# Optimization of NaOH Pretreatment Time on Collagen Production from Milkfish Scales

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**Abstract.** The pretreatment using 0.1 M NaOH is widely used on fish scales in the collagen production stage. This process is necessary to remove non-collagen proteins, fats, and other impurities, but improper pretreatment can reduce collagen levels obtained because prolonged exposure will damage connective tissue on scales, resulting in decreased yield. This research aims to determine the optimum NaOH pretreatment time for the collagen yield produced and identify changes in milkfish scales chemical composition and surface morphology. The stages in this study are: 1) preparation of milkfish scales; 2) pretreatment of milkfish scales with NaOH 0.1 M at various time variations of 0, 1, 3, 6, and 8 hours; and 3) determination of the effect of pretreatment time on collagen yield produced from milkfish scales using proteases from *Bacillus megaterium* TR-10. The determination of optimum conditions is based on the content of water, ash, fat, protein, metal ion content ($Hg^{2+}$, $Pb^{2+}$, $K^{+}$), and surface morphology of milkfish scales before and after NaOH pretreatment is carried out. The results showed that the highest yield of 56.77 ppm was achieved at the pretreatment treatment of NaOH for 6 hours. The longer the NaOH pretreatment time, the water, ash, fat, protein, and metal content in the scales decreases, and the surface morphology of milkfish scales is cleaner, but in the 8-hour treatment, damage to connective tissue fibers in fish scales is observed. The optimal NaOH pre-treatment time for collagen production from milkfish scales is 6 hours of treatment.

**Keywords:** collagen, pretreatment time, milkfish scales, protease.

## 1 Introduction

Collagen is a protein that makes up the connective tissue of an organism which is composed of three polypeptide chains that are twisted together to form a triple helix structure [1]. Collagen acts as an active ingredient in the field of nutritional and functional development such as cosmetics, food, and pharmaceuticals [2]. Along with the development of collagen in the industrial field, it encourages several studies to obtain collagen made from environmentally friendly and halal raw materials.

The halalness of a product is not only in food but also in non-food items [3]. Meanwhile, currently the industrial source of collagen and gelatin comes from mammalian


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bones and skins (mainly pigs and cows). This is clearly related to Muslims who strictly prohibit pork products and their derivatives. While cows are allowed but also need to be considered as long as the slaughter process is in accordance with the prescribed Islamic law [4]

Based on the problems and considerations related to halal raw materials in kolagaen production, fish ingredients have high potential to meet the halal requirements of Muslims [5]. One of the fish materials that can be utilized as raw material is fishery waste in the form of fish scale waste. Fish scales have a low lipid content and a large surface area so they are considered more efficient in the production process [6]. Fish scales have a smaller molecular structure than cow or pig skin, so they have a wide surface [7]. In addition, Indonesia, with its diversity of fish species, is a potential resource, so the productivity value is high. The high amount of production results in an increase in the number of by-products in the form of scales, bones, and skin. Therefore, the use of fish scales as a source of collagen canbe used as well to overcome fishery waste that is accumulating.

Milkfish is a leading commodity, especially in the Sidoarjo Regency area. The Fisheries Office of Sidoarjo Regency said that milkfish production in 2018 reached 34,120,500 kg of milkfish. This large production will certainly result in waste production in the form of large fish scales. In line with this, using milkfish scales as a source of collagen was previouslycarried out and achieved a dissolved collagen yield of 166 mg from 5 grams of milk fishscales using an enzymatic extraction method [8]

The production of collagen from milkfish scales itself can be influenced by several things, one of which is the main focus of this study, the pretreatment process. Thus, an important stage is to remove impurities that can affect the purity of the collagen obtained so that if the process is carried out during an inappropriate time, it can impact the purity of collagen and the low yield of collagen produced [9]. The right pretreatment time can dissolve non-collagen proteins and fats optimally without dissolving collagen in fish scales so that the collagen yield obtained will be more, while extended pretreatment time can cause collagen protein dissolution so that the yield obtained becomes low [10]. Some studies show that the length of time pretreatment of NaOH in raw materials can affect the purity of collagen and impact the yield of collagen produced. One of them is research on collagen production derived from Grass Crap fish skin, where the right pretreatment time can increase the collagen yield obtained [10]. In line with this, this study was conducted to prove the effect of pretreatment time on the yield of collagen production.

