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Characteristics of Milkfish (*Chanos chanos*) Bone Gelatin Using Bromelain Enzyme Hydrolysis Method

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ABSTRACT

Gelatin is a product obtained through partial hydrolysis of collagen from animal skin and bones. Milkfish is a type of fish that contains high protein, milkfish bones have 32% protein which can be used as an alternative for gelatin production. The purpose of this research was to determine the effect of soaking time and soaking temperature on the characteristics of milkfish bone gelatin. This research used the Split Plot Randomized Block Design method which consisted of 2 factors. The first factor was soaking time with 3 levels, namely (1) 12 hours (2) 16 hours and (3) 20 hours. The second factor was soaking temperature consisting of 3 levels (1) 25^o (2) 35^{o}C (3) 45^{o}C. The research results showed that soaking time had a significant effect on yield, ash content, pH, viscosity, L*, a*, b*, *chroma* and clarity. Soaking temperature has a significant effect on yield, ash content pH, viscosity, L*, a*, b*, ^{0}Hue, *chroma* and clarity. There was an interaction of soaking time and temperature on yield, ash content, viscosity, L*, a*, b*, ^{0}Hue, chroma and clarity.

Keywords: bromelain enzyme, gelatin, milkfish bone

INTRODUCTION

Gelatin is a product obtained through partial hydrolysis of collagen from the skin and bones of animals such as pigs and cows (Duconseille *et al*., 2015) Gelatin is used as a stabilizer, emulsifier, binder, thickener, alternative plastic (edible film) and matrix material for implantation in the food industry (Nugrahaeni *et al*., 2021) The use of gelatin was increasing with the use of gelatin as raw materials of food product development such as marshmallow, jelly, yogurt, candy and ice cream (Santosa *et al*., 2018).

The development of gelatin base products needs to consider the halal status of gelatin which became the current halal issue. Because in 2020, global gelatin production reached 516,8 thousand tons, 58% of which came from pork skin, and the rest came from cowhide, cow bones, and other sources such as poultry and fish (Febriana *et al*., 2021). Therefore, the production of gelatin requires halal ingredients, such as those derived from fish, poultry, or cows. One of the potential alternatives for becoming raw materials of gelatin is from fish sources such as milkfish.

The Directorate of the Ministry of Maritime Affairs and Fisheries (KKP) (2020) reported that Indonesia produced 784,941.13 tons of milkfish, with Banten Province contributing 2,891 tons of milkfish, which is processes into milkfish satay, a traditional food specialty of Banten. The processing of milkfish satay only utilizes the flesh and skin, leaving waste in the form of bones and scales that remain unused. Both of the waste still contain high protein content which is potential as gelatin's raw materials. (Fadnan Akhmadi *et al*., 2019) stated that milkfish processing often exclusively utilizes the flesh, while the bones are discarded, resulting in waste. The amount of waste produced from milkfish bones reaches 15 kg or approximately 5.4 tons per year. However, milkfish bones have a nutritional content consisting of phosphorus of 3%, calcium of 4% and protein content of 32%, which can be used as an alternative ingredient for gelatin.

The technique of gelatin isolation involves the use of acid, alkaline, and enzymatic solvents (Hidayat *et al*., 2016). The use of acid solvents increases production costs, making them expensive. Additionally, acids cause protein degradation, which negatively impacts the quality of gelatin. On the other hand, alkaline solvents require lengthy processing times, making the overall process inefficient (Gumilar dan Pratama, 2018). The use of enzymes in gelatin production is still rarely applied. The production of gelatin using enzymatic hydrolysis can produce gelatin with a high level of purity (Sasmitaloka *et al*., 2017). Gelatin production using enzymes can enhance the stability of gelatin at high temperatures (Bello *et al.*, 2020) and does not cause damage to the peptides chain that build amino acids structure in the milkfish bones (Baehaki *et al*., 2015). One of the enzymes that can be used to hydrolyze milkfish bone protein is bromelain. Bromelain is highly effective in hydrolyzing proteins, breaking them down into less complex forms by cleaving peptide bonds (Ilyas *et al.*, 2020). Research on enzymatic processing of fish bone gelatin has been conducted, including a study by Yuliana *et al.* (2021) on snakehead fish bone gelatin using bromelain with different enzyme concentration treatments. The optimal result was achieved with the addition of 6% enzyme, yielding gelatin with a recovery rate of 3.56%. Another study related to enzymatic gelatin production was carried out by (Haryati *et al*., 2019) on siganid fish skin gelatin with varying enzyme concentrations. The best result was obtained with a 2% enzyme concentration, producing a yield of 6.5%. (Hariyati 2021) reported that an enzyme concentration of 50 U/g with a soaking time of 3 hours resulted in a yield of 2.47%.

