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Extraction, Characterization and Application of Gelatin from*Carcharhinus amblyrhyncho* and *Sphyraena barracuda*

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Abstract: *Demand of gelatin for food and pharmaceutical application is increasing because it is one of the most widely used food ingredients. Fish gelatin represents one of the important types of gelatin from other sources other than mammalian gelatins and it may thus be considered as an alternative to mammalian gelatin for use in various food and pharmaceutical applications. The main objective of the study is to extract gelatin from fish skin of Carcharhinus amblyrhyncho (shark) and Sphyraena barracuda (sheela). Gelatin extracted by acid and alkaline treatment and confirmed by gelatinase test and FT-IR analysis. The physico-chemical characters were studied. The skin of shark yielded higher amount of gelatin than the sheela. Similarly protein, turbidity, pH, Gelling and Melting temperature, Foaming properties, Emulsifying properties, colour, amino acid composition of shark gelatin were in better than the sheela.Extracted gelatin was used in the preparation of jelly and thus its application was determined.Therefore, the use of fish skin waste as raw material in the gelatin production is quite potential and plays a major role in recycling of waste.*

Keywords:*Acid and alkaline treatment, Fish skin, Gelatin, Gelatinase test, FT-IR analysis*

I. Introduction

Gelatin is derived by partial hydrolysis of collagen .Hydrolysis can be done either by alkali or acid pre-treatment of collagen. During hydrolysis the cross-linkages between polypeptide chains bonds of the collagen are broken down. This leads to the breakdown of fibrous structure of collagen yielding gelatin **(1).** The source, type of collagen and the processing conditions will influence the properties of the gelatin. Different types of gelatins have varying thermal and rheological properties such as Bloom strength, melting and gelling temperatures. These properties are governed by chain length or molecular weight distribution, amino acid composition and hydrophobicity of a particular gelatin **(2).**There are two types of gelatin, Type A gelatin is derived from acid treatment and has an isoionic point of pH 7.9 to 4 and Type B is derived from the alkali treatment with isoionic point of pH 4.8 to 5.5 **(3).**Gelatin is a water soluble substance. They have high molecular weight polypeptide. They are hydrocolloids in nature. It is tasteless and colourless solid substance **(4).**

In cosmetic and health care products, gelatin is used as a gelling ingredient in shampoos, body lotions, face cream, hair sprays, sun screens and bath salts and bubbles. Pharmaceutical industries it is used for encapsulation of different drug products **(5).**In food industries, it is used as stabilizers, thickeners, emulsifiers, foaming agents, water binder, and crystal growth modifier. They are incorporated in confectionaries, gum drops, sauces and gravies, dairy products such as ice-creams, and in beverages**(6).**

II. Materials and methods

2.1 Sample preparation

Fish samples *Carcharhinus amblyrhyncho (shark) and Sphyraena barracuda* (sheela)were purchased from local market. The fish skin was peeled using sharp knife. After peeling the fish skins were cleaned with running tap water for three times and drained. The skin was stored at -20ºC for further extraction and analysis.

2.2 Extraction of Gelatin

The fish skins were soaked in 0.2% (w/v) sodium hydroxide for 40 minutes. After washing with sodium hydroxide, the fish skin was treated with acid, initially with sulphuric acid 0.2% (v/v) followed by citric acid solution 1%(w/v). The acid solutions were drained and the skins were washed with cold water. The final extraction of gelatin was performed in distilled water at 45°C for 18 hours. Residual water in the gelatin extract was removed by the freeze drying method. The extracts were filtered using two layers of clothes and evaporated at 70°C to remove excess water. The filtrate was dried in hot-air oven at 50°C.
Gelatin yield was calculated as percentage

$$\text{Yield of gelatin (\%)} = \frac{\text{Dry weight of gelatin}}{\text{wet weight of gelatin}} \times 100\%$$

2.3 Gelatinase test

Media required for gelatinase test was prepared and sterilized. About 10ml of media was added to two test tubes namely control and test. The *Pseudomonas sp.*, culture was inoculated into the test tube except control. After 24 hours of incubation at 37°C, the tubes were exposed to -4°C and the results were recorded.

2.4 FTIR spectroscopy

Fourier transform infrared (FT-IR) spectroscopy of gelatin sample was performed using an FT-IR spectrophotometer (PerkinElmer spectrum version 10.4.00).

