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Physicochemical and functional properties of fish skin collagen hydrolysate extracted using microbial protease

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Abstract. The hydrolyzed form of collagen demonstrates various biological activities that are absent in its native structure. Enzymatic hydrolysis is frequently utilized to generate collagen hydrolysate from different animal tissues. However, the application of microbial proteases in this process is not widely documented. The resulting peptide fractions can vary considerably based on the collagen source and the specific enzymes employed. Extracting crude enzymes from microbial sources, which tend to be more economical than commercial proteases, has shown considerable promise for liberating bioactive peptides from collagen. Furthermore, the functional characteristics of the resulting hydrolysate can be improved by selecting appropriate enzymes and optimizing hydrolysis conditions. Despite this potential, the use of microbial proteases for such applications remains largely underexplored. This study intends to identify local microbial protease sources for the preparation of collagen hydrolysate from barramundi fish skin. The collagen hydrolysis process will be evaluated based on physicochemical parameters, including the degree of hydrolysis, hydroxyproline content, morphological analysis via scanning electron microscopy (SEM), and identification of chemical structure using Fourier Transform Infrared (FTIR) spectroscopy. Additionally, the biological activity, particularly the antioxidative potential of the collagen hydrolysate, will be assessed. The effectiveness of collagen hydrolysis using microbial proteases derived from bacterial and fungal sources will be compared with that of commercial proteases.

1 Introduction

The production of freshwater fish through aquaculture has surged significantly in Indonesia and other regions worldwide. Farmed barramundi is considered a more economical and sustainable option compared to other cultured fish, with a consistent supply primarily throughout Southeast Asia, including Indonesia, as well as other countries.

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Barramundi fish is viewed as a commodity with a promising future. In Indonesia, one processing method for barramundi fish involves producing fish fillet products, which are then freeze and dried. The fillet processing yield for barramundi fish is approximately 40-50%, resulting in a considerable amount of waste [1].

The significant waste produced during barramundi fish processing presents an environmental challenge if not properly addressed. One effective strategy for managing this waste is to extract collagen from the fish skin. These skins, which account for approximately 10-12% of the total waste generated after filleting, are abundant in collagen. Collagen is extensively utilized as an additive across various industries, including food, pharmaceuticals, cosmetics, and photography, and constitutes roughly 30% of the body's total protein content. Traditional collagen production primarily relies on the skins and bones of cattle, pigs, and poultry, which can result in problems such as biological contamination and diseases like bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy, foot-and-mouth disease, and tapeworm infections. These issues have led to concerns among health-conscious consumers [2].

Collagen is an excellent precursor for producing protein hydrolysate and peptides with potential bioactivities [3]. Enzymatic hydrolysis is commonly used to extract biologically active peptides from precursor proteins. Most enzymes reported for collagen hydrolysis are commercial proteases such as alcalase [4–6], trypsin [7], papain [8,9], flavourzyme, and bromelain [10]. Previous research has shown that proteolytic enzymes derived from microbial sources can effectively release peptides with promising bioactivities from collagen [11].

However, microbial-derived proteases have been largely overlooked in research concerning collagen hydrolysis. Depending on the enzyme specificity, substrate characteristics, environmental conditions, and the extent of hydrolysis, it may be feasible to identify cost-effective alternative proteases from microbial sources for this purpose. The resulting collagen hydrolysate will display properties influenced by the amino acid composition and structural characteristics of the synthesized peptides [12]. A related study investigating gelatin hydrolysates from cuttlefish skin, produced using various microbial proteases, demonstrated diverse substrate specificity profiles and varying levels of antioxidant activity. It has been hypothesized that the heterogeneity of peptides, encompassing variations in molecular weight, amino acid composition, and sequence motifs, plays a pivotal role in modulating the bioactive properties and functional efficacy of the hydrolysates [13].

