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Research Article

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The Effect of Salt Concentration and Incubation Time on Protein Content and Bacteria Number of Cakalang Fish (*Katsuwonus pelamis*) *Ina sua*

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Abstract *Ina sua* is a traditional salted fish product belongs to Teo Nila Sarua (TNS) community, Maluku, Indonesia. The first aim of this study is to find out the effect of salt concentration on protein content and bacteria number of cakalang fish (*Katsuwonus pelamis*) *Ina sua*. Three salt concentrations (10%, 20% & 30%) are used with incubation time of two weeks at 35° C. The second aim of this study is to find out the effect of incubation time on protein content and bacteria number of cakalang fish (*Katsuwonus pelamis*) *Ina sua*. Four incubation times (2, 3, 4, 5 weeks) at 35°C are used with salt concentration of 20%. For a control, protein content and bacteria number of fresh cakalang fish are analysed. The result shows that *Ina sua* with the highest protein content (30.7 %) and the lowest bacterial number (2.3 x 104 CFU/ml) is obtained from the use of 30% salt concentration. The value of pH decreases with increasing salt concentrations. The result also shows that *Ina sua* with the highest protein content (±30%) is obtained at incubation time of 3 and 4 weeks but for all incubation time used the numbers of bacteria are not significantly different ($2.5 \times 10^6 - 1.2 \times 10^8$ CFU/ml). The values of pH are not significantly different for all incubation time used.

Keywords Ina sua, Salted fish, Bacterial Number, Protein Content

Introduction

Ina sua is a traditional salted fish product to be held in reserve for people in Teo Nila Sarua (TNS) Islands, Central Maluku, Indonesia, to be used during the season where no enough fresh fish is available. In producing *Ina sua*, fish is covered by dry-crystal salt and the mixture is put in a closed plastic jar/container for certain period of time (weeks/months). Coconut sap is often added to the mixture to get certain aroma and flavour of *Ina sua.*

People in TNS used to make *Ina sua* only from fish which contain a high amount of lipid. However, nowadays many different kinds of fish are used as a raw material for *Ina sua*. These include *Samandar fish (Siganatus guttatus), Gala-gala fish (Lutjanus sp.) and Sikuda fish (Lethrinus ornatus)* [1]. *Cakalang fish* (*Katsuwonus pelamis*) is also often used to make *Ina sua* due to its abundance in the coastal waters of TNS and its perishables.

Numerous kinds of microorganisms such as bacteria, yeast and mould can be present in *Ina sua* due to the spontaneous fermentation that occurs in the process. The microorganisms in *Ina sua* can be originated from the fish itself, the salt and also the coconut sap used in the manufacture of *Ina sua*. Bacteria are found in many parts of fresh fish, i.e. in body surface ($10^2 - 10^5$ CFU/ml), in gill (($10^3 - 10^7$ CFU/ml), and in intestinum ($10^3 - 10^8$ CFU/ml) [2]. Only bacteria that can survive in saline environment are found in *Ina sua*. Those bacteria are called halophilic/halotolerant bacteria. Halophilic bacteria are bacteria that only can live in saline environment, while halotolerant bacteria can live in both saline environment and nonsaline environment.

Ina sua is made by TNS people in traditional way without a standard procedure. This leads to different quality of *Ina sua* from different producers. The fermentation that occurs in *Ina sua* processing leads to some biochemical changes

on the fish especially the fish proteins as well as the development of certain aroma and flavour. The fish proteins are degraded by proteases which belong to the fish itself and microbes to simple molecules such as amino acids. The amino acids are then used to support the microbe growth. *Ina sua* taken from TNS community is reported to have halo tolerant bacteria numbers of $1.4 \times 10^6 - 2.8 \times 10^7$ CFU/g [1]. High number of bacteria in *Ina sua* can reduce the quality of *Ina sua* and cause *Ina sua* to become unsafe for consumption. The number of bacteria in salted fish has been found to depend on salt concentration and the length of incubation time [3, 4].

