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Physicochemical Properties, Morphology and Functional Group Analyses of Gelatin Extracted from Croaker Fish (*Pseudotolithus senegalensis*) Scale for Suitability in Biomedical Applications

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ABSTRACT

Challenges are evident during adoption of some biomaterials in tissue engineering (TM), due to their toxicity, immunogenicity, lack of biocompatibility, biodegradability, and bioactivity. Gelatin, a protein-based biopolymer bridges most of those gaps because it possesses good biocompatibility, biodegradability, bioactivity, cell-adhesive structure, and non-toxicity properties that can be adopted for tissue engineering and regenerative medicine (RM). In this study, gelatin was extracted from croaker fish scales using varying hydrochloric acid (HCl) concentrations of 1 M, 2 M, 3 M, 4 M, and 5 M, followed by water bath extraction at 60 °C for 5 hours. The extracts were oven-dried at 60 °C for 12 hours and physicochemical properties of extracts and characterization was carried out via Fourier transform infrared (FTIR) spectroscopy and scanning electron microscopy (SEM). It was observed that the yield, viscosity, pH and gel strength of the gelatin decreased with an increase in acid molarity, with the highest yield, viscosity and gel strength of 56.15%, 3.98 cP, 199.8 g respectively observed at 1 M. FTIR spectra showed O-H, C=O, and N-H bond stretch, while SEM confirmed the presence of spongy porous structure. The properties of the gelatin extracted showed suitability to be adopted for the production of gelatin biomaterial and gelatin-based composites in tissue engineering.

1. INTRODUCTION

Biomaterials are either natural, synthetic or both in origin and they act as a whole or part of a system that augment, replace or repair any worn out tissue or organ of the human body. Biomaterials create the biomolecular and spatial environment that is needed for cell proliferation and vascularization in tissue engineering (TE) and regenerative medicine (RM) as confirmed in the study by Banigo *et al.* (2019).

The human body's reaction to the presence of these biomaterials has led to research concerning their suitability in medical procedures. Natural biomaterials include protein-based biomaterials such as collagen, silk fibroin, gelatin, fibronectin, keratin, fibrin, eggshell membrane) and polysaccharide-based biomaterials such as hyaluronan, cellulose, glucose, alginate, chondroitin, and chitin and its derivative, chitosan. These are promising subsets of biomaterials employed in TE because of their bioactivity, biocompatibility, biodegradability, mechanical kinetics and their intrinsic structural resemblance of native tissue extra cellular matrix (ECM). They promote biological recognition which helps cell adhesion, proliferation, cell differentiation and function, they are usually produced from animal and human sources which include bioactive molecules that mimic the extracellular environment (Banigo *et al.,* 2019).

Synthetic polymers like polypropylene, polyethylene terephthalate (PET)/dacron and nylon which have been used in TE for fixing tendons and ligament, give preferable mechanical steadiness over organic scaffolds, but their non-biodegradation and persistence in the body causes challenges like irritation and synovitis i.e. they induce antigenicity and toxicity to the body cells (Maitz, 2015). Also, their structure and composition are different from that of the original or host tissue hence, their biocompatibility and capability to induce cell regeneration are low. Among the protein-based biomaterials, gelatin has found abundant use owing to recent technological advancements such as additive manufacturing, rapid prototyping, three-dimensional printing and also its cross-linking capability. These have resulted in great strides toward the generation of functional gelatin-based biomaterials and composites for biomedical purposes. Gelatin a natural bio-polymer is produced through partial hydrolysis of collagen from animal parts and by-products such as cartilages, bones, tendons and hides. There are two varieties of gelatin, depending on the processing reagent adopted: namely type A from acid hydrolysis and type B from alkaline hydrolysis (Parasuraman *et al*., 2016; Winarti *et al*., 2021). Recently, the global gelatin production was estimated to be over 300,000 metric tons: 46% was from pigskin, 29.4% from bovine hides, 23.1% from bones, and 1.5% from other parts (Gómez-Guillén *et al.,* 2009).