The soaking time and temperature were used as factors that can influence the properties of the gelatin produced. The longer the soaking time, the higher the yield, however, as the temperature increases, the yield tends to decrease. The performance of the enzyme is influenced by temperature and processing time. High temperatures accelerate the hydrolysis process by denaturing proteins. Prolonged exposure to the enzyme can lead to excessive hydrolysis, causing the gelatin to lose its ability to gel properly.

Therefore, this study aims to determine the effect of soaking time and temperature on the characteristics of milkfish bone gelatin.

MATERIALS AND METHODS

Tools and Materials

The tools used in this research were knives, basins, pots, ovens (B-ONE), strainers, baking pans, glassware (Phyrex), thermometers, gas stoves (rinnai), waterbaths (Memmert), desiccants, cruisers, analytical balances (Excellent H7K), acidity meters, kilns (Suhaterm), aluminum cups, viscometers (Anton Paar type DC 300), stopwatches, centrifuges, funnels, incubator oven (B-One), hot plate (IKA, CMAG HS-7), Genesis 150 type Uv-Vis spectrophotometer (Thermo Scientific), chromameter hunterlab (MahaChem) and mortar.

The materials used in this study consisted of milkfish bones obtained from the by-products of milkfish satay production in Serang City, Banten, water, distilled water, bromelain enzymes (Guangxi), label paper, calico, aluminum foil, white paper, tissues. Polypropylene plastic.

Research Design

This study was conducted using a group random design split plot with 2 factors. The first factor was the soaking time which consists of 3 levels, and the second factor is the soaking temperature which consists of 3 levels, so that in this study 3 x 3 combinations or 9 treatments were obtained which was repeated 2 times. If the result were significantly different then further testing will be carried out with the *Duncan Multiple Range Test (*DMRT).

Research Procedures

The research begins with milkfish bone preparation. The fish bones are cleaned with running water, then soaked in hot water at a temperature of 80^{o}C for 30 minutes with a ratio of 1:1.5 (w/v). The next process was, filtering, washing and size reduction milkfish bone.

A total of 375 grams of milkfish bones were soaked in an enzyme solution with a concentration of 100 U/g substrate in a ratio of 1:2 (w/v) with Soaking time treatment P1 (12 hours), P2 (16 hours), P3 (20 hours) and Soaking temperatures T1 (25^{o}C), T2 (35^{o}C), T3 (45^{o}). After that, filtering and washing are carried out. The pre-treatment process produces soft bones or ossein.

The Ossein is soaked with distilled water in a 1:2 ratio (w/v) at 70^{o}C for 4 hours. Then filtration and separation of filtrate is carried out using a centrifuge. After that, the gelatin filtrate is dried at a temperature of 60^{o}C for 24 hours. Next, the dried gelatin is mashed using a mortar and pestle.

Observation Parameters

The physical characteristics analyze was yield (AOAC 2005), moisture content (AOAC, 2005), ash content (AOAC, 2005), pH (GMIA, 2013), viscosity (GMIA, 2012), color testing using a color reader device (L* value, b*value, a* value, chroma value, ^{0}Hue value) and clarity (Shyni *et al*., 2014). The data from the study was analyzed using Analysis of Variance (ANOVA) fingerprint analysis. If there is a real difference in treatment between treatments, it will be continued with the DUNCAN multiple range test.

RESULTS AND DISCUSSION

Effect of Soaking time and temperature to the gelatin quality

The results of the both treatment of the soaking time (P) and the soaking temperature (T) and its interaction on the gelatin characteristics of milkfish bones using the hydrolysis method with bromelain enzyme are presented in the following Table 1. Table 1 showed that the soaking time (P) had a significant effect ($P<0.01$) on the yield, L* value, a* value, b* value, ^{0}hue value and chroma value and had a significant effect ($P<0.05$) on acidity, viscosity and clarity. The soaking temperature (T) had a very significant effect ($P<0.01$) on the yield, pH, viscosity, L* value, a* value, b* value, ^{0}hue value and chroma value, had a significant effect ($P>0.05$) on clarity. There was an interaction between the soaking time (P) and the soaking temperature (T) on yield, viscosity, L* value, a* value, b* value, chroma value and clarity and there was no interaction on acidity.