Collagen from fish skin has been hydrolyzed using commercial enzymes, yielding collagen hydrolysates with notable antioxidative properties and cell proliferation activity [4]. The combined use of proteases was found to be more effective, resulting in better recovery and a higher degree of hydrolysis compared to when each enzyme was used alone, demonstrating the enhanced efficiency of this protease combination [9]. This research aims to explore the use of microbial proteases for hydrolyzing collagen from barramundi skin. The hydrolyzed collagen plays a crucial role in determining peptide characteristics, allowing enzymatic hydrolysis to create peptides with specific bioactivities. The hydrolysis process using microbial protease will be monitored, and the resulting hydrolysates will be assessed for their antioxidative capacity. Collagen extraction with weak acid results in low yields; hence, protease enzymes are utilized to break peptide bonds in proteins, aiding the extraction process. Enzymes facilitate this by lowering the activation energy, thereby speeding up the extraction.

Protease enzymes from fungal and bacterial cultures are an alternative to the more expensive and commercially available enzymes for collagen extraction. Research by [14] indicates that the collagen yield from barramundi fish skin using fungal and bacterial enzymes was higher, at 44%, compared to the 43.6% yield from using commercial enzymes.

The enzyme concentration directly influences the rate of the enzymatic reaction; at a specific substrate concentration, the reaction rate increases as the enzyme concentration increases.

Research on microbial proteases for collagen hydrolysis has been limited. However, there is potential to identify more cost-effective alternative proteases from microbial sources, depending on factors such as enzyme specificity, substrate characteristics, environmental conditions, and the degree of hydrolysis. The properties of the resulting collagen hydrolysate will be shaped by the amino acid composition and structural characteristics of the synthesized peptides [12]. A related study investigating gelatin hydrolysates derived from cuttlefish skin, produced with various microbial proteases, showed distinct substrate specificity profiles and varying antioxidant activity levels. This peptide diversity, characterized by differences in chain length and amino acid sequences, is believed to contribute to the variations in functional properties and biological activities observed among the hydrolysates [13].

Collagen extracted from seabass skin has been enzymatically hydrolyzed using alcalase and papain, resulting in collagen hydrolysates that exhibit promising antioxidative capacity and cell proliferation activity [4]. Synergistic effects observed between papain and alcalase led to improved recovery and higher degrees of hydrolysis compared to individual enzyme treatments, highlighting the enhanced efficiency of combined protease action [9]. Based on Baehaki's study [15], the optimal degree of protein hydrolysis is achieved with a 10% enzyme concentration treatment. Comparatively, enzyme concentrations of 5% and 10% exhibit noticeable differences in the degree of protein hydrolysis, suggesting distinct outcomes for each treatment. Increasing the concentration of commercial enzymes generally leads to higher degrees of protein hydrolysis [6]. However, beyond a certain concentration, the hydrolysis degree tends to plateau, showing no significant further increase.

The aim of this research was to determine the ideal concentration of microbial enzymes for the extraction of collagen from barramundi fish skin and to assess the physicochemical and antioxidant characteristics of the collagen produced through enzymatic hydrolysis. This study seeks to tackle the problem of fishery byproducts by offering insights into the yield and properties of collagen soluble in enzymes derived from the fish skin.

2 Materials and Methods

The collagen will be used as starting material to produce collagen hydrolysate. Microbial proteases will be provided by Universitas Ciputra, Surabaya. All the chemicals used were of analytical grade. Papain (EC 3.4.22.2; 30000 USP-U/mg) from *Carica papaya* and microbial proteases will be obtained from liquid culture of *Aspergillus oryzae* and *Bacillus natto*.

2.1 Collagen Preparation and Skin Collagen Extraction

Collagen extraction was performed following the modified protocol of Nagai and Suzuki [16]. Fish skins were first sectioned into uniform fragments using sterile scissors. To eliminate non-collagenous contaminants, the skin samples underwent alkaline pretreatment with 0.1 M NaOH at a sample-to-alkali ratio of 1:8 (w/v). This degumming step was carried out under continuous agitation for 24 hours. The samples were then rinsed thoroughly with distilled water until achieving a neutral pH. Next, the defatted skins were subjected to solvent-based delipidation by immersion in 10% butyl alcohol (1:10, w/v) overnight. After fat removal, the skins were washed repeatedly with distilled water to eliminate residual buthyl alcohol. Collagen extraction was conducted using acetic acid at varying molarities (1.0 M) for 3-5 days. The resulting extract was screened through fine mesh cotton cloth, and protein recovery was achieved via salting-out precipitation using NaCl to a final concentration of 2.5 M, in the presence of 0.05 M acetate buffer (pH 5.0). The precipitated collagen was pelleted by ultracentrifugation at 10,000rpm for 15 minutes. Subsequently, the pellet was solubilized