In this study we want to find out the effect of salt concentration and incubation time on protein content and bacteria number of cakalang fish (*Katsuwonus pelamis*) *Ina sua*. *Ina sua* is prepared with the same procedure used by TNS people. However, in this study we used salt with different concentrations and without coconut sap addition.

Materials and Methods

Fish Sample Preparation

Three fresh cakalang fish (*Katswonus pelamis*) with similar weight and size were purchased from the fishermen in Latuhalat village of Ambon Island. The fish were put in a cool box and brought to the Biotechnology Laboratory, Science Faculty, Pattimura University, Ambon. The fish were then eviscerated and washed in running tap water. Each fish was sliced to four similar pieces of fish meat with the weight of 100g/piece. Three pieces, each from different fish, were covered evenly by Iodized fine salt of 10 %. Another three pieces were covered by Iodized fine salt of 20 %. Another three pieces were covered by Iodized fine salt of 30 %. The last three pieces were used as a control where no salts were added and analysed for protein contents, bacteria numbers and pH without any incubation time. Individual fish piece covered by salt was put in a closed plastic jar. All the plastic Jars were placed in an incubator with temperature of 35 °C for 2 weeks.

After 2 weeks of incubation, *Ina sua* made by 10 %, 20 % and 30 % salt were taken from each plastic jar to be analysed for their protein content, bacterial numbers and pH values. *Ina sua* made by 20 % salt was taken from each plastic jar after 3 weeks, 4 weeks and 5 weeks of incubation to be analysed for their protein content, bacterial numbers and pH values.

Protein Analysis

The total protein contents of fresh fish and *Ina sua* were analysed by Kjeldahl. Approximately 1.0 g of crushed and homogenized sample from the fish fillet was weighed and mixed with a mixture of sodium sulfate and mercury oxide (20:1) in a digestion flask (Digestion step). Ten ml of concentrated sulphuric acid was added into the flask and then the flask was heated at 400 °C to get a clear solution in the flask. A small quantity of sodium hydroxide was added into the flask and the end of the condenser was dipped into a boric acid solution (distillation step). The ammonia was determined with a volumetric HCl solution 0.1 N (Titration step).

Bacteria Number Analysis

A total of 4 g sample was crushed and homogenized with 36 ml of sterile aquadest. Approximately 1 ml of the homogenate of each sample was diluted serially and spreaded on the surface of marine agar plates and the plates were placed in a 35 °C incubator for 1-3 days. The number of bacteria colony grown on the agar was counted with a colony counter. Only plates with 30 – 300 colonies were counted.

pH Measurements

The pH values of *Ina sua* and fresh fish were determined using homogenized fish fillets with a digital pH meter. Before starting measurements, the pH meter was calibrated for pH-4 and pH-7. The pH of homogenate was recorded by immersing electrode of digital pH meter in the homogenate.

Data analysis

Analysis of Variance (Anova) and Tuckey test were used to analyse the data to find the significance of differences between means ($\alpha = 5$ %).

Results and Discussion

The Effect of Salt Concentration

The results of protein content analysis of fresh fish and *Ina sua* made by different salt concentrations with 2 weeks incubation time are shown in Figure 1. The protein content of the fresh fish (0 % salt) used in this research is 25 %, which indicates that the fish used to make *Ina sua* are still fresh. Protein content of fresh cakalang fish is around 25 % [5]. The protein content of *Ina sua* made with 10%, 20% and 30% salt are 26.1 %, 27.9 % and 30.7% respectively. The result shows that *Ina sua* made with salt concentrations of 20% and 30% have higher protein content than that of either 0 % salt (fresh fish) or 10 % salt ($p < 0.05$). The increase of protein content of the *Ina sua* is a result of the osmotic process where water moves out from the inside of the fish fillet to outside of the fish due to the difference of salt concentration between the inside and outside of the fish fillet. The water discharge from fish fillet occurs simultaneously with the ingress of salt into the fish fillet until a state of equilibrium is obtained where the concentration of salt outside and inside the fish fillet is equal [6]. The ingress of salt into fish fillet and the discharge of water from fish fillet cause the significant thickening of the remaining fluids in fish fillet. This condition leads to the increase in protein content of the *Ina sua* made with 20 % and 30% salt compared to the protein content of the fresh fish. On *Ina sua* made with 10% salt, the thickening of the fluids in the fish fillet is not sufficient enough to produce a significant increase of the fillet protein content.