The characteristics of gelatin extracted from Milkfish (C*hanos chanos*) scales and bones with variation in acid and base concentrations was investigated in Isriany *et al.* (2019). Scales and bones of the milkfish were pre-treated with 0.01 N, 0.1 N and 1 N of sodium hydroxide and acetic acid. Extraction was carried out by soaking ossein in distilled water using different equipment namely water bath at 60 °C for 8 hours, sonicator at 50 °C for 3 hours, microwave at 100 °C for 1 hour, and autoclave at 121 °C for 1 hour. Finally drying was done using dry hot air at 60 °C and freeze dryer at -40 °C. The characteristics of gelatin from milkfish bone: viscosity, pH and yield were 3.389 cP, 4.6, and 1.9% respectively, while those from milkfish scales were 4.6759 cP, 5.4 and 3.7% respectively. Studies on the physico-chemical properties of gelatin from fish waste as an alternative source was conducted in Merina *et al.* (2017) due to its increasing demand and its excellent biocompatibility, biodegradability properties in biomedical application. The gelatin was extracted from the scales of freshwater fish, *Labeo rohita.* After extraction, the proximate analysis and physicochemical analysis of the fish scale gelatin was carried out. This functional polymer was also characterized using different analytical methods, such as UV-vis spectroscopy, scanning electron microscopy (SEM), and X-ray diffraction (XRD) for the evaluation of crystalline and surface morphology, and Fourier transform infrared spectroscopy (FTIR) for structural determination. The scales of *L. rohita* yielded 24% (dry weight basis) of gelatin, indicating this fish species as a potential source of gelatin. The proximate analysis showed low moisture content of 4.2%, ash (1.4%) and high protein content of 90%.

Gelatin quality relies to a great extent upon its rheological properties, aside from essential physio-chemical properties such as composition parameters, solubility, transparency, appearance, smell, and taste. The principal traits that best characterize the general commercial quality of gelatin are gel strength and thermal stability (gelling and liquefying temperatures). Gelatin based hybrid composites for wound dressing were reviewed in terms of their performance in the treatment of infected, exuding, and bleeding wounds in Ndlovu *et al.* (2021). It was concluded that when gelatin is used in combination with other polymers, it resulted in excellent mechanical properties that are required for ideal wound dressings for biomedical applications. Isriany *et al.* (2022) produced fish gelatin composite by mixing milkfish scale gelatin with several types of

starch, namely, maize (MS), cassava (CS), and potato (SS). This improved viscosity by limiting water absorption and also increased the thermal stability of the milkfish scale gelatin composite. Gelatins from bovine and porcine are becoming less acceptable because of increasing allergen cases, tradition and religious beliefs (Alfaro *et al.,* 2014; AL-Kahtani *et al*., 2017). This has called for production of gelatin from other sources, therefore in this study, gelatin was extracted from croaker fish (*Pseudotolithus senegalensis*) scales by adopting the acid pretreatment route, and the acid concentration was varied from 0 M, 1 M, 2 M, 3 M, 4 M, and 5 M in order to investigate its effect on the properties of the extracted gelatin samples.

2. MATERIALS AND METHODS

2.1. Material Collection and Preparation of Samples

The materials adopted were croaker fish scales, Hydrochloric acid (HCl), distilled water, Whatman 200 mm filter papers. Croaker fish scale was sourced from Ijora fish market in Lagos, Nigeria. It was weighed and packaged in zip lock bags, after which it was transported to the laboratory for processing. The scales were rinsed several times in running water to remove attached flesh and dirt. The washed fish scales were degreased by soaking in hot water of about 70 ^{o}C for 30 minutes as shown in Figure 1. The scales are washed again thoroughly to remove superfluous materials and fat before finally being dried at 40 ^{o}C for 2 days as shown in Figure 2. Hydrochloric acid (HCl) of analytical grade was adopted for pre-treatment to facilitate a good yield of Type A gelatin.