The coefficient of variation (CV) in this study ranged from 0.88% to 10.82%, indicating that the CV values fall into the moderate category, meaning that the accuracy of the experiment was quite good. (Delgado *et al*., 2019) stated that the larger the coefficient of variation inline with the greater the variability of a characteristic due to environmental errors. A smaller coefficient of variation indicates higher reliability and precision and is leading to better conclusions.

Gelatin yield

The efficiency and effectiveness of the method can be seen from the yield value according to (Haryati *et al*., 2019). The yield results of the research are listed in Table 2. The gelatin product's yield ranged from 1.02% -1.69%. The yield of the P2 sample (1.51%) had a significant difference with the P3 sample (1.36%) and P1 (1.08%) sample. The optimum gelatin yield value was at 16 hours of soaking (P2). Soaking for more than 16 hours did not show an increase in the yield value. (Miskiyah *et al*., 2022) in her research revealed that the increase in yield with longer soaking time occurred because the longer the soaking time, the more soaking solution penetrated into the chicken feet, leading to more collagen being broken down into gelatin. Furthermore, the low decrease in yield at 20 hours of soaking is suspected to be due to collagen bonds breaking and dissolving in the solution that was lost during the neutralization process. This is supported by research from (Arima dan Fithriyah, 2015), who conducted a study with soaking times of 12, 24, 36, 48, and 60 hours. A decrease in yield occurred as soaking time increased from 36 to 48 hours. This was caused by the longer soaking time, during which collagen with broken hydrogen bonds dissolved in the acidic solution, as evidenced by the loss of very soft ossein during the neutralization process.

Table 2 Showed that the yield of T_2 (1.40%) samples had a significant effect on T_3 (1,29%) and T_1 (1.25%) samples. The yield value past the temperature of 35^{o}C tends to decrease. This is suspected because at a temperature of 35^{o}C the removal of calcium and mineral salts to soften the bones contained in collagen has occurred perfectly so that the breakdown of collagen into gelatin produces a high yield. Moreover, the decrease in yield in T3 is likely due to the high temperature used. (Mulia Jaya dan Rochyani, 2020) It was revealed that the low yield of gelatin was likely also caused by the high temperature used in the extraction process of ossein into gelatin.

The yield analysis of gelatin from milkfish bone waste obtained in this study was lower compared to the research by Yuliana (2021), which found that the yield of gelatin from snakehead fish bones using bromelain enzyme as the soaking solution ranged from 1.34% to 3.56%, and (Haryati *et al*., 2019b), who conducted research on gelatin from siganid fish skin using bromelain enzyme, with a yield reaching 6%. The difference in yield values is suspected to be due to the differences in raw materials and enzyme concentrations used. Utomo *et al*., (2023) stated that, in addition to soaking time, enzyme concentration also affects the yield produced. The higher the enzyme concentration added, the more peptide bonds are hydrolyzed.

Moisture Content

Moisture content refers to the amount of water in a material which can be expressed based on dry and wet weight basis. It is a critical parameter that must be considered as it directly impacts the shelf life. Excessive moisture content can affect the texture, flavor, and microbial growth in gelatin (Utomo *et al.,* 2023). The moisture content results are presented in Table 3.

The moisture content in Table 3 ranged from 6.59-7.47%. The moisture content value increased with the length of soaking time P1 (6.70%), P2 (6.83%) and P3 (7.00%). This is thought to be because the longer the soaking time will cause the water molecules gelatin molecules to create a bond to.

the water to bind more to the gelatin, making it difficult for the water to evaporate during the drying process. (Ridhay *et al*., 2018a) stated that the longer the soaking time, the more water bound in the gelatin solution or the attraction of water to gelatin compounds is very strong, the strength of the attraction of water is what makes it difficult for water to come out when evaporated.

Table 3 shows that the moisture content decreased along with the increase in soaking temperature T1 (6.98%), T2 (6.88%), T3 (6.68%). Decreasing in water content value was hypothesized to occur due to the movement of water in the gelatin structure in the gelatin protein tissue structure of milkfish bones. (Suryati dan Kunci, 2015) revealed that the moisture content decreased with the increase in hydrolysis temperature caused by the movement of water in the gelatin structure

Ash Content

Ash content is an inorganic residue from the combustion process or oxidation of organic components of a foodstuff. The more minerals in a food, the higher the ash content produced (Syahputra *et al.*, 2022).