in 0.5 M acetic acid. Finally, the acid-soluble collagen was lyophilized to obtain a purified collagen powder. Process optimization was carried out by systematically varying extraction conditions, including acetic acid concentration, pH, temperature, and extraction time, to enhance yield, purity, and bioactivity [17].

2.2 Enzymatic hydrolysis of fish skin collagen

Collagen sample prior to enzymatic hydrolysis was prepared based on [18]. The skin collagen will be dispersed in 0.5 M phosphate buffer (pH 7.0) and subjected to microbial proteases hydrolysis for 3 h under optimal temperature and pH conditions with an enzyme/substrate ratio of 1:25 (w/w). The reaction will be inactivated by heating at 100°C for 10 min. After enzyme inactivation, the mixture will be centrifuged at 5000 rpm at 4°C (Beckman Coulter, Inc., USA) for 10 min to remove the debris and the supernatant will be collected and lyophilized for further analysis.

2.3 Proximate Analysis

2.3.1 Moisture Content

The procedure begins by preheating a porcelain crucible in an oven at 100-110°C for one hour. After heating, the crucible is placed in a desiccator for 15 minutes to prevent moisture absorption and then weighed repeatedly until a constant mass is achieved. Next, 2 grams of the sample are precisely measured and transferred into the crucible, which is subsequently dried at 100-110°C for three hours. Once dried, the crucible is allowed to cool in a desiccator for 30 minutes to stabilize, followed by repeated weighing until a consistent weight is attained, following the guidelines of SNI 2354.2:2015 [19].

2.3.2 Protein Content

A digestion flask is filled with two grams of the sample, two catalyst tablets, and a boiling stone. To this mixture, 15 ml of concentrated H_2SO_4 and 3 ml of H_2SO_4 are gradually added, and it is allowed to sit for 10 minutes in a fume hood. The sample is then digested at 410°C until the solution clears. Once digested, it is cooled and diluted with 50-75 ml of distilled water. An Erlenmeyer flask is prepared with 25 ml of a 4% H_3BO_3 solution containing an indicator to capture the distillate. The digestion flask is connected to a steam distillation apparatus, with the addition of 50-75 ml of $Na_2S_2O_3$ solution, and distilled. The distillate is collected in the Erlenmeyer flask until the total volume reaches 150 ml, after which it is titrated with 0.2 N HCl until a neutral gray color is observed (SNI 01-2354.4-2006) [19].

2.3.3 Ash Content

Two grams of the homogenized sample are placed in a porcelain crucible and dried in an oven at 100°C for 24 hours. The crucible is then moved to a muffle furnace, where the temperature is gradually increased to 550°C ± 5°C and maintained until white ash is produced. Once the ashing process is complete, the crucible is cooled in a desiccator for 30 minutes. The ash is then gently moistened with distilled water, dried on a hot plate, and ashed again at 550°C until its weight becomes stable. The temperature for ashing is then lowered to ±40°C, and the crucible is returned to the desiccator for another 30 minutes before being weighed immediately after cooling (SNI 01-2354.4-2006) [19].

2.3.4 Fat Content

Three grams of the sample, along with 20 ml of concentrated HCl, 30 ml of water, and boiling stones, are placed in a 250 ml beaker and boiled for 15 to 20 minutes. After boiling, the sample is filtered to achieve a neutral pH and then dried in an oven at 100°C for 10 minutes. The extraction process continues with a conventional Soxhlet extractor, where the filter paper containing the sample is placed in a fat sleeve, and 50 ml of diethyl ether is added to a round-bottom flask. The fat sleeve is inserted into the extractor, and extraction is performed in approximately 5-minute cycles for a total of 3 hours. The diethyl ether solvent is then evaporated, and the round-bottom flask containing the fat is dried in an oven at 105°C for about 2 hours. After being cooled in a desiccator for 30 minutes, the flask is weighed until a stable weight is reached (SNI 2354-3:2017) [19].