2.2. Acid Pretreatment

Acid conditioning was adopted followed by washing out of the acid and extraction in polar solvent (water) as shown in Figures 3 to 5, this technique gave Type A gelatin. The dried fish scales were weighed and ground to about 150-300 microns, 30 grams of ground fish sample was weighed out in six places to be pretreated with varying concentrations of HCl acid: 0 M, 1 M, 2 M, 3 M, 4 M, and 5 M for 2.5 hours where 0 M is the sample where pretreatment was not done. The product of pretreatment was then washed in distilled water until pH was 6.5-7.0. The samples were designated as B sample depending on the acid concentration adopted for pretreatment. The product from the pretreatment of samples of B is called ossein. This process of demineralization is known as maceration and facilitates the release of collagen for the extraction.

2.3. Gelatin Extraction and Yield

The ossein (pretreatment product) was soaked with distilled water at 60 ^{o}C for 5 hours using water bath (DK 420 U-Clear) located at the Metallurgical and Materials Engineering Laboratory in the Faculty of Engineering of the University of Lagos, Nigeria. The mixture was then filtered and the filtrate as shown in Figure 6 was collected for drying. The drying was carried out in an oven at 60 ^{o}C for 12 hours. The flow chart of the pretreatment process followed with extraction and filtration are shown in Figure 7. The dried product was then ground, the yield was calculated as the ratio of weight of dried product to the initial weight of dried fish scales as expressed in Equation (1).

$$\%\,Yield = \frac{weight\ of\ dried\ gelatin}{weight\ of\ dried\ fish\ scales} \times 100 \quad (1)$$

Figure 1: Croaker fish scales from fish market

Figure 2: Washed and dried Croaker Fish scales

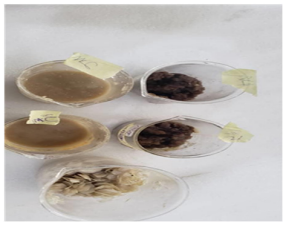


Figure 3: Ground and pretreated samples

Figure 4: Extracted product before oven drying

Figure 5: Water bath extraction at 60 °C

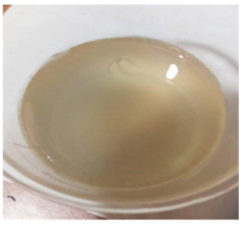


Figure 6: Oven drying of gelatin filtrate at 60 °C

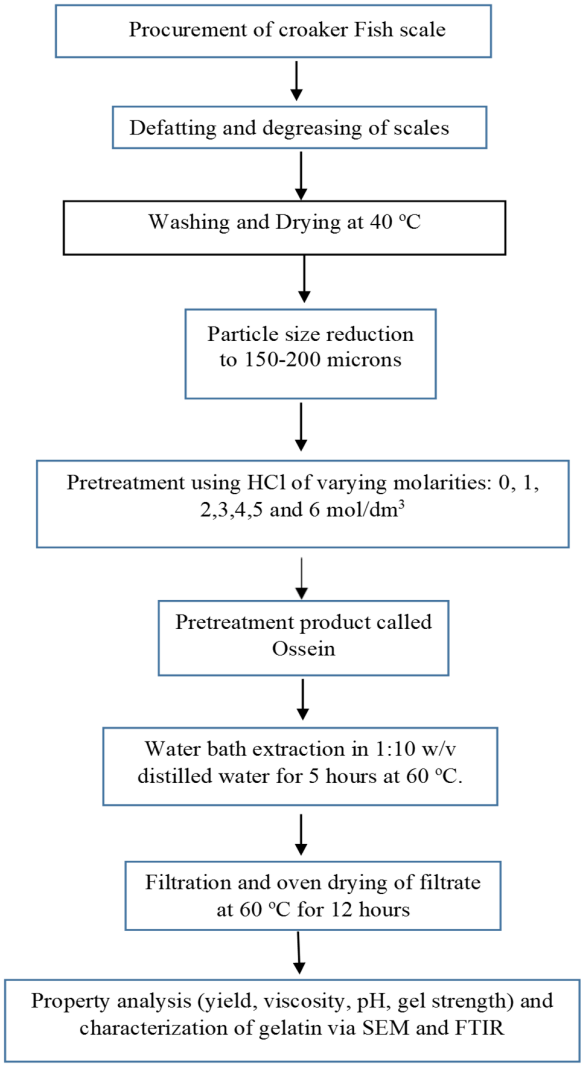


Figure 7: The flow chart for the methodology adopted for gelatin extraction from croaker fish scales

