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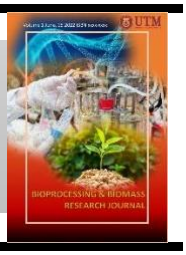

Research Article

Optimization and Characterization of Fish Protein Hydrolysate (FPH) from Milkfish Scales (*Chanos chanos Forsskal*) Using Papain Enzyme

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ABSTRACT

This study determined that optimum fish protein hydrolysed (FPH) production results from milkfish scales using crude extract of papain can be achieved by mixing 0.305 units of papain crude extract with 1 g of milkfish scales previously treated with 0.1 M NaOH (1:10 w/v). The incubation process comprised three stages: incubation at room temperature for 3 h, incubation at 75 °C for 1 h, and continued incubation at 90 °C for 5 min, with a resulting FPH yield of 45.70%. FTIR characterization revealed that FPH derived from milkfish scales includes amide A, amide I, amide II, and amide III groups. Additionally, SDS-PAGE analysis indicated that the FPH molecules measure between 10-35 kDa. The results of the study showed that FPH can inhibit the activity of *E. coli* and *S. aureus*, forming clear zones measuring 2.438 mm and 1.563 mm, respectively. Furthermore, FPH exhibited antioxidant activity against DPPH with an IC50 value of 81.91 ppm.

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INTRODUCTION

Fish protein hydrolysate (FPH) is a product derived from the breakdown of proteins from fish body parts into simple peptides (2-20 amino acids) (Chalamaiah et al., 2012). In 2019, the global FPH market reached $243.7 million and is expected to increase by 5.1% from 2020 to 2027 (Grand View Research, 2019). The high FPH market is partly because FPH can be used as an antibacterial and antioxidant agent, which is safe to consume as it can be metabolized in the body (Aditia et al., 2018). In addition, FPH also has bioactive properties such as antihypertensive, anticancer, immunomodulatory, and anti-inflammatory (Chalamaiah et al., 2018). In the food sector, FPH is used as an additive to increase nutritional value (Idowu et al., 2021), and it was recently discovered that FPH can be used as a cryoprotectant for frozen fish products (Jenkelunas & Li-Chan, 2018). The increasing demand for FPH needs to be balanced with the development of economical and environmentally friendly production methods.

FPH can be acquired through either enzymatic or chemical processes that involve the use of acids or bases (Nurilmala et al., 2018). Enzymatic techniques are preferable over acid or base methods because they can generate FPH without any chemical residues (Yuniarti et al., 2021). Residues of acids and bases from the chemical manufacturing process are harmful to the environment and necessitate extra expenses to neutralize them. Moreover, enzymatically produced FPH exhibits superior nutritional qualities. The use of acids for protein hydrolysis generally leads to the loss of crucial methionine, and cysteine (Das et al., 2021). Hydrolysis using bases can reduce the amino acid content of cystine, lysine, arginine, serine, threonine, and isoleucine and can produce undesired residues, such as lantionine and lysinoalanine. This can ultimately diminish the protein's digestibility (Tavano, 2013).

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Enzymes such as pepsin (Ahmad et al., 2021), bromelain (Amini et al., 2018), zingibain (Habtu et al., 2020), ginger protease (Zheng et al., 2018), actinidin (Ahmad et al., 2019), and papain (Deviarni et al., 2021) can be used for FPH production. Out of these various enzymes the papain enzyme has several unique properties, including its ease of obtainment, stability at high temperatures (60-75 °C) and and hight activity in broader acidic range (4.5-7.0) (Bernadeta et al., 2012). A study by Ngo et al. (2011) demonstrated that protein hydrolysates produced from Pacific cod skin (*Gadus macrocephalus*) using papain had higher antioxidant ability against DPPH compared to hydrolysates produced using alkalase, trypsin, neutrase, pepsin, and chymotrypsin. Hydrolysed protein from chum salmon skin (*Oncorhynchus keta*) using papain showed potential as a preventive agent against hydrogen peroxide-induced oxidative injury in rat hepatocyte cell lines (BRL/buffalo rat liver cells) through increased cell viability and antioxidant enzyme activity (Fu & Zhao, 2015). The study suggests that papain exhibits good protease activity in the process of hydrolysing collagen into protein hydrolysates. Another study by Bahari et al. (2020) reported that the protein hydrolysate produced from the papain enzymatic hydrolysis of sea cucumber (*Actinopyga lecanora*) shows a high amount of hydrophobic amino acids (286.40 mg/g sample) that might be responsible for antioxidant and antityrosinase activities.