2.5 Estimation of protein

Protein was estimated by Lowry's method (7).

2.6 Purification of gelatin by dialysis

Dialysis was carried out to remove any inorganic salts left after pre-treatment process. Two Dialysis bags were cut to an approximate length of 30cm and soaked in NaCl for few minutes. One end of the bag was tied and 10ml of the sample was added through the other end onto both dialysis bag and left in phosphate buffer saline for 24 hours under continuous stirring

2.7 Determination of turbidity

The turbidity of gelatin samples at different pH (3-10) were determined by dissolving the sample in distilled water and kept at 60°C for few minutes. The pH was adjusted either with 6N NaOH or HCl. Absorbance were measured at 360nm.

2.8 Determination of pH

The pH of extracted gelatin was determined using pH pen calibrated to an acidic pH range of 5.

2.9 Determination of gelling temperature and melting temperature

10ml of gelatin sample was taken onto test tubes kept in deep freezer (-20°C). The gelling temperature was noted.The freezed gelatin was kept in water bath at 40°C and the time period corresponding to the melting temperature was recorded.

2.10 Determination of colour

The colour of gelatin solutions extracted were observed visually and recorded.

2.11 Determination of emulsifying stability index

Emulsion stability index (ESI) of gelatin samples were determined by taking Oil (2ml) and gelatin solution (6ml) mixed using motor and pestle. Emulsions were pipetted out at every 0 and 10 minutes and 100-fold diluted with 0.1% SDS. The mixture was mixed thoroughly for 10 seconds using a vortex mixer. ESI was determined by spectrophotometer at A500.

$$\text{ESI (min)}=\frac{A0}{(A0-A10)}\times\Delta t$$

Where, $A_{0=}A_{500}$ at time of 0 min, $A_{10=}A_{500}$ at time of 10 min and Δt=10 min.

2.12 Determination of foaming properties

Foam expansion (FE) and Foam stability (FS) of gelatin sample were determined. Gelatin solutions (1, 2, and 3%) were transferred onto test tubes and mixed well using vortex mixer for one minute at room temperature. The samples were allowed to stand for 0 and 60 minutes.

FE and FS calculated using the following formula given below

$$\text{FE (\%)} = (VT/V0) \times 100$$

$$\text{FS (\%)} = (Vt/V0) \times 100$$

Where, V_T = total volume after whipping; V_0= the original volume before whipping and V_t = total volume afterleaving at room temperature for 60 min.

2.13 Amino acid composition

The extracted gelatin was hydrolysed for 16 hrs in 15 ml of 6N HCl at 110ºC. The sample was dissolved in deionised water and filtered. The amino acid composition was obtained using a high performance liquid chromatography (HPLC) Waters 410 Scanning Florescence and AccQ Tag column (3.9 × 150 mm).

2.14 Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)

Acrylamide & Bis Acrylamidegel polymerization was allowed to complete for about 15-30 minutes using APS & TEMED. In the meantime, the sample was prepared. The test sample was mixed with the sample

buffer in the ratio of 3:1 and it was boiled for 5-10 minutes. After polymerization was over, the comb was carefully removed, the wells were rinsed with tank buffer and the bottom spacer was removed. The gel plate was clamped and it was tightened with the vertical gel electrophoresis apparatus. Using a micropipette, the sample was loaded to each well. Slowly the top chamber was filled with tank buffer and tank buffer was added to the top and bottom reservoir. The tank was connected to the power supply and the power was turned on. The power supply was set to constant current mode (100 mV). The system was continued till the marker dye reaches the bottom of the gel, it was turn off and the power supply was disconnected. The gel from the glass plate was carefully taken and transferred to a plastic tray. The CBB R-250 staining solution was poured into the gel tray and it was placed on the rocker for overnight. After overnight incubation the excess amount of stain was removed by using destaining solution. The gel was transferred onto the transilluminator and the protein bands were visualized.

2.15 Application-Preparation of jelly using extracted gelatin

Gelatin jelly was prepared by dissolving gelatin in instant juice mixture and exposing to refrigeration temperature (4° C) for few minutes.

III. Result and Discussion

3.1 Extraction of Gelatin

The extracted gelatin from the different fish species of fresh water are tabulated on (TABLE 1). The highest yield of 8% was obtained from the *Carcharhinus amblyrhyncho* compared to the yield of 6% from *Sphyraena barracuda* shown in (Fig 2).