2.4. Physico-chemical Property Evaluation

Gelatin solution was prepared by mixing 0.5 g of the gelatin sample with distilled water at 60 ^{o}C. The viscosity was evaluated at room temperature with the Ostwald viscometer and the efflux time was recorded using a stopwatch. For gel strength/bloom value, 6.67% w/v granulated gelatin samples were prepared by mixing 3.5 g of the extracted gelatin and 50 ml of distilled water and heated at 60 ^{o}C with constant stirring for 30 min. The gelatin solutions were then transferred into small bottle containers of (50 × 80 mm, flat bottom), then kept in a refrigerator at 10 ^{o}C overnight for gel maturation. The gel strength (g) was determined using the Texture Analyzer armed with a load cell, using a 12.5 mm plunger pressed 4 mm into the gelatin gels at a speed of 1 mm/s. The maximum force (in g) at the penetration depth of 4 mm was recorded (See *et al*., 2010; GMIA 2019; Yuliani *et al*., 2019). The color of the gelatin extracted was observed visually and recorded. For the pH evaluations, gelatin of 0.2 g was dissolved at 20 ml of distilled water at 80 °C, then the pH was taken with a pH meter and values was analyzed and tabulated. Other physical properties like smell, texture of gelatin were observed organoleptically.

2.5. Characterization of Extracted Gelatin

2.5.1. Fourier transform infrared spectroscopy (FTIR)

The functional groups present in the ossein (collagen samples prior to extraction) and gelatin samples were analyzed using Nicolet iS10 Fourier Transform Infrared (FTIR) Spectroscope. The samples (2 g) were mixed with 100 mg of potassium bromide (KBr) and placed on the crystal cell of the FTIR spectrometer. The samples were analyzed for different functional groups in the region of 4400 – 350 cm^{-1} at room temperature.

2.5.2. Scanning electron microscopy (SEM)

A scanning electron microscope (JOEL-JSM 7600F), was adopted to determine the morphology of the extracted gelatin samples.

3. RESULTS AND DISCUSSION

3.1. Yield of the Extracted Gelatin

The sample designations and yield (Equation 1) of extracted gelatin from B samples with concentration of HCl acid adopted is shown in Table 1. The highest yield of 56.15% for sample B1 was obtained by adopting 1 M pretreated collagen compared to the yield of other molarities. The lowest yield of 21.75% could be attributed to larger degree of swelling resulting from the high acid concentration and consequently the possibility of collagen loss during washing out of HCl with distilled water after the pretreatment. However, for sample B0 where pretreatment was bypassed prior to water bath extraction it was observed that gelatin was not produced for that sample as the fish sample remained almost the same before and after the treatment, with the weight of 19.55 g compared with 20 g which was used for the water bath treatment. This indicates that the cross-linkages between polypeptide chains in the fish scale raw material need to be broken down during pretreatment, a crucial process that aids production of collagen to yield gelatin during the water bath extraction process (See *et al.,* 2010).

Table 1: Yield of extracted gelatin with concentration of HCl

Sample designation	Concentration of HCl (M)	Mass of fish scale (g) before pretreatment	Mass (g) of extracted gelatin	Yield (%)
B0	0.00	20	19.55	0.00
B1	1.00	20	11.23	56.15
B2	2.00	20	7.09	35.45
B3	3.00	20	6.04	30.20
B4	4.00	20	5.40	27.00
B5	5.00	20	4.35	21.75

3.2. Color of Gelatin Extracts

The variations in the color of the extracts based on the varying acid concentrations are shown in Table 2. The color variations can be attributed to the acid concentrations. In the study by Jamilah and Harvinder**,** (2002), it was concluded that factors such as fish species, raw material and extraction conditions influence the final color of the extracted gelatin.

Table 2: Color of Color of extracted gelatin with concentration of acid.