2.4 Spectroscopy Observation using FTIR

The collagen sample will be subjected to attenuated total reflectance Fourier transform infrared spectroscopy (FTIR). Its Spectra will be recorded using a Thermo Fisher FTIR spectrometer. A resolution of 4 cm^{-1} and the mean value of 64 automated scans from 400 to 4000 cm^{-1} will be used to obtain the FTIR spectra. FTIR spectra were obtained from discs containing collagen sample in approximately potassium bromide (KBr). Infrared spectra was obtained in the range between 4000-500 cm−1 using spectrophotometer (Shimadzu Scientific Instruments' IR-Prestige-21, The instrument's software (OMNIC 8, Thermo Scientific) was used for the analysis of acquisitioned spectrum data.

2.5 Scanning electron microscopy (SEM)

Prior to analysis, the specimens were meticulously affixed to sample stubs and treated with gold sputter-coating to improve electrical conductivity. The morphological features of the collagen samples were examined using a SEM. Each specimen was mounted onto a standard SEM holder equipped with glow-discharged carbon adhesive films prepared through a 20-second discharge process. Gold ions were deposited onto the samples using an automated fine coater. The coated specimens were then placed into the SEM's specimen chamber, where surface morphology was analyzed under an accelerating voltage of 20 kV.

2.6 Analysis of Possible Heavy Metals

Heavy metal analyses of the samples were conducted at Angler BioChem Lab, Co. Ltd, located in Surabaya, Indonesia. The levels of cadmium, lead, arsenic, and mercury in the samples were analyzed using an inductively coupled plasma mass spectrometer (ICP-MS), specifically the Perkin Elmer ICP-OES Optima 8000, following the method 5.4/IK/2/2.8.10. The measured values, units, wavelengths, and RL values.

3 Results and Discussion

3.1 Determination of microbial protease activity

Qualitative testing of A. oryzae and B. natto strains showed protease activity, characterized by the formation of clear zones around the colonies in skim milk media. The A. oryzae strain exhibited the largest clear zone in the tested media (Fig. 1, *left*). Additionally, the B. natto strain also displayed clear zones around the colonies grown on the medium containing skim

milk (Fig. 1, *right*). This indicated that both A. oryzae and B. natto strains could hydrolyze the protein in fish skin collagen, associated with protease enzyme activity.

The extracted collagen was hydrolyzed using enzymatic methods with crude microbial protease solution or enzyme precipitated with acetone. The resulting collagen hydrolysate was then utilized for further experiments.

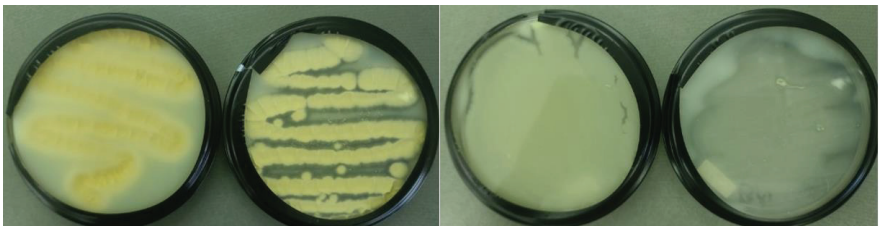

Fig. 1. Qualitative tests of *A. oryzae* (*left*) and *B. natto* (*right*) strains on selective media showed that bacterial and fungal colonies were surrounded by clear zones indication proteolytic activity exhibited by both strains on media containing skim milk.

3.2 Chemical and Enzymatic hydrolysis of collagen

The preparation of the collagen sample prior to enzymatic hydrolysis was conducted following the methodology outlined by [18]. Skin collagen was dispersed in a 0.5 M phosphate buffer solution at pH 7.0 and subjected to hydrolysis using crude proteases for a duration of 3 hours, maintained under optimal temperature and pH conditions, with an enzyme-to-substrate ratio of 1:25 (w/w). To terminate the enzymatic reaction, the mixture were heated to 100°C for 10 minutes. Following enzyme inactivation, the samples were undergo centrifugation at 5000 rpm at 4°C using a Beckman Coulter centrifuge (Palo Alto, CA, USA) for 10 minutes to eliminate any debris. The resulting supernatant was then be collected and subjected to lyophilization for subsequent analysis. This method ensures the efficient extraction of collagen peptides for further biochemical investigations.