The lower yield could be due to the loss of extracted collagen due to incomplete hydrolysis of the collagen. *Carcharhinus amblyrhyncho* has comparatively more connective tissue and hence has more collagenous material than *Sphyraena barracuda* fish.

Samples	**Wet weight of fish skin(gms)**	**Yield of the gelatin (gms)**
Carcharhinus amblyrhyncho	10 gms	0.8gms
Sphyraena barracuda	10gms	0.6gms

Table 1: Yield of the gelatin in percentage

Figure 2:*Sphyraena barracuda***Figure 3:***Carcharhinus amblyrhyncho*

Figure 4: Alkaline treatment**Figure 5**:Extracted Gelatin

3.2 Confirmatory test

3.2.1 Gelatinase test

The gelatinase test performed using extracted gelatin shows positive result (Fig 6a, b). The extracted gelatin liquefied by the gelatinase enzyme produced by the *Pseudomonas sp.*

Figure 6(a) **Figure 6(b)**

Figure 6: Gelatinase test for extracted gelatin (*Sphyraena barracuda* (a) and *Carcharhinus amblyrhyncho* (b)).

3.2.2Fourier transform infrared spectroscopy (FT-IR)

The peaks were characterised using extracted gelatin using FT-IR spectra. The range peaks in each sample are tabulated below (TABLE 2). Samples showed three major peaks ranging between 3600-2700 cm^{-1}, 1900-900 cm^{-1}, 900-400 $cm^{-1.}$

In FT-IR spectrum analysis, both the samples show peaks at 3339.36cm^{-1}, 1637.81cm^{-1}, 668.50cm^{-1}. The peak at 3339.36 cm^{-1} corresponds to the amines (N-H), Stretch with medium intensity, peak at 1637.81cm^{-1} corresponds to the Amides (N-H), Stretch with strong intensity and peak at 668.50 cm^{-1} corresponds to the Alkyl halide (C-Cl) Stretch with strong intensity. The presence of major peaks 3339.36cm^{-1}, 1637.81 cm^{-1}, 668.50cm^{-1}confirmed the structure of the gelatin.

Sample	**FT-IR spectra peak wave length^{-1}**	**Vibration mode**
Sample1 *(Carcharhinus amblyrhyncho)*	3339.36cm^{-1} 1637.81cm^{-1} 668.50cm^{-1}	Amines (N-H),Stretch with medium intensity Amides (N-H), Stretch with strong intensity Alkyl halide (C-Cl)Stretch with strong intensity
Sample2 *(Sphyraena barracuda)*	3339.54cm^{-1} 1638.40cm-^{1} 669.00cm^{-1}	Amines (N-H),Stretch with medium intensity Amides (N-H), Stretch with strong intensity Alkyl halide (C-Cl)Stretch with strong intensity

Table 2:FT-IR analysis of *Carcharhinus amblyrhyncho* and *Sphyraena barracuda*.