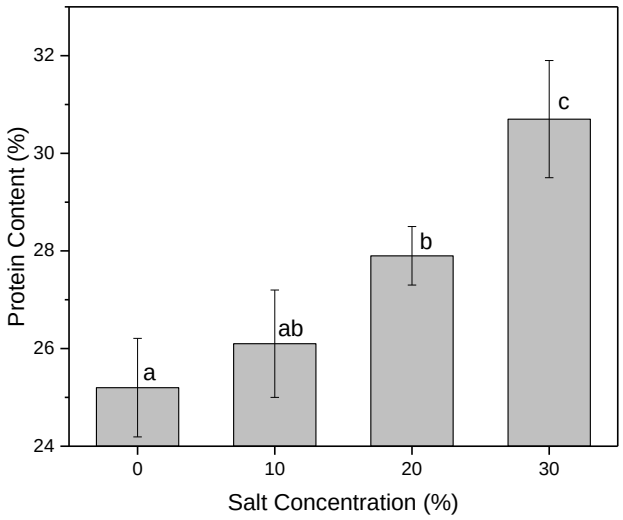

Figure 1: Protein content of fresh fish (0%) and Ina sua made with different salt) with 2 weeks- incubation time. The same letters on the graph indicate that there is no significant difference, while different letters indicate that there is a significant difference

Among the salt concentrations used to make *Ina sua*, salt concentration of 30 % produces *Ina sua* with the highest protein content. This may be due to the higher rate of salt entry into fish fillet and the release of water from fish fillet in *Ina sua* made with 30% salt compared to *Ina sua* made with 20%and 10 % salt. It has been reported that the rate of salt and water diffusion in the manufacture of salted fish from mackerel fish was found to be positively correlated with the increased salt concentrations [7].Higher salt and water diffusion rates at 30% salt concentrations lead to higher body fluid thickening which in turn leads to higher protein concentration of *Ina sua* compared to other salt concentrations.

Another possibility to explain the highest levels of protein in *Ina sua* made with 30% salt is the lowest autolytic activity of protease enzymes in *Ina sua* made with 30% salt compared to that of *Ina sua* made with 20% and 10 % salt. It has been reported that the activity of protease enzymes found in fish meat decreases with the increase of salt [8,9].In addition to the work of proteases derived from the fish itself, degradation of fish protein during fermentation also occurs due to the work of microbial proteases. Fish proteins are degraded by proteases into amino acids which

are either used to build new proteins and enzymes for microbial growth and metabolisms or subsequently degraded into volatile compounds such as ammonia, amines, aldehydes, phenols, indoles and alcohols)[9]. These volatile compounds cause *Ina sua* to have a distinctive aroma.