Fig. 2. Pre-treatment for separating fat and non-collagen compounds (*left*) and crude collagen (*right*).

3.3 Proximate Analysis

The proximate analysis results revealed that the collagen samples exhibited elevated protein levels prior to treatment, with a total protein content of 85.96%, which increased to 97.9% post-treatment. The study indicated a correlation between the protein content in fish skin collagen and the collagen yield, as protein serves as the primary constituent of collagen. Prolonging the enzymatic extraction time led to higher protein concentrations and increased its yield. [20] Observed that collagen constitutes total protein found in fish skin. As a connective tissue protein, collagen was inherently more difficult to extract than other proteins, necessitating the optimization of the extraction process to improve its solubility.

In other study, the protein content exceeded that of catfish skin collagen (64.74%) [21], yet was marginally lower than that of snakehead fish skin collagen (96.21%) [22] and stingray skin collagen (86.97%). However, the protein levels met the standards established by BSN [19] for crude collagen derived from fish scales, which mandates a minimum of 75%. The collagen extraction procedure comprised multiple steps, including NaOH pre-treatment, acetic acid soaking, and hydro-extraction, all of which contributed to the final protein content. The NaOH treatment facilitates the separation of collagen fiber strands. According to [23], the use of bases primarily targets the telopeptide region during the pretreatment phase, thereby enhancing the solubility of collagen. This optimization of the extraction process is crucial for maximizing the yield and quality of collagen for various applications.

Acid soaking causes the skin to swell as water enters the collagen fibers. This swelling is due to electrostatic interactions between the collagen fiber polar groups and H+ ions from the acid or hydrogen bond formation between non-polar groups and H+ ions [24]. Swelling is crucial as it helps disrupt the collagen fiber structure by breaking non-covalent bonds, facilitating extraction, and improving collagen solubility. [25] observed that collagen protein swells due to interactions with acid solutions, indicated by an increase in fish skin weight after soaking. Subsequent heating in warm water can further damage hydrogen and covalent bonds, as initially affected during the acetic acid soaking [21] as shown in Table1.

The analysis results showed that the moisture content of both crude collagen and its enzymatic hydrolysate was 7.24% and 5.42%, respectively. These figures fall within the limits established by the Indonesian National Standard (SNI), which stipulates that collagen moisture content should not exceed 12% [19]. The low moisture content observed in this study can be attributed to the drying process performed using an oven set at 40ºC. Moisture content is a critical factor in assessing the freshness and shelf life of food ingredients [26]. Generally, collagen with high moisture levels has a shorter shelf life, whereas collagen with lower moisture content tends to maintain its quality for a more extended period [27].

Ash content analysis reflects the mineral content present in fish scale collagen. In this study, the ash content of crude collagen was found to be 0.52%, while that of the enzymatic hydrolysate was 0.27%. Both values comply with the SNI standard's maximum ash content limit of less than 5% [19]. The ash content is influenced by the demineralization process and the concentration of the solvents employed; a lower ash content is indicative of higher quality and purity. Overall, these findings underscore the importance of controlling moisture and ash content to ensure the quality and stability of collagen products for various applications.

Table 1. Proximate analysis of fish scale collagen hydrolysate.