The properties and quality of FPH are also subject to the impact of multiple factors such as the concentration of enzyme used and the duration of hydrolysis, aside from the enzyme type (Korkmaz & Tokur, 2021). The production of FPH from the hydrolysis of milkfish scales waste using papain crude extract will undergo optimization in terms of the amount of enzyme used (in units) and the hydrolysis duration (in minutes). The obtained FPH is further evaluated for functional groups and molecular weight using Fourier-transform infrared spectroscopy (FTIR) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Additionally, the FPH is tested for its antibacterial and antioxidant abilities.

MATERIALS AND METHOD

Materials

Milkfish scales utilized in the study were sourced from Bandengan Pond located in Kedungpeluk Village, Candi District, Sidoarjo, East Java, Indonesia. The study also used other materials, such as papaya fruit latex. Chemicals with pro-analysis (p.a) are sodium metabisulfite ($NaHPO_4.2H_2O$), sodium hydroxide (NaOH), trichloroacetic acid, Folin-Ciocalteu, tyrosine, casein, bovine serum albumin, copper (II) sulphate ($CuSO_4.5H_2O$), $Na_3C_6H_5O_7.2H_2O$, sodium carbonate (Na_2CO_3), dimethyl sulfoxide (DMSO) and ethanol were purchased from Sigma Aldrich.

Milkfish Scale Preparation

The milkfish scales were soaked in boiling water in Malang City (between 95.66 °C to 97.14 °C with an altitude of 440 to 667 meters above sea level) and then rinsed with tap water in order to obtain the clean white scales. This process was repeated approximately 10 times. The fish scales were then dried in a Memmert oven at 65-70 °C for about 6-7 h after being cleaned. To prepare the milkfish scales, they were immersed in a 0.1 M NaOH solution (1:10 w/v) and left to soak for six hours. The NaOH solution was changed every two hours. The scales were then rinsed with sterile distilled water again until the wash water reached a neutral pH. After the pretreatment, the scales were dried in an oven at 65-75 °C for about 6-7 h (Liu et al., 2015).

Isolation of Papain Crude Extract

Crude papain extract was obtained from the latex of young papaya fruit through vertical incisions of around ± 1 to 2 mm deep. The latex was collected in a plastic container, and $Na_2S_2O_5$ was added at a ratio of 0.5% (2/b). The mixture was then dried in an oven at 45 °C until it attained a constant weight. The dried papaya latex was dissolved in 0.1 M phosphate buffer pH 7 at a 1:10 (w/v) ratio. It was then filtered with Whatman paper no. 1 and centrifuged using Tomy MX-105 High-Speed Refrigerated Microcentrifuge for 30 min at 4 °C and 10000 rpm. The resulting supernatant was the crude extract of papain (Urgessa et al., 2019).

Measurement of Specific Activity in Papain Crude Extract

The papain crude extract was diluted in a 1:1 ratio using a phosphate buffer pH 7 before measuring its specific activity. The specific activity of the papain crude extract was determined using **Equation 1** (Urgessa et al., 2019).

$$Specific\ Activity\ (unit/mg) = \frac{Enzyme\ Activity\ \left(\frac{unit}{mL}\right)}{Protein\ content\ \left(\frac{mg}{mL}\right)} \quad (1)$$