Sample designation	Molarity (M)	Color before pretreatment	Color after pretreatment
B0	0.00	Gray	Gray
B1	1.00	Gray	Gray
B2	2.00	Gray	White-gray
B3	3.00	Gray	Dark-gray
B4	4.00	Gray	Brown
B5	5.00	Gray	Brown

3.3. pH of Gelatin Extracts

The plot of pH versus acid concentration for the gelatin extracts from the B samples is shown in Figure 8. The values of pH ranged from 4.4 – 5.7, and these values met the requirement as stipulated in the standard recommendation for gelatin used in numerous applications including medical applications by the Gelatin Manufacturers Institute of America (GMIA 2012). The plot shows that as concentration of the pretreatment acid increased, there was a decrease in pH of the samples. Higher molarity of the acids gave more H^+ ions in solution which consequently decreased the pH of the pretreatment product (ossein) and impacted on the pH of the gelatin extracts as seen in Figure 8. This trend is in agreement with the findings in Santiz-Gomez *et al.* (2019) and See *et al.* (2015).

3.4. Gel Strength of Gelatin Extracts

The plot of Gel strength with the concentrations of HCl adopted for the pretreatment is shown in Figure 9. The gel strength ranged from 199.8 g for 1 M concentration to 88.3 g for 5 M concentration of HCl, this shows a downward trend in gel strength with increased acid concentration. Increased acid concentration lead to increase in H^+ ions in solution during pretreatment process causing further hydrolysis, this phenomenon further splits up the bonds in the collagen compound causing reduction in its gel strength of the final sample. This trend is also in agreement with findings observed in Winarti *et al.* (2021). Gel strength is essentially one important property of gelatin as it influences its ability to change from liquid into solid or to change the solution form into a reversible gel. This ability has gained gelatin its wide use in both in the field of food, pharmaceutical and biomedical applications. The gelatin derived from 1 M acid pretreatment was found to be the strongest with a bloom strength value of 199.8 g which is close to the value of gel strength of bovine gelatin observed by Sulaiman *et al.* (2015), this property makes it suitable for bone implants application.

3.5. Viscosity of Gelatin Samples

The plot of the viscosity of gelatin extracts from the B samples with concentration of HCl adopted is as shown in Figure 10. The viscosity of the samples ranged from 1.86 cP to 3.98 cP for B5 and B1 samples respectively. These values met the viscosity requirements as stipulated in the standard recommendation for gelatin used in various applications including medical applications by the Gelatin Manufacturers Institute of America (GMIA 2012). It was observed that higher acid concentrations gave gelatin samples with lower viscosity values, and this can be attributed to increased hydrolysis, thus causing breaking of collagen polypeptide bonds into shorter gelatin amino acids which consequently reduced the viscosity of the extracts.