Parameter	Crude Collagen (%)	Collagen Hydrolysate (%)	Standard of BSN 8076:2014
Moisture	7.24	5.42	<12
Ash	0.76	0.27	<5
Protein	85.96	97.91	>75
Fat	1.5	1.34	1%

The result of analysis revealed that the moisture content of both crude collagen and enzymatic hydrolysate of collagen was 7.24% and 5.42%, respectively. These values are within the limits set by the Indonesian National Standard (SNI) for collagen, which should not exceed 12% [19]. The low moisture content observed in this study is attributed to the

drying process conducted using an oven at 40ºC. Moisture content is a crucial factor in determining the freshness and shelf life of a food ingredient [26]. Collagen with high moisture content tends to have a shorter shelf life, while collagen with low moisture content generally has a longer shelf life [27]. Ash content analysis indicates the mineral content in fish scale collagen. In this study, the ash content in crude collagen and enzymatic hydrolysate of collagen was 0.52% and 0.27%, respectively. These values meet the SNI standard's maximum ash content limit of <5% [19]. The ash content is influenced by the demineralization process and the concentration of the solvent used; lower ash content indicates higher quality and purity.

Fat is a primary component found in fish skin, alongside protein and water. The fat content in fish scale collagen is higher, at 1.5% and 1.34%, compared to the standard collagen fat content, which is a maximum of 1%. This increase in fat content is linked to the rise in protein content in collagen derived from enzymatic extraction. This phenomenon is likely due to the dissolution of fat bound to protein (lipoprotein) during the extraction process, causing it to be deposited between collagen proteins. [28] found that the denaturation process, involving acidic compounds and high temperatures, leads to the separation of lipoproteins from tissue and their dissolution into the extractor solution. [29] Also noted that the increase in fat content is directly proportional to the increase in treatment time, as more protein molecules bound to fat (lipoprotein) dissolve between collagen proteins during the process. Overall, the fat content in this study was lower compared to the fat content of catfish skin collagen, which was 2.13% according to [20], but higher than the fat content of shark skin collagen, which was 0.37% as reported by [3]. According to Safandowska and Pietrucha [30], some proteins degrade or denature at temperatures above 40°C, but cannot exceed the melting point of stearic fatty acid (70°C), resulting in less fat being degraded.

3.4 FTIR Spectroscopy for Structural Analysis of Collagen Hydrolysates

Fourier Transform Infrared (FTIR) spectroscopy was utilized to perform a detailed molecular fingerprinting and functional group analysis of the collagen samples. This technique measures the absorption of infrared radiation across a broad spectral range, typically between 4000–400 cm^{-1}, allowing the identification of vibrational modes associated with covalent bonds. Key absorption bands, such as amide I (~1650 cm^{-1}), amide II (~1550 cm^{-1}), and amide III (~1240 cm^{-1}), were analyzed to confirm the presence of peptide linkages and evaluate the secondary structures, such as α-helices and β-sheets.

FTIR spectral data offered insights into the physicochemical integrity of collagen, enabling the detection of molecular interactions, including hydrogen bonding and cross-linking between polypeptide chains. Additionally, spectral shifts or intensity variations provided information on denaturation, hydrolysis, or chemical modifications resulting from extraction and processing conditions. This non-destructive analytical method is critical for assessing collagen's purity, structural conformation, and potential functional modifications.

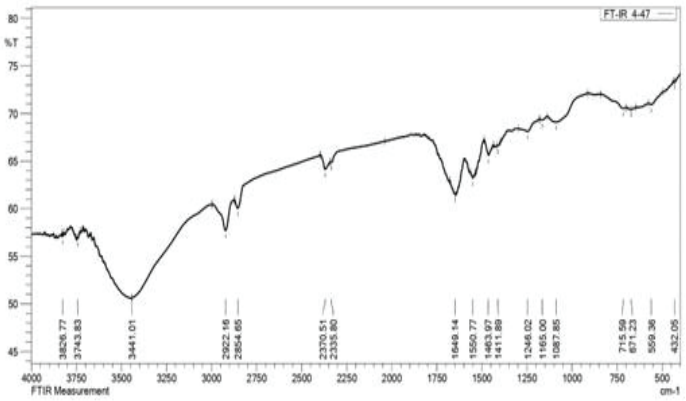

Fig. 3. FTIR spectra collected for fish skin crude collagen (control).