The protease activity test of the papain crude extract refers to Anson's method which includes sample treatment, negative control, and blank. The sample was treated by adding 0.5 mL of casein into a 0.2 mL test tube containing diluted papain crude extract. The sample was then incubated for 20 min at 40 °C. 1 mL of 10% trichloroacetic acid was added to the sample tube, and the mixture was then incubated for 15 min at room temperature. The mixture was centrifuged at 10000 rpm for 10 min at 4 °C. The supernatant was collected and then homogenized by adding 4 M Na_2CO_3. Following that, 1 mL of Folin-Ciocalteu reagent (1:10) was added to the sample tube, and it was kept in the dark for 30 min at 37 °C for incubation. To prepare the negative control, 1 mL of 10% trichloroacetic acid was added to 0.2 mL of diluted papain crude extract and kept for incubation at room temperature for 15 min. After that, 0.5 mL of casein was added to the negative control tube, which was then incubated at 40 °C for 20 min. Lastly, the mixture was centrifuged at 10000 rpm for 10 min at 4 °C. The supernatant was extracted, and 4 M Na_2CO_3 was added, followed by homogenization. 1 mL of Folin-Ciocalteu reagent (1:10) was added to the negative control tube. It was then incubated for 30 min at 37 °C in a dark room. The blank preparation was similar to the sample treatment, except that distilled water replaced the crude papain extract. After incubation with Folin-Ciocalteu, each treatment's absorbance was measured at the wavelength of 660 nm using a Spectronic 20 50DA Spectrophotometer. A standard curve for tyrosine was created by using tyrosine solutions with concentrations of 0, 10, 30, 50, 70, 90, and 110 parts per million (ppm). The formula in **Equation 2** was used to calculate the protease activity of the crude papain extract.

$$Enzyme\ Activity = \frac{C \times V}{Ve \times t} \quad (2)$$

Where:
C = Tyrosine concentration (µg)
V = Substrate and enzyme volume (mL)
t = Incubation time (min)
Ve = Enzyme volume (mL)

The protein content of the papain crude extract was determined using the Lowry method. Diluted papain crude extract of 0.5 mL was added to 2.5 mL of biuret solution and homogenized. The mixture was left to stand for 10 minutes. 0.25 mL of Folin-Ciocalteu reagent was added and then the mixture was homogenized. It was incubated once more for 20 min in a dark room. The control solution was prepared using the same procedure but replacing the crude papain extract with distilled water. The mixture was subsequently measured for absorbance at a wavelength of 750 nm. The protein content of the papain enzyme was obtained by inputting the absorbance of the samples into the Bovine serum albumin (BSA) standard curve that had been created.

Optimizing Enzyme Quantities in FPH Production

To produce FPH, 1 g of milkfish scales pretreated with NaOH was added to 8 mL of phosphate buffer (pH 7) in an Erlenmeyer flask. Papain crude extract was added to the mixture in varying amounts of 10 µL, 20 µL, 30 µL, 40 µL, and 50 µL, which is equivalent to 0.077, 0.153, 0.229, 0.305, and 0.382 units. The Erlenmeyer flask was covered with aluminium foil and incubated for 3 h at room temperature. The purpose of this stage is to extract collagen, and the room temperature was selected to ensure that the activity of papain is not compromised during the process. Subsequently, the mixture underwent hydrolysis at 75 °C for 15 min to break down collagen into FPH followed by an additional incubation at 90 °C for 5 min to deactivate the crude papain extract. The resulting mixture was then filtered with the filtrate collected. The filtrate was subsequently oven-dried at 70 °C for 24 h. The product obtained from the process described above is FPH (Urgessa et al., 2019).

The FPH yield was calculated by comparing the mass of dried FPH obtained to the mass of milkfish scales used. One can obtain a percentage yield by using **Equation 3** (Shi et al., 2013).

$$Yield(\%) = \frac{dried\ HPI\ mass}{milkfish\ scale\ mass} \times 100\% \quad (3)$$

Incubation Time Optimization in FPH Production

The optimization of incubation time in FPH production is discussed in **Optimizing Enzyme Quantities in the FPH Production** subchapter. However, variations in incubation time for 30, 60, 90, and 120 min were conducted at a temperature of 75 °C.

Identifying FPH Functional Groups Using FTIR

The Advanced Minerals and Materials Laboratory (Central Laboratory) at the State University of Malang performed the analysis of functional groups in FPH. FPH underwent FTIR analysis using FTIR type IR Prestige Shimadzu and the method was followed the protocol book of this tool that already KAN-accredited IK.M.F.1.

FPH Molecular Weight Identification Using SDS-PAGE

The analysis of FPH molecular weights was performed at the Laboratory of Cellular and Molecular Biology, Brawijaya University. The method utilized in the analysis of FPH molecular weights of FPH molecular weights was followed the protocol book on the SDS-PAGE kit from BIO-RAD brand.