The production of collagen from milkfish scales carried out in this study refers to re- search [11]. that uses protease enzymes from Bacillus megaterium TR-10 to hydrolyze peptide bonds in connective tissue so that dissolved collagen can be obtained. Collagenproduction from milkfish scales conducted in research [11] has stag-es, namely prepara-tion and pretreatment of milkfish scales with 0.1 M NaOH, inoculation and productionof Bacillus megaterium TR-10, and collagen extraction from milkfish scales using Ba-cillus megaterium TR-10. In the study [11], the NaOH pretreatment process was carriedout for 6 hours, while this study wanted to know whether 6 hours was the optimal timeor before and after 6 hours which was the optimal time, and it wanted to see the effect on the collagen yield obtained. Based on this, pre-treatment time optimization was car-ried out with time variations: 0, 1, 3, 6, and 8 hours.

In addition to being determined by the yield of collagen produced, the optimum time

also considers changes in the chemical composition of milkfish scales. Therefore, it is also necessary to identify whether, during the collagen production process, especially after the NaOH pretreatment stage, there are changes in chemical composition such as water content, ash content, fat content, protein content, and metal ion levels in milkfish scales. This information is useful for identifying the safety of collagen produced from raw materials during the production process to the final product.

## 2 Materials and Methods

### 2.1 Preparation of Milkfish Scales

Milkfish fish scales that have been obtained are washed first to remove non-specific impurities such as soil and mucus using hot water and running water. This process was repeated 10 times until clean white fish scales were obtained. After that, clean fish scales were dried using an oven at 65-70 °C for 6-7 hours. Fish scales that are not used immediately can be stored in a container and put in the freezer.

### 2.2 Pretreatment of Milkfish Scales

Milkfish scales that had been cleaned were soaked in 0.1 M NaOH solution (1:10 b/v) with time variations of 0, 1, 3, 6, and 8 hours (every 2 hours NaOH was replaced), then the scales washed with sterile distilled water until a neutral pH is obtained in the used washing water. Next, it is dried in the oven at 65-70 °C for 6-7 hours.

### 2.3 Extraction of Dissolved Collagen from Milkfish Scales at Various Times

Milkfish fish scales pretreated at various times of 0, 1, 3, 6, and 8 hours were taken 5 grams each and suspended in 1.56 U crude protease extract and phosphate buffer pH 6 solution to a total volume of 36 mL. The crude protease extract used in this study used the crude protease extract from Bacillus megaterium TR-10, which was isolated according to [12]. The mixture was incubated in a water bath shaker at room temperature (25 °C) at 100 rpm for 30 hours. The resulting suspension was centrifuged cold at a temperature of 4 °C, speed of 10,000 rpm for 60 minutes. The supernatant obtained is Protease Soluble Collagen (PSC) which can then be measured using the Lowry method.

### 2.4 Determination of Collagen Yield

Determination of dissolved protein levels in PSC was carried out according to the Lowry method, by means of 0.5 mL of sample, added with 2.5 mL of Biuret solution, then homogenized with a vortex and incubated for 10 minutes. After that, 0.25 mL of Folin-ciocalteu reagent was added and incubated in the dark for 20 minutes. Then a blue solution will be obtained. Then the absorbance was measured using a UV-VIS spectrophotometer at a wavelength of 750 nm.

### 2.5 Determination of Moisture Content

Each scale of milkfish fish pre-treated was determined by taking 2 grams of it and putting it into an evaporating dish whose weight was known. Then dried in the oven at 100-105 ℃ for 4 hours. Next, it was put into a desiccator and weighed. Reheated for 30 minutes and put back into the desiccator. this treatment repeated until a constant weight is obtained.

### 2.6 Determination of Ash Content

Each pre-treated milkfish scales were determined by placing 2 grams into a crucible cup whose weight was known. After that, it is dried in a furnace for 3 hours at a temperature of 400-600 ℃ until an ash sample is obtained. Weigh the ash formed after cooling.

### 2.7 Determination of Fat Content

Each pre -treated milkfish scales was determined for total N-content referring to the Soxhlet extraction method [13].

### 2.8 Determination of N-total

Each pre-treated milkfish scales was determined for the total N-content referring to the Kjedhal method [13].

### 2.9 Determination of Metal Content

As much as 1 gram of each pre-treated milkfish scales was destroyed using HNO3 reagent or regia reagent. Then the absorbance was measured using Atomic Absorption Spectrophotometry (AAS) at the respective wavelengths (Pb : 223.8 nm, Hg : 253.7 nm, Na : 589.0 nm, K : 766.5 nm).