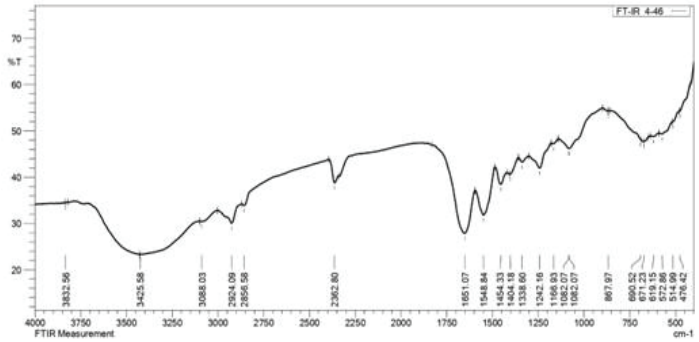

Fig. 4. FTIR spectra collected for fish skin enzymatic collagen hydrolysates.

3.5 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) images of the cross-section of crude collagen (B) and collagen hydrolysates (A) derived from fish skin are presented in Figure 5. The SEM images reveal a dense morphology for both crude collagen and collagen hydrolysate materials. The materials appeared compact with a non-porous structure. The morphological structures of the extracted lyophilized (freeze dried) collagens materials were visualized SEM under different magnifications ×3000 and ×6000 [31].

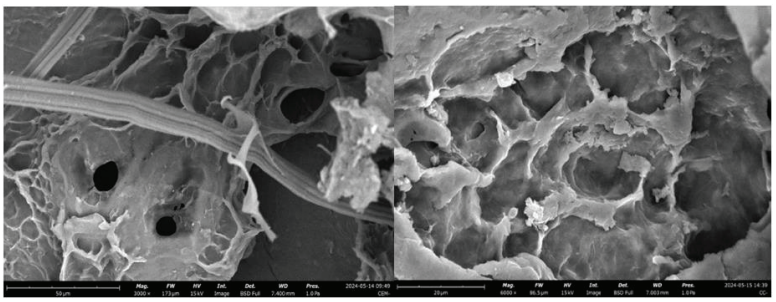

Fig. 5. SEM images of collagen hydrolysates (*left*) x3000 and crude collagen (*right*) ×6000.

3.6 Analysis of possible heavy metal content.

The amounts of heavy metals tested from the samples are shown in Fig. 6. No cadmium, lead or mercury residues were found in the analyzed collagen hydrolysates samples prepared through enzymatic hydrolysis extraction. However there was slight arsenic level residue detected in fish skin collagen hydrolysates was 0.125 mg/kg. However, higher levels of lead and arsenic, measuring 0.373 mg/kg and 0.205 mg/kg respectively, were detected in the crude fish skin collagen. The cadmium level in the crude fish skin collagen sample was undetectable, while the mercury level was found to be quite low at 0.057 mg/kg.

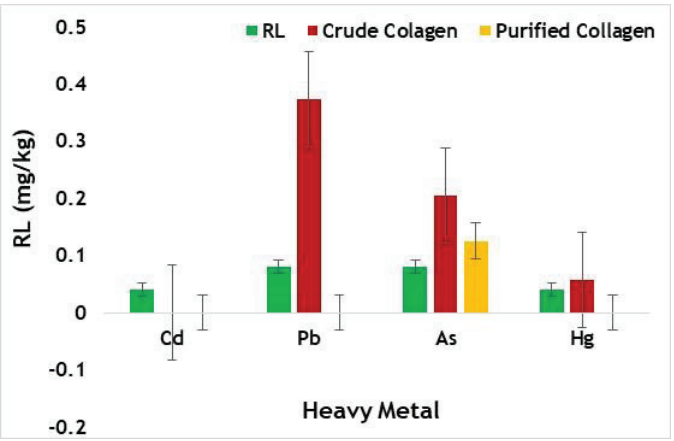

Fig. 6. Analysis of possible heavy metal content.

4 Conclusion

The hydrolyzed form of collagen offers biological activities not found in its native state. Typically, enzymatic hydrolysis is used to produce collagen hydrolysate from animal tissues, but microbial proteases are not widely used in this process. Crude enzymes from microbes, which are more cost-effective than commercial proteases, show potential for producing bioactive peptides from collagen. Using specific enzymes and controlled conditions can enhance the functional properties of the hydrolysate. However, the use of microbial proteases for collagen hydrolysis is underexplored.

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