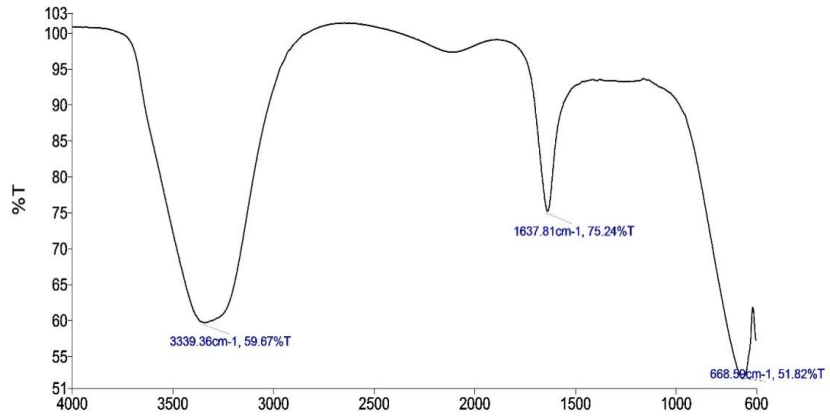

Figure 7: FT –IR analysis for *Carcharhinus amblyrhyncho*

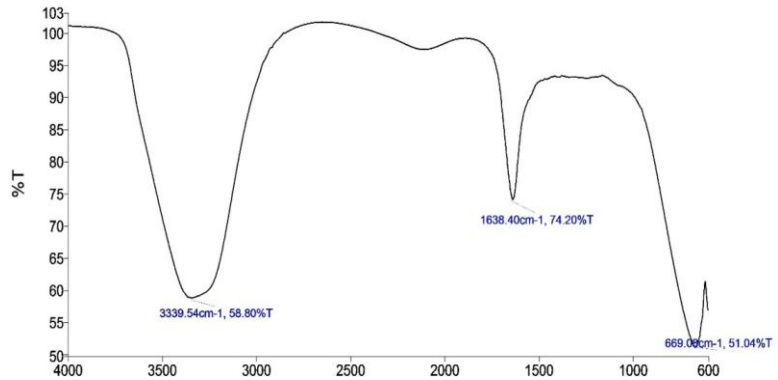

Figure 8: FT –IR analysis for *Sphyraena barracuda*.

3.3 Estimation of protein

The protein content in extracted gelatin found to be 55mgfor *Carcharhinus amblyrhyncho* and 50mg for *Sphyraena barracuda*by plotting it in a standard graph also depicted in (Fig9).

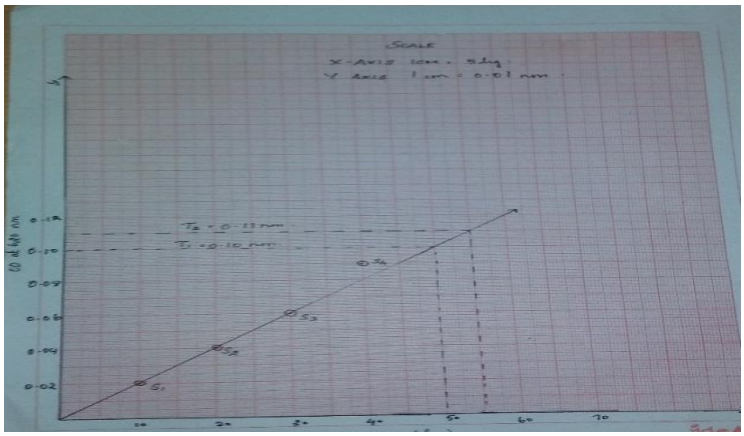

Figure 9: Estimation of protein by Lowry's Method

3.4 Purification

The purification of extracted gelatin carried out using dialysis method using PBS buffer. Activity of the gelatin is correlated with the purity of extracted gelatin.(Fig 10)

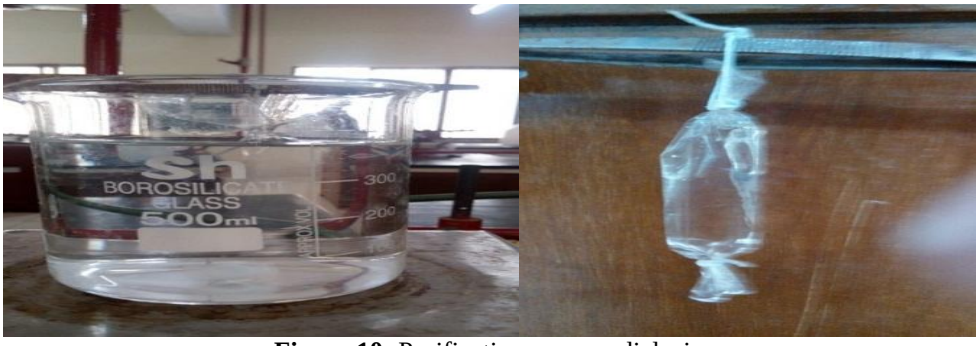

Figure 10: Purification process-dialysis

3.5 Turbidity

The turbidity of the extracted gelatin was determined by measuring the absorbance at 360nm. The turbidity of the gelatin gradually increases depending on the pH range. Turbidity values are dependent on efficiency of the clarification process. Turbidity values of the extracted gelatin were tabulated (TABLE 3) and (Fig 11).