Analysis of Antibacterial Activity using Disc Diffusion Method

Test bacteria, including *Staphylococcus aureus* and *Escherichia coli*, were inoculated into sterile nutrient broth media. They were then incubated at 37 °C for 24 h until the optical density (OD) reached 0.6. The bacterial inoculum was evenly spread on nutrient agar media using a cotton swab. To create a positive control, a paper disc containing chloramphenicol was attached at the center of the NA media surface that was previously spread with bacteria. To test the FPH samples, sample treatments were carried out in the same way, but chloramphenicol was substituted with FPH samples at concentrations of 10000 ppm, 1000 ppm, and 100 ppm, which were dissolved in DMSO. Then, the dish was incubated at 37 °C for 48 h. The diameter of the zone of inhibition around the paper disc was measured using vernier calliper (Nurhayati et al., 2020).

Antioxidant Activity Test Using the DPPH Method

Antioxidant activity testing is based on the study by Mustika (2022) with minor modifications. To determine the maximum wavelength, the DPPH 0.1 mM solution's absorbance was measured at a wavelength was measured at a wavelength range of 400-700 nm. Additionally, FPH samples were dissolved in ethanol pro analysis with concentrations ranging from 0 ppm to 100 ppm in increments of 20 ppm. 1 mL of the FPH sample is measured and transferred into each bottle that had been previously wrapped in aluminium foil. Next, 1 mL of 0.1 mM DPPH solution was added to each bottle containing the FPH samples and incubated for 30 min. Subsequently, the absorbance was measured at wavelength 517 nm. The obtained absorbance data was utilized in determining the IC50 value.

RESULTS AND DISCUSSION

The extraction of crude papain from papaya fruit latex is typically done in two stages: latex extraction and drying. The purpose of the drying process is to decrease the water content in papaya latex, thereby inhibiting microbial growth and prolonging the shelf life of papaya latex (Hestyani Arum et al., 2014). In this particular study, fresh papaya latex was dried using the oven method at 45 °C to minimize harm and decrease the protease activity of the crude papain extract. **Figure 1** displays fresh latex collected by tapping and dried latex.

(A) **(B)**

Figure 1 Fresh papaya latex (A) and dried papaya latex (B)

To isolate the papain crude extract from papaya latex, the dried latex was dissolved in phosphate buffer (pH 7). This aimed to separate the papain crude extract from cell organelles (Macalood et al., 2013). Next, the mixture was centrifuged to precipitate the cell organelles. The resulting supernatant, which contained the papain crude extract, was measured for protease activity and protein content. This calculation helped determine the specific activity of the isolated papain crude extract. Upon measuring the protease activity of the papain crude extract and its protein content, a specific activity of 50.957 ± 5.536 U/mg was obtained using **Equation 1**. The protease activity was found to be 10.839 ± 0.764 U/mL, and the protein content was 0.213 ± 8.171 mg/mL. Finally, the crude papain extract was used in the FPH production process from milkfish scales.

To maximize the FPH yield from milkfish scales, it is necessary to optimize the amount of enzyme. Yield is an essential parameter in FPH production. The treatment given during the production process is more efficient when the FPH yield value is higher. The obtained results demonstrated that among the variations in the amount of papain crude extract used, the highest FPH yield of 45.70% ± 0.594% resulted from using the amount of 0.305 units (40 µL). When the amount of enzyme was increased from 0.077 units to 0.305 units, there was an increase in the FPH yield, as shown in **Figure 2**. This occurred because increasing the enzyme amount can hydrolyse more peptide bonds into short peptides or amino acids, thereby increasing the FPH yield. Nevertheless, the addition of papain crude extract caused a decrease in FPH yield of up to 0.382 units (50 µL). This is believed to happen because the increase in enzyme amount without a corresponding increase in substrate amount results in suboptimal FPH formation. Khattak et al. (2006) stated that enzymes catalyse one substrate, so if the available substrate is limited but the enzyme is in excess, enzyme activity will cease when the substrate is exhausted. Thus, no further increase in the product occurs.

