and cause fish sauce to be flavourless.

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