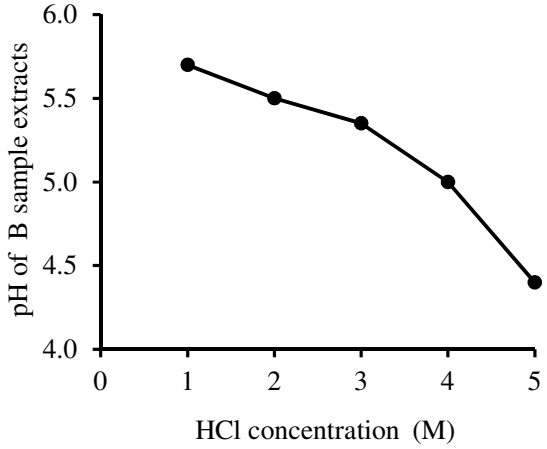


Figure 8: Plot of % pH versus HCl concentrations for B samples

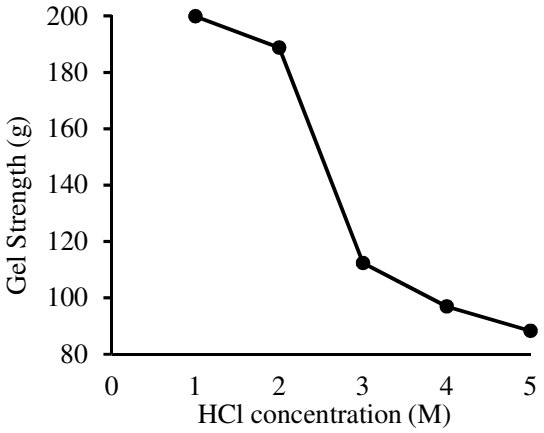


Figure 9: Plot of Gel strength versus HCl concentrations for B samples

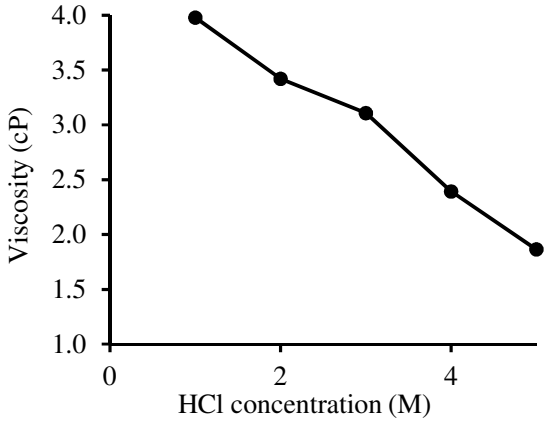
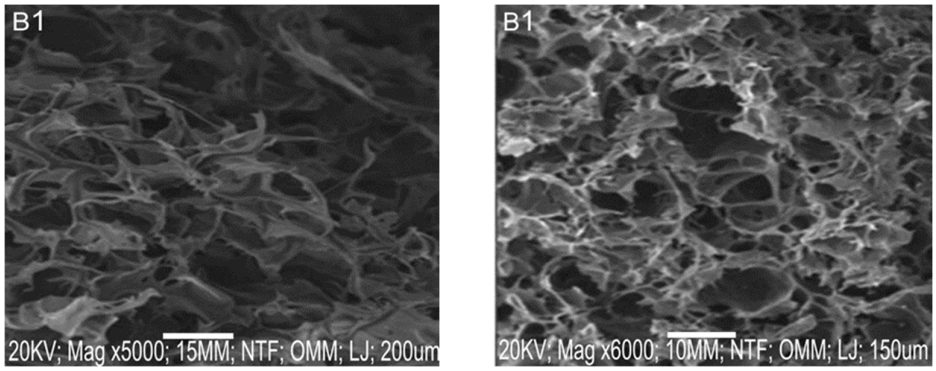


Figure 10: Plot of viscosity versus HCl concentrations for B samples

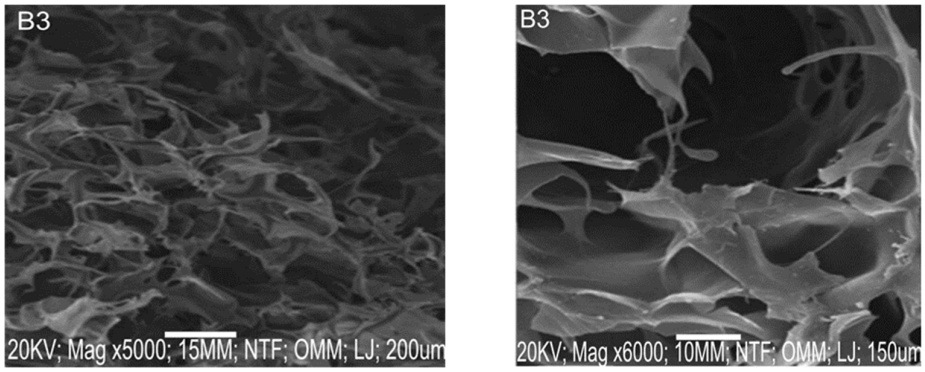
3.6. Scanning Electron Microscope (SEM) Analysis of samples B1, B3 and B5.