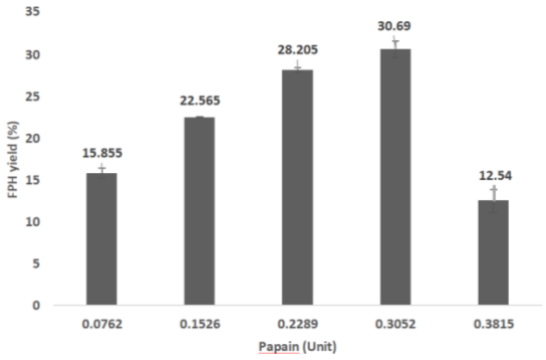

Figure 2 Graphic of the correlation between the concentration of the enzyme and the FPH yield of milkfish scales

Apart from the enzyme quantity, the FPH yield obtained is also influenced by the incubation time in the thermal hydrolysis process. Pitpreecha & Damrongsakkul (2006) found that papain crude extract works optimally at 75 °C and pH 7. Therefore, the incubation time variations for producing FPH using 0.305 units of papain crude extract were carried out under these optimized conditions in this study. An incubation time of 60 min at 75 °C resulted in the highest FPH yield, which was 45.70%. The yield increased during incubation times of 30-60 min, but decreased at 90 and 120 min, as presented in **Figure 3**. The decrease is attributable to the hydrolysis of peptide bonds into short-chain peptides or amino acids, as the incubation time increases. In this research, the papain crude extract optimally hydrolysed collagen to produce FPH after 60 min of incubation. However, over a longer period, the protein hydrolysate's peptide bonds will also be hydrolysed into amino acids with higher solubility, leading to a decrease in the yield of FPH produced (Srikanya et al., 2017).

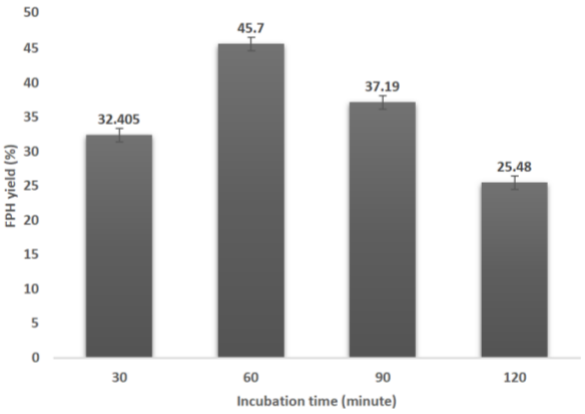

Figure 3 Graphic of relationship between FPH yield and incubation time at 75 °C using 0.305 units of papain crude extract

The FPH product obtained from the optimal production process was analysed by FTIR to ensure that the produced compound was protein. The operating principle of FTIR is recognizing the functional group of a compound from the performed infrared absorbance. Each compound absorbs a different absorbance pattern, allowing them to be distinguished and quantified (Kristoffersen et al., 2019). FTIR analysis spectra of FPH from milkfish scales are presented in **Figure 4**. Moreover, characterization is available in **Table 1**. Typical IR absorption of amide A occurs at wave numbers 3600-2300 cm^{-1}, and amide III at 1300-1200 cm^{-1} (Maryam et al., 2019).

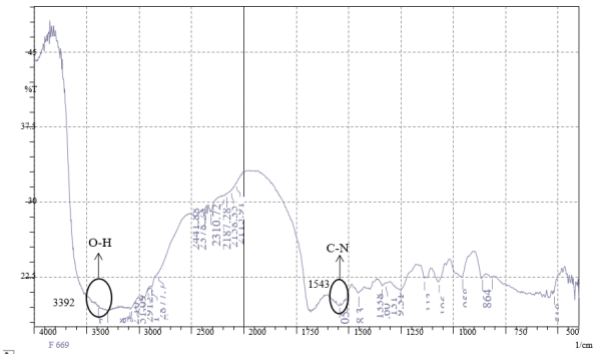

Figure 4 The fourier transform infrared (FTIR) spectrum of FPH milkfish scales

Table 1 Characterization of functional groups in milkfish tissue

Absorption Area	Measured Frequency of FPH from Milkfish Scales (cm^{-1})	Vibration Type
Amida A	3600-2300	It is a wavelength that indicates vibrations from N-H stretching
Amida I	1700-1600	It is the wavelength of the C=O stretching
Amida II	1480-1575	It is a wavelength that shows N-H bending vibrations plus CN stretching vibrations
Amida III	1229-1301	It is a wavelength that shows N-H bending vibrations plus CN stretching vibrations