Different pH	Sample1*(Carcharhinus amblyrhyncho)*	Sample2*(Sphyraena barracuda)*
3	0.09	0.08
4	0.10	0.09
5	0.11	0.10
7	0.12	0.11
10	0.32	0.17

Table 3: Absorbance value of the gelatin for turbidity at 360nm

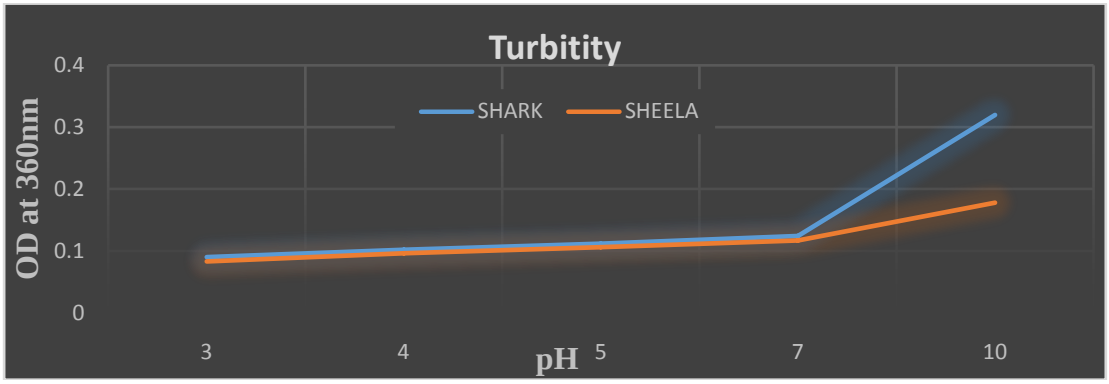

Figure 11: Graphical representation of turbidity for extracted gelatin.

3.6pH

In the present study the pH of extracted gelatin was 5 for *Carcharhinus amblyrhyncho*and 5.1 for *Sphyraena barracuda*. The pH of the gelatin influenced the turbidity of the extracted sample in turn correlated with the application of gelatin. The pH of the extracted gelatin from fish sample was similar to the pH of the commercial gelatin which was 5-6 reported in the study **(8).**

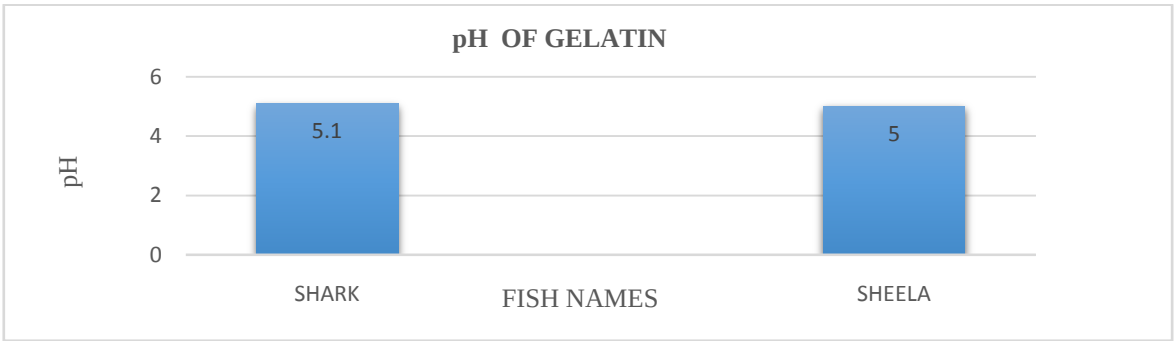

Figure 12:pH of gelatin

3.7 Gelling temperature and Melting temperature

The gelling temperature of the extracted gelatin represented in the (Fig 13). The gelling time for *Carcharhinus amblyrhyncho* at -20°C was found to be 15 minutes whereas for *Sphyraenabarracuda* was 20 minutes. The extracted gelatin has higher gelling temperature compared with commercial gelatin due to the presence of impurities. The melting temperature of the extracted gelatin was found to be 35°C.

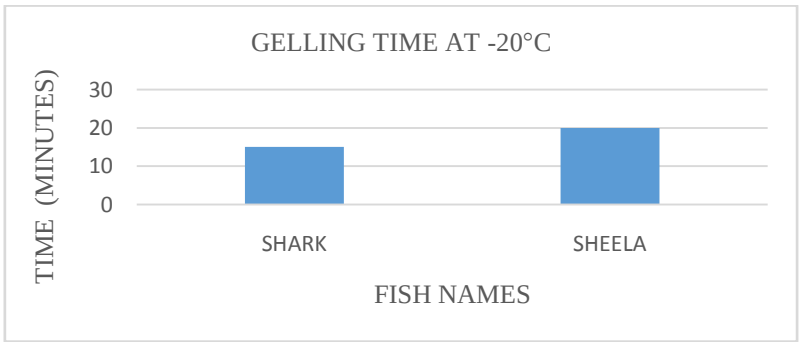

Figure 13: Gelling time at -20°C

3.8 Colour

The extracted *Carcharhinus amblyrhyncho* fish skin gelatin appeared white in colour and *Sphyraena barracuda* fish skin gelatin appeared yellow in colour. The colour of the gelatin depends on the raw material and the colour does not influence the functional properties of the gelatin.