The ash content produced ranged from 2.91-3.63%. The ash content value in Table 4 P1 (3.30%) is significantly different from the soaking time of P2 (3.14%) and P3 (3.02%). The gelatin ash content of milkfish bones decreased with the longer the soaking time. This is suspected because the longer the soaking time, the more minerals will be released from the collagen, therefore reducing the ash level. This study is in accordance with (Ridhay *et al.*, 2018) which revealed that the gelatin ash content of tilapia fish bones produced is getting smaller with increasing soaking time. Because each time difference will cause the mineral salts to be released so that it affects the ash content produced.

The test results in Table 4 show that the ash content of T1 (3.33%) is significantly different from T2 (3.18%) and T3 (2.96%). The ash content of this study shows a decrease with the higher the immersion temperature, this is suspected when the temperature increases the amount of minerals also decreases which makes the ash content produced low. This research is supported by (Siregar *et al.*, 2015) in their research stating that the higher the temperature, the lower the ash content, this is because the higher the temperature, the less minerals contained in ossein so that the lower the ash content.

Potential hydrogen (pH)

Potential hydrogen is one of the important properties of gelatin. The acidity value is a parameter applied in determining the quality standards of gelatin. Factors that have a great influence on the pH value of gelatin obtained are the type of pre-treatment solution used and the washing process carried out after the soaking process (Iqbal *et al.*, 2015). The pH results obtained in the research are listed in Table 5.

The acidity ranged from 6.77-7.15. P3 (7,12) was significantly different from P2 (6.94) and from P1 (6.82). The pH of gelatin increases with the length of soaking time. It was suspected that the longer the soaking time will make the enzymes trapped in the bones and finally extracted during the heating process so that the pH value becomes high. The pH of gelatin becomes high because during the soaking process, the soaking solution was trapped in the raw materials and finally extracted during the heating process (Sutyasmi, 2015).

T_3 (6,98) was significantly different from T1 (6.87) and T2 (6.79). The pH value has increased along with the higher temperatures. The difference in pH value produced is thought to be influenced by the washing process after the Soaking process of the enzyme solution. (Gunawan dan Suptijah, 2017) It is stated that the pH value of gelatin approaching neutral (pH 7) reflects the efficiency of the washing process after soaking in an acid solution, although the pH results in this study do not meet the quality standards for gelatin.

Viscosity

One of the parameters that is the quality standard for gelatin is viscosity, which indicates the viscosity level of gelatin as a solution at a certain concentration and temperature (Putri *et al*., 2023). Viscosity is affected by several factors such as temperature, pH and gelatin concentration. At high temperatures of more than 40°C, it will cause the viscosity value to decrease (Febriana *et al*., 2021). The results of the viscosity measurements are presented in Table 6.

The results of the viscosity value measurement in Table 4 showed that the viscosity value ranges from 135-265 cP. The difference in soaking time shows that the longer the soaking time of the resulting viscosity decreases. The soaking time of P1 (228.33 cP) was significantly different from P2 (203.33 cP) and P3 (163,33 cP). The decrease in viscosity value along with the increase in soaking time is suspected that the longer the soaking of the bromelain enzyme solution will break the peptide bonds of amino acids into short molecular chains, so that the viscosity value becomes low. (Febriana *et al*., 2021) stated that the longer the soaking time causes the low viscosity value obtained, this is due to the break of the amino acid chain from the gelatin and causes the chain to become shorter.

Table 6 shows that T1 (252,5 cP) is significantly different from T2 (225 cP) and T3 (170 cP). The difference in soaking temperature shows that the higher the temperature, the lower the viscosity produced. This decrease in viscosity value is suspected to be that the gelatin of this milkfish bone is easily degraded at high temperature, so that the molecular chain becomes shorter which causes the viscosity value to decrease. This research is supported by (Capriyanda *et al*., 2020.) who stated that the higher the temperature, the lower the viscosity of gelatin. This is because the structure of gelatin is easily degraded at high temperatures, resulting in shorter chains.

The viscosity analysis results from this study did not meet the gelatin quality standard, which is a maximum of 6 cP (GMIA, 2012). This is suspected to be related to the relatively high moisture content of the dry gelatin, which affects the viscosity value. (Pantow *et al*., 2016) stated that the viscosity or thickness of the gelatin solution is closely related to the moisture content of the dry gelatin. The lower the moisture content of the dry gelatin, the higher its ability to bind water (to form a gel). The more water bound by the gelatin, the thicker the gel will be, which directly affects the higher viscosity value measured.