Milkfish scale-derived FPH exhibits a peak in the amide absorption region A with peaks observed in the 3392.79-3000 cm^{-1} range, as reported in **Table 1**. Absorption at the peak is generated by the N-H stretch of the amide group that is associated with hydrogen bonds and the presence of OH groups. The widened-shaped absorption confirms the presence of the hydroxyproline OH group. The shift of the NH group absorption of a peptide towards lower wave numbers is due to the presence of NH groups involved in hydrogen bonding, with a possibility of overlapping NH bonds with OH groups in the nearby area. The second part of the amide A band corresponds to absorption between 2900-2300 cm^{-1}. **Table 1** shows the presence of a peak at the second part of the amide A band, indicating its presence in the sample. Absorption at these wave numbers indicates the presence of NH groups in amides, which can bind to the CH_2 stretch if the carboxylate group is stable. Therefore, the tested FPH has been shown to contain an OH group, an NH stretch, and a CH_2 stretch.

Next is the unique amide I group, producing absorption between 1661-1636 cm^{-1}. Both samples absorb within the range of 1700-1600 cm^{-1}. The lack of wave numbers indicates that the % T value is too small to be detected in the measurement results. The absorption at these wave numbers shows the double bond stretching of the C=O carbonyl group, NH bond bending, and CN stretching. The results indicate that the FPH of milkfish scales displays a unique amide I group.

The amide II absorption region refers to the absorption between 1560-1335 cm^{-1}. **Table 1** shows that the FPH sample has a peak in the amide II absorption region. The amide II absorption region is attributed to the deformation of NH bonds in proteins. This suggests that FPH milkfish scales exhibit N-H bond deformation. The last region of FPH absorption is amide III. The absorption peak of amide III is at 1300-1200 cm^{-1}. Milkfish scales' FPH has a peak indicating the absorption of amide III, as displayed in **Table 1**.

SPS-PAGE was used to analyze FPH obtained from milkfish scales and determine its molecular weight and purity. SDS-PAGE measurements yield protein bands separated by molecular weight, which is proportionate to the length of the protein chain. On SDS-PAGE, the migration of protein bands is inversely proportional to their molecular weight, resulting in large protein molecules positioned at the top of the electropherogram. **Figure 5** demonstrates SDS-PAGE results of FPH from milkfish scales with polished protein bands ranging from 10-35 kDa in molecular weight. The molecular weight indicates that the protein present has a small size. Dinakarkumar et al. (2022) research suggests that fish protein hydrolysate, produced via papain enzyme extraction from trawl bycatch fish, has a molecular weight of 15 kDa and ranges from 25-75 kDa. Baehaki et al. (2015) show that protein hydrolysates extracted from catfish bones using papain enzymes had molecular weights that varied between 11.90-65.20 kDa. This implies that papain enzyme effectively cleaves complex protein peptide bonds into short peptides and amino acids with low molecular weight.

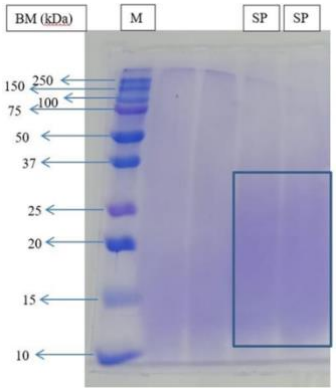

Figure 5 SDS-PAGE results of FPH from milkfish scales

The FPH antibacterial activity test was performed against *Escherichia coli* and *Staphylococcus aureus* bacteria using the disc diffusion method. This method was employed due to its relative simplicity. The antibacterial activity is indicated by the formation of a transparent zone around the FPH-infused paper disc. **Table 2** and **Figure 6** present the results of the antibacterial test.