Figure 14: Colour of the extracted gelatin *Carcharhinus amblyrhyncho* and *Sphyraena barracuda*

3.9 Emulsifying stability index

ESI was determined by spectrophotometer at A500. Emulsifying stability index of extracted gelatin was increased with increasing concentration tabulated in (TABLE 4). Long chain was able to form the stronger and stiffer films surrounding oil droplets, thereby increasing the stability towards emulsion collapse.

Fish samples	**1 gram**	**2 grams**	**3 grams**
Carcharhinus amblyrhyncho	29.42	30.81	31.35
Sphyraena barracuda	31.65	35.14	36.94

Table 4: Absorbance value of ESI

Figure 15: Emulsifying properties at 0 minute and 10 minutes

3.10 Foaming properties

Foam expansion (FE) and foam stability (FS) of extracted gelatin are tabulated in (TABLE 5). The FE and FS of extracted gelatin increased with increasing gelatin concentrations. A protein must be capable of migrating rapidly to the air water interface, unfolding and rearranging at the interface to express good foaming ability.

Fish names	**Foam expansion**		**Form stability**	
	3 grams	**6 grams**	**3 grams**	**6 grams**
Sphyraena barracuda	85%	93.75%	93.7%	98.7%
Carcharhinus amblyrhyncho	95%	87.5%	97%	100%

Table 5: Percentage of Foam expansion and Foam stability

Figure 16: Forming properties at 0 minute and 60 minutes

3.11 Amino acid analysis

The amino acid composition plays a main role in the physical properties of gelatin. The contents of glycine and imino acid are important for gel strength. A low amount of imino acids indicates a poor gelling ability. Glycine (Gly) being the most predominant one. Glycine (Gly) content is higher than other amino acids. Gelatin properties will also strongly depend on the preservation of raw materials. The amino acid composition tabulated in (TABLE 6).

Amino acid composition	***Sphyraena barracuda***	***Carcharhinus amblyrhyncho***
Aspartic acid	2864	2108
Glutamic acid	Not detected	1159
Serine	2515	2445
Histidine	2355	1328
Glycine	4617	11946
Threonine	1283	10062
Arginine	3808	16140
Alanine	1942	4688
Tyrosine	10771	12100
Methionine	1638	3987
Valine	1303	1429
Phenylalanine	2284	3184
Isoleucine	1852	3439
Leucine	2499	10198
Lysine	1066	3779