Gelatin Color

Color is one of the important parameters in the specifics of gelatin, where in general the color of gelatin is expected to be white, because high-quality gelatin is usually colorless. Whiter the gelatin better the application. The brightness of gelatin is determined by the raw materials and the gelatin manufacturing process (Gunawan dan Suptijah, 2017). The color of the gelatin obtained can be seen in Fig. 1.

L* Value

The L value indicates lightness with a value of 0 (black)-100 (white). The L* value ware shown in Table 7. The L* The value shown in table 7 ranges from 49.06-60.69 which shows that the gelatin color obtained was getting brighter. Dewantoro *et al* (2019) revealed that L* values (brightness) with low numbers (0-0) indicate color darkness, while L* values with high numbers (51 to 100) indicate color brightness. The soaking time of 12 hours to 16 hours decreased in brightness from 55.94 to 53.13, indicating that the color was getting darker.

In addition, Table 7 also showed that the

L* value has decreased with the increasing temperature. T1 (56.85) is significantly different from T2 (54.71) and T3 (52.04). This value shows that the color of the gelatin gets darker with the higher the Soaking temperature.

Redness (a* Value)

Redness (a*) expresses the value of red on an object. The red-green color interval is calculated by the value a* with the notation a*:+ (red); - (Green). The notation a* produces a red chromatic color for a positive value from 0 to 80 and a green chromatic color for a negative value from 0 to (-80) (Juliasti *et al.*, 2015). The results of the redness analysis (a*) are shown in Table 8.

The redness value (a*) ranged from 0.08-9.75. Based on Table 8, it shows that the value of P2 (5.06) is significantly different P1 (2.71). The soaking time from 12 hours to 16 hours increased, the redness value (a*) decreased.

Table 8 also Showed that the redness value (a*) decreases along with the increase in temperature P1 (7.31), P2 (3.00) and P3 (1.34) which can be interpreted as the reddish color of gelatin decreases with increasing temperature.

(b* Value)

Yellowness (b*) expresses the value of the indication of blue to yellow on an object, with the value of b* with the notation b*; + (Yellow); - (blue). The notation b* states the chromatic color yellow for positive values from 0 to 70 and the blue color for negative values from (-70) to 0 (Juliasti *et al.*, 2015). The results of the yellowness test (b*) can be seen in Table 9.

Table 9 Showed that the yellowness value (b*) ranges from 25.09-38.73. The value of P2 (31.63) is significantly different from P1 (28.27). The b* the value obtained shows that the yellowish color time from 12 hours to 16 hours is increasing. This increase in yellowish color is suspected to come from the gelatin of milkfish bones.

The results of Table 9 also showed that the b* value has decreased along with the increase in temperature, T1 (35.81), P2 (28.71) and P3 (25.33). This means that the yellowish color decreases with the increasing temperature. The resulting color is related to mineral residues from a less than perfect washing or neutralization process that can increase the polysaccharide components in the gelatin

^{o}Hue Value

^{o}Hue is the result of a value that indicates a specific color. Sari *et al.* (2020) revealed that the decrease in value is influenced by the drying time which can turn the pigments oxidized into brown color. The results of the chroma test are shown in Table 10.

The ^{o}hue value in table 10 ranges from 21,01-29,36. P2 (25,30) is significantly different from P1 (24,81). The Soaking time of 12 hours to 16 hours increases. This is suspected to be due to the influence of the drying time which changes the color to browner.

The Soaking temperature in table 10 shows a significant effect ($P<0.05$) on the ^{o}Hue gelatin value of the milkfish bones produced. The ^{o}Hue value decreases as the temperature increases. T1 (28,05), T2 (24,56) and T3 (22,54).

Chroma Value

The value of C (chroma) indicates the intensity of the color or the clarity of the color. A low chroma value indicates a weak or faded color, whereas a high chroma value indicates a sharp color. Chroma is obtained from the coordinates of the values a* and b*. The higher the chroma value, the stronger the color intensity produced (Susanti Thamrin *et al.*, 2022). The results of chroma value calculation are presented in Table 11.