### 2.10 Identification of the Surface Morphology of Milkfish Scales

Each of the pretreated milkfish scales was dried in an oven for 2 hours at 100 ℃. Then the sample was inserted into the Structural Equation Model (SEM) tool using 25000x magnification and the images obtained were observed.

## 3 Results and Discussion

### 3.1 Milkfish Scale Preparation and Pretreatment

The milkfish scales used in this study were randomly obtained from Tambak Sidoarjo Regency and Malang City. Milkfish scales used as a source of collagen in this study have a white morphology, a diameter of 0.5 cm, and a flattened shape. The purpose of

preparation on scales is to remove impurities attached to the time of sampling fish scales. Before preparation, the scales of the fish obtained are brownish, slimy, and smell fishy. After the preparation, clean fish scales were obtained that were white, not slimy, and odorless (Figure 1).

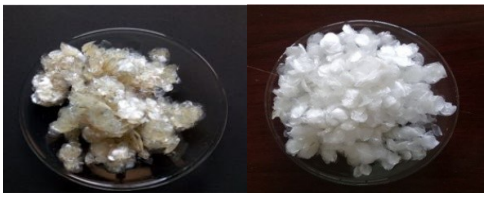

**Fig. 1.** Morphology of milkfish scales before preparation (A), after preparation (B)

Next, the pretreatment stage is carried out. This stage aims to remove non-collagen proteins, fats, and other impurities other than collagen that can affect production results [10] [9]At this stage, a NaOH concentration of 0.1 M is used in the pretreatment process because 0.1 M is the optimum concentration to dissolve non-collagen proteins and other impurities without degrading collagen proteins in fish scale tissue [10]. The levels of dissolved collagen or PSC (Protease Soluble Collagen), shown in Figure 2, increase until the 6th hour but decrease in the 8th hour. The decrease is suspected at the 8th hour. NaOH damages the structure of scales so that the amount of protein in the scale tissue decreases as a result of which the number of PSCs due to the activity of crude protease extracts also decreases. This hypothesis is proven through proximate analysis of the scales of the retreat results.

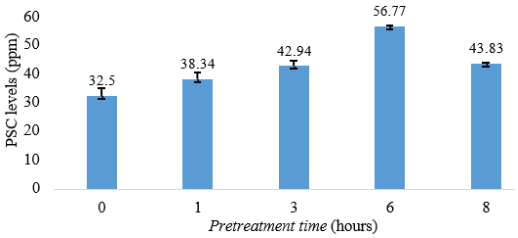


**Fig. 2.** Protease Soluble Collagen Levels of Milkfish Scales Pretreatment Results

### 1.1. Effect of Pretreatment Time on the Proximate Composition of Milkfish Scales

The results of the proximate analysis that has been carried out can be seen in Table 1, indicating that the longer the pretreatment time, the proximate composition of milkfish scales decreased. In identifying the water content, it can be seen that there has been a decrease in the level in accordance with the provisions of SNI 8076-2020, that the maximum water content is 14%. This decrease in water content is thought to be due to the breaking covalent bonds between amino acids in non-collagen proteins [14]. Theoretically, the longer the pretreatment process for non-collagen proteins will dissolve first. The longer the process, the higher the solubility of non-collagen proteins. The solubility of non-collagen proteins is caused by the breaking of peptide bonds between amino

acids in alkaline conditions, and this breaking requires water molecules around these bonds so that the water content in fish scales decreases.

**Table 1.** Proximate composition of milkfish scales

| *Pretreatment Time of* Milkfish scales | Moisturecontent (%) | Ash content (%) | Fat content(%) | Protein content (%) |
|---|---|---|---|---|
| 0 Hours | 25,10 | 29,45 | 0,55 | 61,30 |
| 1 Hours | 9,37 | 25,07 | 0,40 | 57,07 |
| 3 Hours | 8,09 | 17,72 | 0,29 | 54,15 |
| 6 Hours | 7,25 | 14,98 | 0,01 | 50,30 |
| 8 Hours | 6,6 | 13,51 | 0,039 | 42,44 |

The decrease in ash content is in line with several journals which state that the pretreat-ment process dissolves minerals and other impurities present in fish scales so that the ash content decreases. However, the ash content obtained is above the maximum level attached to SNI 8076-2020. The lowest ash content for milkfish scales is 13.51%, while the maximum ash content for SNI 8076-2020 provisions is 1%. This high ash content indicates that the scales of milkfish contain high minerals, so it is necessary to confirm whether it is a dangerous metal ion and the need for other efforts to reduce the ash content of milkfish scales, one of which is the demineralization procedure [15]. The reduction in fat content due to the pretreatment process is in accordance with the purpose of the process [16]. Based on the data obtained, it shows that the longer the pretreatment process, the lower the crude protein content contained in milkfish fish scales. During immersion with NaOH, it can cause the protein to undergo hydrolysis and cause damage to its constituent amino acids [17]. Therefore, the longer the pretreatment process is carried out, the lower the levels.