The log total numbers of bacteria in fresh fish (0% salt) and in *Ina sua* made by 10%, 20% and 30% salt are 4,56 (3.6 X 10^4 CFU/ml), 8.98 (9.6 x 10^8 CFU/ml), 7.49 (3.1 x 10^7 CFU/ml) and 4.37 (2.3 x 10^4CFU/ml) respectively (Figure 2). The results show that the total number of the bacteria in *Ina sua* made with 30% salt is not significantly different from that in fresh fish, but is significantly lower than that in *Ina sua* made with 20% and 10% salt. An increase in salt concentration resulting in a decrease in the number of bacteria was also reported in viscera tuna incubated with different salt concentrations [3]. Salt pulls out water from bacterial cells and eventually leads to the death of the cells. Therefore, the more salt used in making *Ina sua* the more microbes that do not survive in *Ina sua*. The low number of bacteria in *Ina sua* made with 30 % salt may be correlated with its high protein content due to its low microbial activity. Only halophilic and halotolerant bacteria can survive in saline environment like *Ina sua*. Halotolerant bacteria found in *Ina sua* are *Bacillus* sp., *Propioni bacterium* sp., *Leuconostoc* sp., and *Lactobacillus* [10]. Halophilic bacteria often found in salted fish product are Halobacterium and Halococcus [11,12], which are the cause of the appearance of a pink-coloured slime in salted fish products. The pink-coloured slime and pink-coloured colonies were not found in *Ina sua* and Marine agar respectively during the experiments indicating the absence of Halobacterium and Halococcus in the *Ina sua*. Halotolerant bacteria have special adaptations to survive in saline environment, whereas halophilic bacteria need saline environment to function well. The numbers of bacteria in 10%salt-*Ina sua*(1.2 x 10^9CFU/ml)and 20% salt-*Ina sua*(4,1 x 10^7 CFU/ml)are above the Indonesian National Standard and International standard of microbial food numbers which are 5 x 10^5 CFU/ml and 10^5 CFU/ml respectively [13,14]. The number of bacteria in 30% salt-*Ina sua* (4.8 x 10^4CFU/ml) is below the standards.

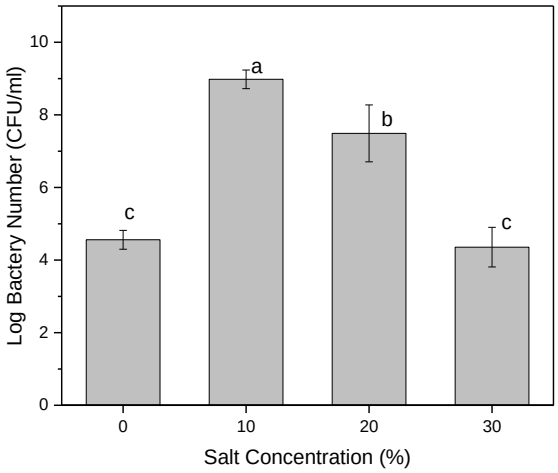

Figure 2: Log bacteria number of fresh fish (0%) and Ina sua made with different salt concentrations and 2 weeks-incubation time. The same letters on the graph indicate that there is no significant difference, while different letters indicate that there is a significant difference

The pH values of *Ina sua* made with different salt concentrations are shown in Figure 3. The results show that the value of pH in 10% salt-*Ina sua* (pH 7.1) is higher significantly than that in fresh fish (pH 5.6) and it approaches the pH value of base indicating the presence of compounds such as ammonia, trimethylamine, and other volatile compounds resulted from amino acid degradation by proteases. The volatile compounds cause the *Ina sua* to give off an unpleasant odor. The result also shows that the pH values in 20% salt-Ina sua (6.3) and 30% salt- Ina sua (6.1) are lower significantly than that in 10% salt-Ina sua. The pH decrease in 20% salt-Ina sua and 30% salt- Ina sua is thought to be due to the presence of large amounts of acids such as lactic acid, format acid, acetic acid produced by lactic acid bacteria in the Ina sua. Palludan-Muller et al. [15] found that during fermentation of *plaa-som*, a

fermented fish originated from Thailand, there is a decrease in value of pH along with an increase in the number of lactic acid bacteria. Using fish sauce and *peda*, an Indonesia fermented fish, Desniar *et al*., [16,17] also found the same results. The decrease of pH during the fermentation of *Ina sua* also has been reported [11]. The formation of organic acids in *Ina sua* provides a typical taste and aroma of *Ina sua*. They also function as a preservative in *Ina sua* since the pH value of *Ina sua*is very influential on the types of microbes that can grow in *Ina sua*.