Results of the chroma analysis presented in Table 11 shows that the chroma value ranged from 25,11-39,94. The soaking time has a significant effect (P<0.05) on the gelatin chroma value of the milkfish bones produced. The value of P2 (32.14) is significantly different from P3 (29,59) and P1 (28.43). The soaking time from 12 to 16 hours showed an increase. However, when the soaking time was extended to 20 hours, the chroma value decreased. This means that a soaking time of 16 hours produced a more intense color compared to soaking times of 12 and 20 hours. This is likely due to the higher b* value, which results in a deeper color. (Susanti Thamrin *et al*., 2022) stated that the higher the b* value, the more intense the color (chroma) will be.

The soaking temperature in Table 11 shows a significant effect (P<0.05) on the chroma value of gelatin of milkfish bones produced. The value of chroma decreases as the temperature increases. T1 (36.59), T2 (28.89) and T3 (25.37).

Clarity

Clarity is the part of light that is transmitted through a solution. The clarity of a gelatin solution is one of the desired properties. High clarity indicates that the solution has no particles that are insoluble with water. The higher the clarity level value, the lower the clarity level (Windyasmara *et al*., 2019). The results of the clarity analysis are presented in Table 12.

The results of the clarity analysis in Table 12 showed that the clarity value ranged from 0.15%-0.42%T. The soaking time had a significant effect (P<0.05) on the clarity (%T). The soaking time of P3 (0.45) was significantly different from P2 (0.32) and P1(0,18). This indicates that the difference in soaking time affects the level of gelatin clarity, which is shown by the longer time of soaking will make clarity value higher, which means that the longer the soaking time causes a decrease of the clarity level.

The soaking temperature in Table 12 shows a significant effect (P<0.05) on clarity. The value of T1 (0.36) is significantly different from T2 (0.31) and T3 (0.28). The temperature difference shows that the higher the temperature will decrease on the clarity level, meaning that the higher the temperature causes increasing on the clarity level of gelatin. This is likely because an increase in soaking temperature produces aggregates with high molecular weight, which affects the clarity level. Mostafa *et al* (2015) stated that a cloudy gelatin solution may result from high temperatures that produce high molecular weight aggregates, impacting the clarity of the gelatin. Additionally, according to Nada (2017), a high clarity value can be achieved when there are no insoluble particles in a solution that scatter light, thereby causing cloudiness.

CONCLUSION

Based on the results, it can be concluded that both soaking time and temperature significantly affected the yield, ash content, pH, viscosity, color characteristics (L*, a*, b*, chroma) and clarity of gelatin. There were interactions between soaking time and temperature that affected the yield, viscosity, ash content, color characteristics (L*, a*, b*, ^{0}hue, chroma), and clarity.

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Table 1. Recapitulation of the results analysis of variance the effect of soaking time and temperature on the gelatin characteristics of milkfish bones using the hydrolysis method with bromelain enzyme.

No	Observation Parameters	Treatment			CV (%)
		P	T	P*T	
1	Yield	**	**	**	0,03
2	Moisture content	tn	tn	tn	2,20
3	Ash content	*	**	**	0,38
4	Ph	*	**	tn	0,10
5	Viscosity	*	**	**	6,03
6	Color				
	L* value	**	**	**	0,28
	a* value	**	**	**	0,10
	b* value	**	**	**	0,01
	^{0}Hue value	tn	**	**	0,45
	Chroma value	**	**	**	0,31
7	Clarity	*	*	**	0,80

Description:
P = Soaking time
T = Soaking temperature
P*T = Interaction between Soaking time and Soaking temperature
KK = Diversity coefficient
* = significant effect on the 5%
** = significant effect on the 1%
tn = No significant effect

Table 2. Yield response to soaking time and soaking temperature

Soaking time (P)	Soaking temperature (T)			Average (%)
	25° (T_1)	35° (T_2)	45° (T_3)	
12 hours (P_1)	1,02 ± 0,04^g	1,08 ± 0,00^f	1,14 ± 0,01^e	1,08^C
16 hours (P_2)	1,37 ± 0,03^c	1,69 ± 0,01^a	1,46 ± 0,00^b	1,51^A
20 hours (P_3)	1,37 ± 0,03^c	1,45 ± 0,01^b	1,26 ± 0,01^d	1,36^B
Average	1,25^R	1,40^P	1,29^Q	1,31

Description: Numbers followed by the same letter in a column or row showed a significant difference based on the 5% DMRT test.