### 1.2. Effect of Pretreatment Time on the Metal Content of Milkfish Scales

Collagen is generally used as an additive in developing industrial fields such as food, pharmaceuticals, and cosmetics [18]. Previous data showed that the ash content of the milkfish scales used was still on the threshold of the provisions of SNI 8076-2020, so the content of dangerous heavy metal ions (Hg and Pb) and harmless metal ions such as K and Na were also determined, which were abundant in water and pond soil (Table 2).

**Table 2.** Data on Hg, Pb, and K Metal Levels in Milkfish Scales

| *Pretreatment Time of* Milkfishscales | Hg (mg/kg) | Pb (mg/kg) | K (mg/kg) | Na (mg/kg) |
|---|---|---|---|---|
| 0 Hours | 11,85 ± 0,62 | Not detected | 3,30 ± 0,02 | 438,62 ± 0,90 |
| 1 Hours | Not detected | Not detected | 1,43 ± 0,01 | 1561,29 ± 0,72 |
| 3 Hours | Not detected | Not detected | 0,78 ± 0,00 | 1333,78 ± 1,26 |
| 6 Hours | Not detected | Not detected | 0,28 ± 0,00 | 1224,68 ± 1,54 |
| 8 Hours | Not detected | Not detected | 0,13 ± 0,00 | 1047,73 ± 0,00 |

Table 2 shows that the pretreatment process was able to affect the heavy metal contentfound in fish scales. The scale sample used previously detected the heavy metal Hg

which exceeded the threshold for SNI 8076-2020. The maximum limit for Hg metal contamination is 1.0 mg/kg. Then after the pretreatment process, Hg metal was not detected. This can happen because the pre-treatment process is effective in removing impurities, one of which is heavy metals. In addition, for the heavy metal Pb, the fish scale samples used were not detected to contain the heavy metal. Table 2 also shows that the milkfish scales sample used as a source of collagen contains potassium metal. The longer the pretreatment time, the lower levels of metal K decreased to the lowest level at 8 hours of pretreatment, which was 0.13 ± 0.00 mg/kg. But a unique phenomenon occurs in the determination of Na content. The results showed an increase in Na levels after pretreatment, presumably this was due to the addition of Na ions thought to have come from the distilled water used during the study. However, this hypothesis needs to be proven empirically.

### 1.3. Effect of Pretreatment Time on the Surface Morphology of MilkfishScales

The morphological structure of the surface of milkfish scales in this study was analyzed using the SEM instrument at a uniform magnification of 25000x. The following is a surface image of the milkfish fish scales before and after the NaOH pretreatment (Figure 3-7), showed that the longer the pretreatment time, the surface of the milkfish scales showed a cleaner and smoother morphology. At the 6-hour pretreatment time, it showed the cleanest and smoothest morphology compared to the others. Theoretically, the proper pretreatment process can remove impurities other than collagen found in fish scales to obtain a clean and smooth surface. In addition, the longer the pre-treatment time showed significant swelling on the surface of the fish scales. This is in accordance with several other journals which stated that the pretreatment process with NaOH was abe to cause changes in the structure of collagen where most of the telopeptides of the collagen molecule were broken down, resulting in swelling of the fish scales [10][19][20]. Whereas at the pre-treatment time above 6 hours, it showed that the surface of milkfish fish scales had been damaged. This is presumably because the longer pretreatment time can cause damage to collagen crosslinks in alkaline conditions [9]. Remove impurities without affecting the collagen content found in fish scales.