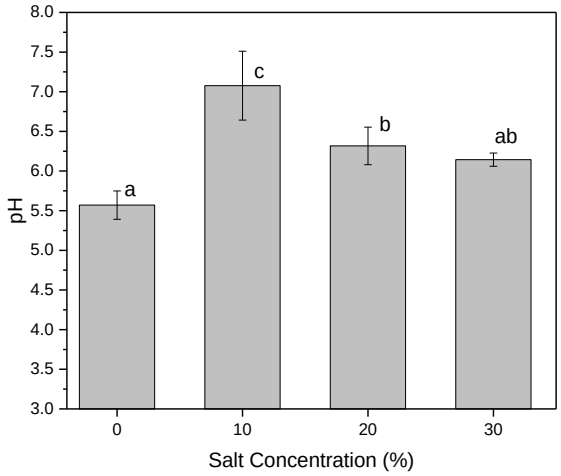

Figure 3: The pH value of fresh fish (0% salt concentration) and Ina sua made with different salt concentrations and 2 weeks-incubation time. The same letters on the graph indicate that there is no significant difference, while different letters indicate that there is a significant difference

The Effect of Incubation Time

Protein content of *Ina sua* made in different incubation time is summarized in Figure 4.The protein content of *Ina sua* with 0, 2, 3, 4 and 5 weeks of incubation time are 25%, 27.9%, 29.8%, 30, 4% and 23.7% respectively.

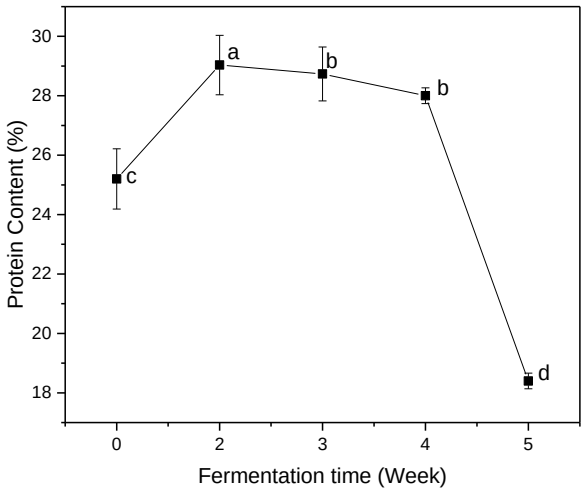

Figure 4: Protein content of fresh fish (0%) and Ina sua made with different incubation time on 35°C with 20 % salt concentration. The same letters on the graph indicate that there is no significant difference, while different letters indicate that there is a significant difference

The results show that there is an increase in protein content of *Ina sua* during incubation time of 2, 3, and 4 weeks in comparison to protein content of fresh fish. The increase may be due to high osmotic process and low fish protein degradation during fermentation. The highest protein content is found in *Ina sua* with incubation time of 3 and 4 weeks. The 3 and 4 week protein content are found to be not significantly different ($p < 0.05$). As incubation time reaches 5 weeks, the protein content of *Ina sua* declines by 6.7% (from 30.4% to 23.7%). High protein degradation by proteases and microbial activity as explained above and also the release of amino acids and peptides along with the release of fluids from fish meet are thought to be the cause of the decline. The liquids collected during fermentation of fish in the presence of salt have been reported containing amino acids and peptides by 12% [18]. Decreased protein content during spontaneous fermentation in other fermented fish products have also been reported [18,19, 20].