Table 3. Moisture content response to soaking time and soaking temperature

Soaking time (P)	Soaking temperature (T)			Average (%)
	25° (T_1)	35° (T_2)	45° (T_3)	
12 hours (P_1)	6,39 ± 0,04	7,03 ± 0,02	6,69 ± 0,35	6,70
16 hours (P_2)	7,08 ± 0,13	6,82 ± 0,10	6,59 ± 0,92	6,83
20 hours (P_3)	7,47 ± 0,04	6,78 ± 0,02	6,76 ± 0,12	7,00
Average	6,98	6,88	6,68	6,84

Description: Numbers followed by the same letter in a column or row showed a significant difference based on the 5% DMRT test.

Table 4. Ash content response to soaking time and soaking temperature

Soaking time (P)	Soaking temperature (T) 25° (T_1)	35° (T_2)	45° (T_3)	Average (%)
12 hours (P_1)	3,63 ± 0,09[a]	3,26 ± 0,02[b]	3,00 ± 0,07[de]	3,30[A]
16 hours (P_2)	3,29 ± 0,05[b]	3,18 ± 0,02[bc]	2,96 ± 0,05[de]	3,1[B]
20 hours (P_3)	3,07 ± 0,07[cd]	3,08 ± 0,02[cd]	2,91 ± 0,16[e]	3,02[C]
Average	3,33[P]	3,18[Q]	2,96[R]	3,12

Description: Numbers followed by the same letter in a column or row showed a significant difference based on the 5% DMRT test.

Table 5. Potential hydrogen (pH) response to soaking time and soaking temperature

Soaking time (P)	Soaking temperature (T) 25° (T_1)	35° (T_2)	45° (T_3)	Average
12 hours (P_1)	6,85 ± 0,02	6,77 ± 0,15	6,84 ± 0,04	6,82[C]
16 hours (P_2)	6,89 ± 0,1	6,82 ± 0,01	7,12 ± 0,01	6,94[B]
20 hours (P_3)	7,12 ± 0,02	7,09 ± 0,08	7,15 ± 0,02	7,12[A]
Average	6,95[PQ]	6,89[Q]	7,04[P]	6,96

Description: Numbers followed by the same letter in a column or row show a significant difference based on the 5% DMRT test.

Table 6. Viscosity response to soaking time and soaking temperature

Soaking time (P)	Soaking temperature (T) 25° (T_1)	35° (T_2)	45°(T_3)	Average (cP)
12 hours (P_1)	265 ± 14,14[a]	245 ± 21,21[b]	175 ± 21,21[d]	228,33[A]
16 hours (P_2)	240 ± 14,14[b]	205 ± 28,28[c]	165 ± 21,21[d]	203,33[B]
20 hours (P_3)	205 ± 14,14[c]	150 ± 7,07[e]	135 ± 14,14[f]	163,33[C]
Average	236,67[P]	200[Q]	158,33[R]	193,33

Description: Numbers followed by the same letter in a column or row showed a significant difference based on the 5% DMRT test.

Table 7. L* value response to soaking time and soaking temperature

Soaking time (P)	Soaking temperature (T) 25° (T_1)	35° (T_2)	45°(T_3)	Average
12 hours (P_1)	60,69 ± 0,13[a]	58,08 ± 0,43[b]	49,06 ± 0,65[f]	55,94[B]
16 hours (P_2)	53,01 ± 0,51[d]	51,34 ± 0,70[e]	55,03 ± 0,98[c]	53,13[C]
20 hours (P_3)	57,70 ± 0,39[c]	57,76 ± 1,29[b]	53,64 ± 0,79[d]	56,36[A]
Average	57,13[P]	55,73[Q]	52,58[R]	55,14

Description: Numbers followed by the same letter in a column or row showed a significant difference based on the 5% DMRT test.

Table 8. a* value to soaking time and soaking temperature

Soaking time (P)	Soaking temperature (T) 25° (T_1)	35° (T_2)	45°(T_3)	Average
12 hours (P_1)	4,88 ± 0,07[c]	1,66 ± 0,10[f]	1,60 ± 0,11[f]	2,71[C]
16 hours (P_2)	9,75 ± 0,04[a]	4,35 ± 0,15[d]	1,08 ± 0,10[g]	5,06[A]
20 hours (P_3)	6,73 ± 0,09[b]	2,15 ± 0,01[e]	0,78 ± 0,19[h]	3,22[B]
Average	7,12[P]	2,72[Q]	1,15[R]	3,66