Table 2 Antibacterial activity test results on *Escherichia coli* and *Staphylococcus aureus* test bacteria

No.	FPH sample concentration (ppm)	Diameter of clear zone (mm) against *E. coli*	Diameter of clear zone (mm) against *S. aureus*
1	Control (+)	28.163±0.477	22.825±0.636
2	10.000	2.438±0.407	1.563±0.477
3	1.000	1.725±0.884	1.350±0.106
4	100	1.300±0.283	1.285±0.028

The results of the antibacterial test indicate that the greatest inhibition zone occurred in the FPH treatment at a 10,000 ppm concentration on both types of bacteria used in the experiment. The diameter of the inhibition zone generated increases as the concentration of FPH increases. Aditia et al. (2018) classified FPH antibacterial activity by the diameter of the inhibition zone formed as follows: 0-3 mm diameter inhibition zone (weak), 3-6 mm (moderate), and >6 mm (strong). Considering this classification, the FPH milkfish scales' antibacterial activity falls into the weak category.

Next, the antioxidant activity test of FPH milkfish scales is conducted against the compound 1,1-diphenyl-2-picrylhydrazyl (DPPH). Antioxidant activity is expressed as

the IC50 value, representing the sample concentration required to inhibit 50% of DPPH activity. A sample with a smaller IC50 value exhibits higher antioxidant activity (Zheng et al., 2018).

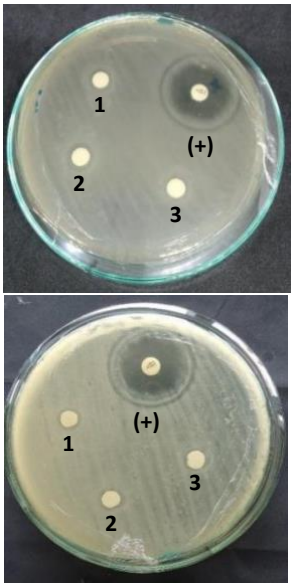

Figure 6 FPH antibacterial activity test results against bacteria (a) *E. coli* and (b) *S. aureus*

The highest percent inhibition (55.46%) was obtained from the 100 ppm concentration of FPH milkfish scales in the sample, as shown in the test results. The graph illustrates that increasing the concentration of FPH milkfish scales in the sample leads to an increase in the percent inhibition. This finding supports the research carried out by Nurjanah et al. (2021), which indicates that the percent inhibition of free radical activity increases with the sample concentration. The test also provided the data on the IC50 value of FPH from milkfish scales, which was determined as 81.91 ppm. Li et al. (2018) classified the antioxidant ability of a compound into the following categories based on the IC50 value: less than 50 ppm as strong, 100-150 ppm as medium, and 150-200 ppm as weak. Therefore, it can be concluded that the FPH from milkfish scales has the potential to have antioxidant properties.

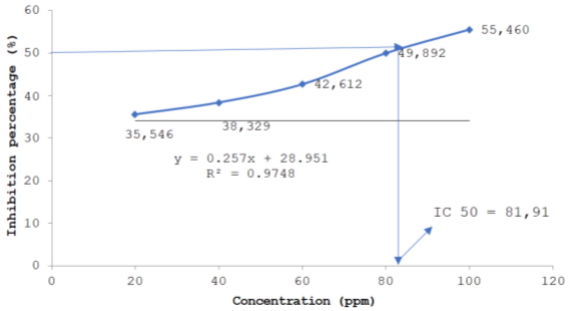

Figure 7 Concentration relationship of FPH sample of milkfish scales with percent inhibition against DPPH

CONCLUSION

This research examines the milkfish scale-derived fish protein hydrolysate (FPH) production utilizing papain enzyme. The optimal conditions yielded a peak FPH output of 45.70 ± 0.60% through 1 g of pre-treated NaOH milkfish scales and 0.305 U papain within an 8 mL phosphate buffer at pH 7. Sequential incubations spanned ambient temperature for 3 h, followed by 75 °C for 1 h, and 90 °C for 5 min. FTIR analysis authenticated typical FPH peptide bonds and the SDS-PAGE analysis resulted FPH with molecular weight within 10-35 kDa. The resultant FPH exhibited notable antibacterial activity against *E. coli* and *S. aureus* and antioxidant activity with an IC50 value of 81.90 ppm.

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