The numbers of halophilic/halotolerant bacteria in *Ina sua* counted in different incubation time are shown in Figure 5. The log numbers of the bacteria are in the range of 6.4 - 8.1($2.5x\ 10^6 – 1.2\ x\ 10^8$ CFU/ml) and already above the Indonesian National Standard and International standard of microbial food numbers. The numbers of bacteria for all incubation time used in this research are not significantly different ($p>0.05$). However, the pink-halophilic bacteria were only found in 5 weeks-*Ina sua* indicating the presence of Halobacterium and Halococcus in the *Ina sua*. Halobacterium and Halococcus grow well at salt concentration around 20-25%, and require at least 5-10% salt for growth. These kinds of bacteria are found in solar salt and solar salt environments [21,22]. The composition of bacteria in fermented fish and shrimp has been found to depend on the salt concentration used and the duration of fermentation time [4,23]. Since the numbers of bacteria in all incubation time are not significantly different, bacterial activity may not be the cause of significantly decreased protein content in *Ina sua* with 5 weeks-incubation time. Other microbes that may be found in *Ina sua* are yeast and mold since they have been found in other salted fish [24, 25,26].

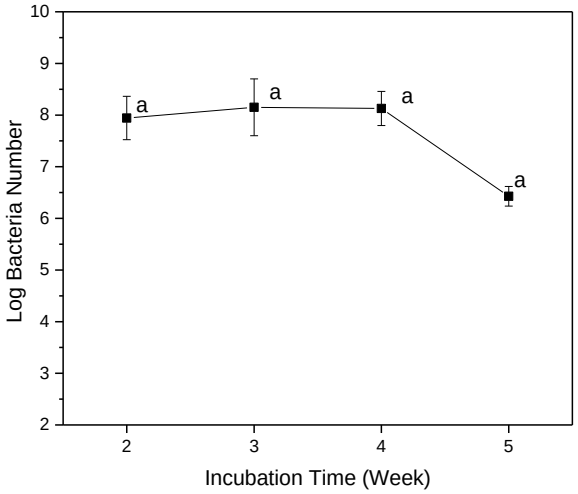

Figure 5: Log bacteria number of Ina sua made with different incubation time on 35°C with 20% salt concentrations. The same letters on the graph indicate that there is no significant difference, while different letters indicate that there is a significant difference

Results from pH measurements are shown in Figure 6. The value of pH of the *Ina sua* with incubation time of 2, 3,4 and 5 weeks are6.28, 7.19, 6.64, and 6.51 respectively. The pH values of these four *Ina sua* were not significantly different ($p>0.05$) indicating the same amount of acids in the four *Ina sua*.

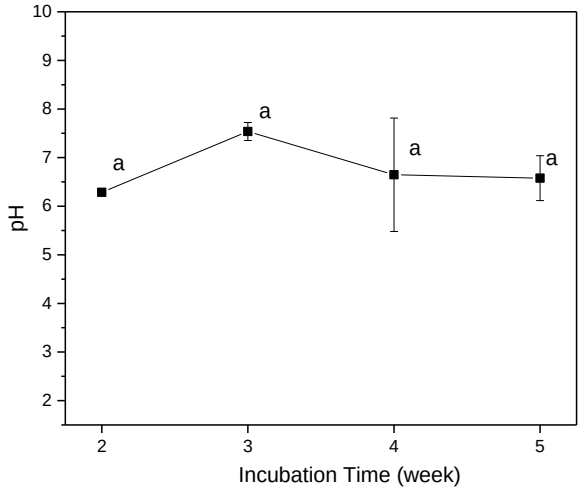

Figure 6: The pH value of Ina sua made with different incubation time and 20% salt concentration. The same letters on the graph indicate that there is no significant difference, while different letters indicate that there is a significant difference

Conclusions

- Among the salt concentrations (10 %, 20%, 30%) used in the preparation of Ina sua with incubation time of 2 weeks, salt concentration of 30 % yields Ina sua with the highest protein content (30.7 %) and the lowest bacterial number (2.3 x 10^4 CFU/ml).
- Among the incubation times (2,3,4,5 weeks) used in the preparation of Ina sua with salt concentration of 20%, Incubation time of 3 and 4 weeks yield Ina sua with the highest protein content (±30%) but the numbers of bacteria are not significantly different for all incubation time used (2.5x 10^6 – 1.2 x 10^8 CFU/ml).

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