Description: Numbers followed by the same letter in a column or row showed a significant difference based on the 5% DMRT test

Table 9. b* value response to soaking time and soaking temperature

Soaking time (P)	Soaking temperature (T) 25° (T_1)	35° (T_2)	45°(T_3)	Average
12 hours(P_1)	32,89 ± 0,15[c]	26,35 ± 0,01[f]	25,58 ± 0,28[fg]	28,27[C]
16 hours (P_2)	38,73 ± 0,92[a]	31,08± 0,70[d]	25,09 ± 0,26[g]	31,63[A]
20 hours (P_3)	34,78 ± 0,04[b]	27,75 ± 0,11[e]	25,51 ± 0,16[fg]	29,34[B]
Average	35,46[P]	28,39[Q]	25,39[R]	29,75

Description: Numbers followed by the same letter in a column or row showed a significant difference based on the 5% DMRT test.

Table 10. °Hue response to soaking time and soaking temperature

Soaking time (P)	Soaking temperature (T) 25° (T_1)	35° (T_2)	45°(T_3)	Average
12 hours (P_1)	26,75 ± 0,16[e]	23,61 ± 0,13[c]	24,07 ± 0,18[c]	24,81
16 hours (P_2)	29,36 ± 0,19[g]	25,52 ± 0,38[e]	21,01 ± 0,24[b]	25,30
20 hours (P_3)	27,66 ± 0,13[f]	23,81 ± 0,05[d]	23,50 ± 1,41[a]	24,99
Average	27,92[a]	24,31[b]	22,86[c]	25,03

Description: Numbers followed by the same letter in a column or row showed a significant difference based on the 5% DMRT test.

Table 11. Chroma response to soaking time and soaking temperature

Soaking time (P)	Soaking temperature (T) 25° (T_1)	35° (T_2)	45°(T_3)	Average
12 hours (P_1)	33,24 ± 0,14[c]	26,40 ± 0,01[f]	25,63 ± 0,29[fg]	28,43[C]
16 hours (P_2)	39,94 ± 0,91[a]	31,38 ± 0,28[d]	25,11 ± 0,26[g]	32,14[A]
20 hours (P_3)	35,42 ± 0,02[b]	27,83 ± 0,11[e]	25,52 ± 0,17[fg]	29,59[B]
Average	36,20[P]	28,54[Q]	25,42[R]	30,05

Description: Numbers followed by the same letter in a column or row showed a significant difference based on the 5% DMRT test.

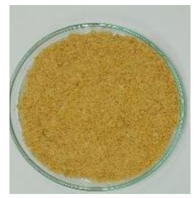
Table 12. Clarity response to soaking time and soaking temperature

Soaking time (P)	**Soaking temperature (T)**			**Average (%T)**
	25^o (T_1)	35^o (T_2)	45^o(T_3)	
12 hours (P_1)	0,20 $\pm$ 0,01^{cd}	0,20 $\pm$ 0,00^{cd}	0,15 $\pm$ 0,02^{d}	0,18^{C}
16 hours (P_2)	0,28 $\pm$ 0,01^{c}	0,25 $\pm$ 0,04^{c}	0,42 $\pm$ 0,12^{b}	0,32^{B}
20 hours (P_3)	0,61 $\pm$ 0,02^{a}	0,47 $\pm$ 0,02^{b}	0,28 $\pm$ 0,02^{c}	0,45^{A}
Average	0,36^{P}	0,31^{Q}	0,28^{Q}	0,32

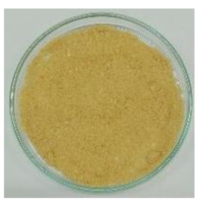
Description: Numbers followed by the same letter in a column or row showed a significant difference based on the 5% DMRT test

P_1T_1

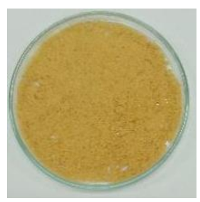
P_2T_1



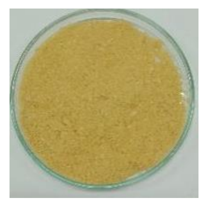
P_3T_1



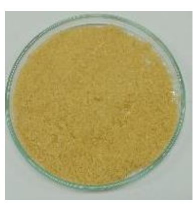
P_1T_2



P_2T_2



P_3T_2



P_1T_3

P_2T_3

P_3T_3

Figure 1. Gelatin of milkfish bones