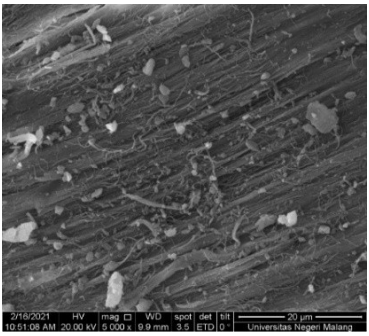


**Fig. 3.** Surface of milkfish scales after 0 Hours of Pretreatment

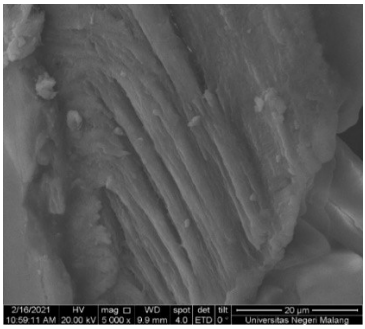


**Fig. 4.** Surface of milkfish scalesafter 1 Hours of Pretreatment

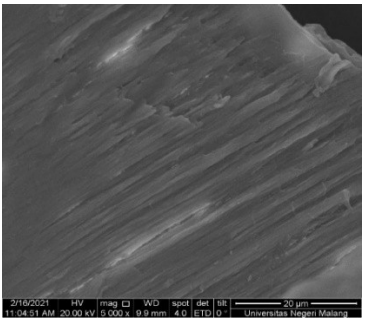


**Fig. 5.** Surface of milkfish scales after 3 Hours of Pretreatment

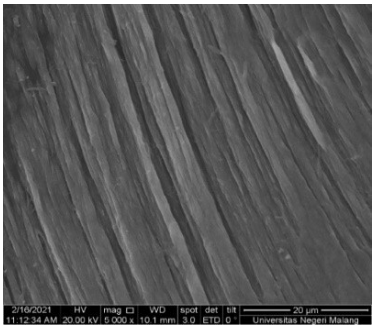


**Fig. 6.** Surface of milkfish scales after 6 Hours of Pretreatment

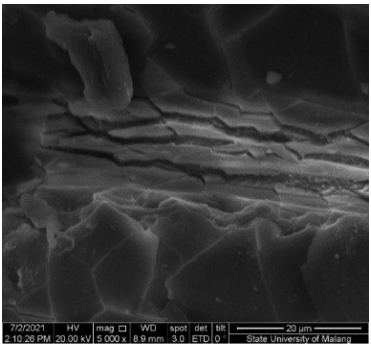


**Fig. 7.** Surface of milkfish scales after 6 Hours of Pretreatment

The optimum time was determined based on several parameters, including the obtainedProtease Soluble Collagen (PSC) levels and milkfish fish scales' surface morphology. Figure 1 shows that the 6-hour pretreatment time produced the highest PSC levels. Above the 6-hour pretreatment time, the levels decreased. Theoretically, too long a pre-treatment time can cause collagen dissolution, thereby affecting the yield of collagen obtained [10]. The length of pretreatment time also affected the surface morphology of milkfish fish scales. The surface of milkfish scales without the pretreatment process depicts the tissue covered by impurities that are considered fat, non-collagen protein, or other compounds. Meanwhile, after the pre-treatment process, the impurities disap- peared with the long pre-treatment time. Until the 6-hour pretreatment time showed thatthe surface of milkfish scales was cleaner and smoother than before (Figure 5). Theo- retically, the correct pre-treatment time can dissolve existing impurities without causingdamage to the collagen molecules, which impacts changes in fish scale tissue [10]. whereas the 8-hour pre-treatment time shows the surface morphology of damaged fishscales. Theoretically, during the pre-treatment process, swelling of the fish scales oc- curs due to damage to the telopeptide collagen molecule so that water can enter and dissolve the existing impurities [19]. So that if the pretreatment process is carried out too long, it can cause damage to the collagen cross-links in an alkaline environment and damage the surface of the raw material [9]. So that the surface morphology of dam-aged fish scales will be obtained, as shown in Figure 6.

Based on these data it is explained that the longer the pretreatment time can minimize the presence of impurities and increase the yield of collagen obtained up to 6 hours as shown in Figure 2, whereas after 6 hours the yield of collagen obtained becomes lower.In line with this, it can be concluded that the optimum pretreatment time for collagen production from milkfish scales is 6 hours.

## 4 Conclusion

The length of time of pretreatment affected the yield of collagen obtained. The optimum time for the pretreatment process for collagen production was 6 hours because the highest dissolved collagen content was obtained, namely 56.77 ppm. The surface morphology of milkfish scales at 6 hours of pretreatment gave the best results by showing a clean and smooth surface compared to the surface at other pretreatment times. In addi-

tion, the longer the pre-treatment time, the lower the water, ash, fat, protein, and metal content.

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