The micrographs for scanning electron microscopy of samples B1, B3 and B5 are shown in Figures 11, 12 and 13 respectively for x5000 and x6000 magnifications respectively. The microstructure obtained by in this study shows that biopolymers have an array of large hollow and porous cells, this structure is in agreement with that observed in the study of Merina *et al.* (2017). The presence of porosity is desired as this property will aid cell attachment and proliferation when eventually adopted for the production of gelatin-based composites for implants in tissue engineering applications. This structure is also in agreement with the study in Campodoni *et al.* (2020), where the researchers highlighted the importance of pore morphology and distribution as these two parameters play crucial roles as they impact (a) the cellular adhesion, proliferation and growth, (b) the permeation of nutrients and oxygen for the cells from the surface towards the core of the scaffold and the elimination of CO_2 and other metabolites from the core towards the surface and (c) the resulting mechanical behavior of the whole scaffold. The pore sizes increased with increase in acid concentration from B1, B3 and B5, i.e. B5 had larger pores when compared to B3 which in turn showed larger pores than B1.



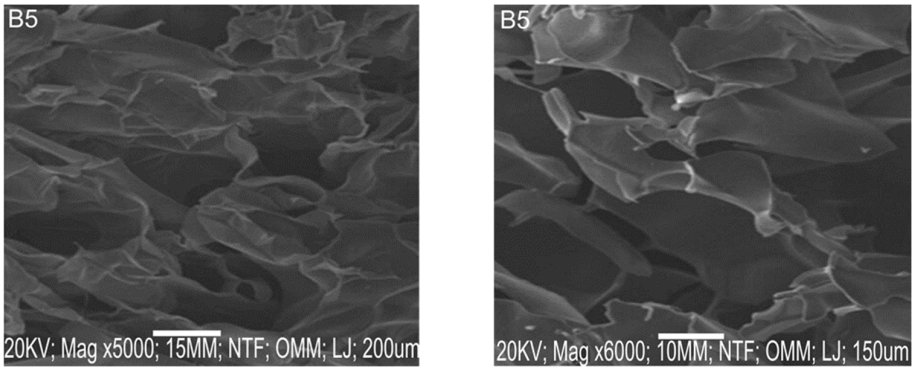
(a) (b)

Figure 11: SEM micrograph for sample B1 at magnifications of a) x5000 and b) x6000



(a) (b)

Figure 12: SEM micrograph for sample B3 at magnifications of a) x5000 and b) x6000



(a) (b)

Figure 13: SEM micrograph for sample B5 at magnifications of a) x5000 and b) x6000

3.7. Fourier Transform Infrared (FTIR) Spectroscopy Analysis

This was adopted to determine the functional groups and secondary structures of extracted gelatin, which is particularly crucial in the production of gelatin-based composites which serve as biomaterials in RM and TE. It helps determine potential reinforcing compounds and also possible chemical reactions towards adequate cross linking and production of composites with good mechanical properties (Ochulor *et al.,* 2021). The band shape, wave number at the band, intensity and chart shape were used to characterize the structure of the gelatin extracts. Typically, gelatin contains functional groups found in proteins such as hydroxyl (O-

H), carbonyl (C=O), and amine (N-H) groups. The FTIR spectrum of samples B1, B2, B3, B4 and B5 are shown in Figure 14. The absorption bands of gelatin in the IR spectra are situated in the amide band region; amide-I represent C=O stretching/hydrogen bonding while amide-II represents bending vibration of N-H groups and stretching vibrations of C-N groups, Amide-III is related to the vibrations in plane of C-N and N-H groups of bound amide (Nur-Hanani *et al.,* 2011; Pradini *et al.,* 2018).

The FTIR spectra for sample B1 as seen in Figure 14 shows peaks at 3250 cm^{-1} attributed to the presence of hydrogen bond in water and amide-A, peaks at 2922 cm^{-1} which represent amide B while the peaks of the gelatin at 1636 cm^{-1} indicates amide I, representative of C=O stretching, hydrogen bonding coupled with COO-). The peaks at wavenumber between 1524-1435 cm^{-1} represents amide II, representative of NH bending, coupled with CN stretching. The result in this study confirms the same functional groups present in commercial gelatin in the study of Wulandari *et al.* (2016). The peaks present in B2 as shown in Figure 14 are the peak at wavenumber 3300-3400 cm^{-1} which is attributed to the presence of hydrogen bond and amide-A, the peaks at (3000-2922) cm^{-1} which represents amide B while the peaks at 1636 cm^{-1} indicate amide I, representative of C=O stretching, hydrogen bonding coupled with COO-). The peaks at 1524-1435 cm^{-1} represents amide II, representative of NH bending, coupled with CN stretching.

The FTIR spectra for sample B3 show the peak at 3300 cm^{-1} attributed to the presence of carboxyl group, the peaks at wavelength 3000-2922 cm^{-1} which represents amide B, while the peak at 1625 cm^{-1} indicates amide I, representative of C=O which are strongly polar bonds that produce strong bands. The peak at 1233 cm^{-1} indicate the presence of an ester (C-O). The peak at 3276 cm^{-1} for sample B4 is attributed to the presence of carboxyl group. The peak at 1625 cm^{-1} indicates amide I, representative of C=O. The peak at 1230 cm^{-1} indicates the presence of ester C-O. The peak at 1334 cm^{-1} indicates an alkane. The FTIR spectra for sample B5 shows the peak at 3198 cm^{-1}, indicative of the presence of carboxyl group. The peak at 1625 cm^{-1} which indicates amide I, representative of C=O. The peak at 1233 cm^{-1} indicates the presence of an ester C-O. The peak at 1338 cm^{-1} indicates an alkane. Thus, the extracted samples contain functional groups such as C-N, N-H, C-H and OH as exists in the structure of gelatin. The result in this study confirms the functional groups present in gelatin produced in the study of Wulandari *et al.* (2016); Das *et al.* (2017) and Bahar *et al.* (2020).

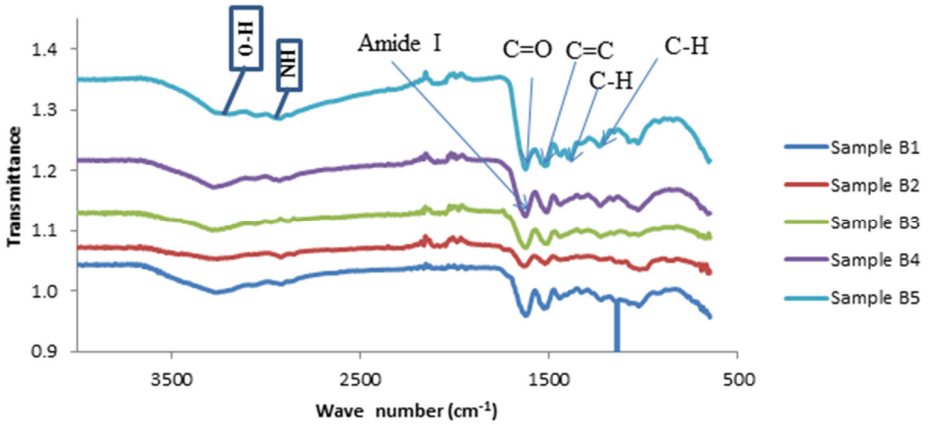


Figure 14: FTIR Spectrum of B1-B5

4. CONCLUSION

In this study, Type A gelatin was successfully extracted from croaker fish scales so as to serve as an alternative source to bovine gelatin. During the extraction, varying concentrations of hydrochloric acid (HCl) was adopted to determine the impact of the acid concentration of physico-chemical properties, morphology and functional groups present in the gelatin extracts. The yield, color, pH, viscosity and gel strength were all impacted by the concentration of the pretreatment acid as these properties reduced as the acid concentration

was increased. FTIR analysis showed the existence C-N, N-H, C-H, O-H, Amide I and Amide III functional groups which are characteristic peaks in the structure of gelatin. The SEM micrographs of the gelatin extracts showed adequate porosity necessary for cell adhesion and proliferation and that the pore sizes increased with increase in acid concentration from B1, B3 and B5, i.e. B1 had smaller pores when compared to B3 which in turn showed smaller pores compared to B5. Hence, this study has shown that croaker fish scale is a viable source for production of gelatin biomaterial and gelatin-based bio composites for RM and TE applications.

5. ACKNOWLEDGMENT

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6. CONFLICT OF INTEREST

There is no conflict of interest associated with this work.

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