Table 6: HPLCanalysis for estimation of amino acids

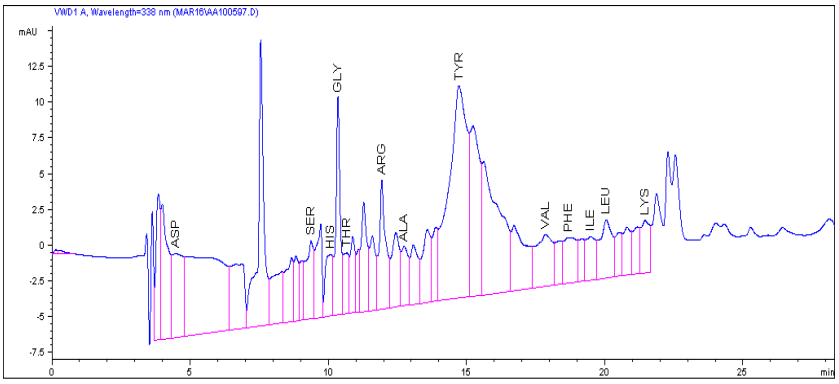

Figure 17:HPLC analysis of *Sphyraena barracuda*

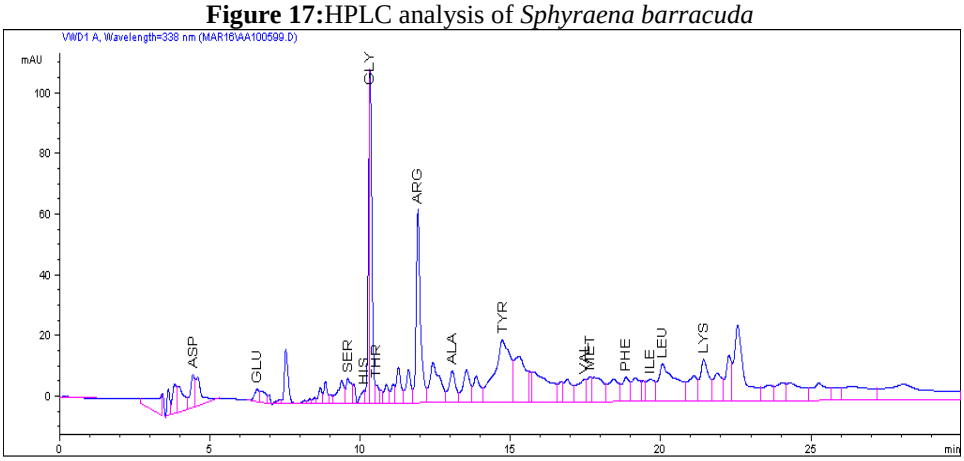

Figure 18:HPLC analysis of*Carcharhinus amblyrhynch*

3.12SDS- PAGE

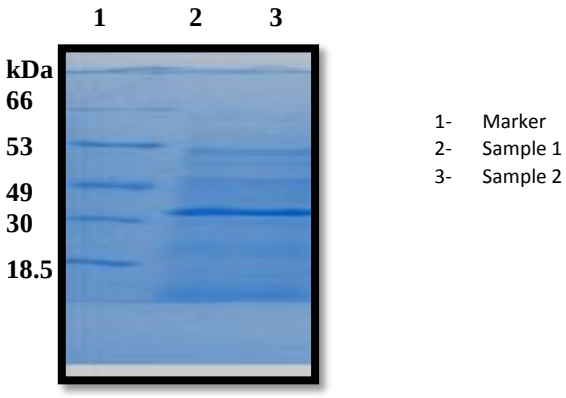

Figure 19: SDS – PAGE.

The molecular weight of the test samples 1 & 2 had two peptides with molecular weight of 30 kda and 58kd

3.13 Application of gelatin

Preparation of jelly

Orange and mango jelly prepared from extracted gelatin shown in the (Fig 21). Orange jelly prepared from the extracted gelatin *Carcharhinus amblyrhyncho,* mango jelly prepared from the extractedgelatin *Sphyraena barracuda*.

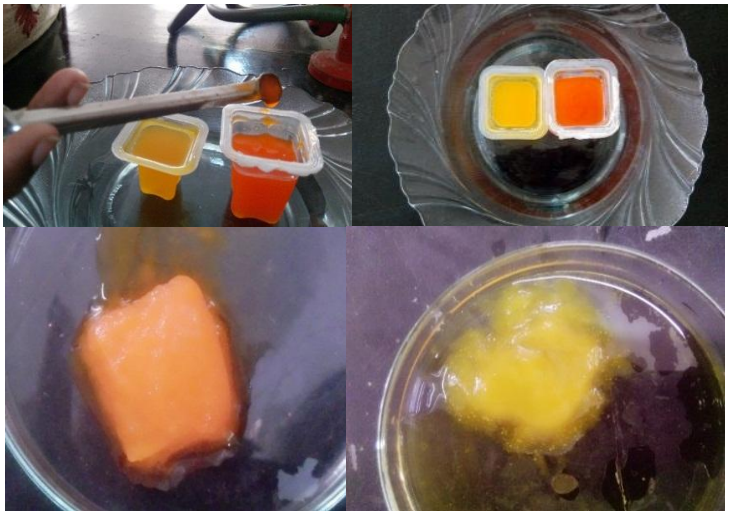

Figure 20: Preparation of jelly using extracted fish gelatin

IV. Conclusion

Gelatin is one of the most popular biopolymers. It is tasteless and colourless solid substance derived from the fibrous protein collagen. It is widely used in food, pharmaceutical, cosmetic and photographic applications because of its unique functional and technological properties these properties increases the demand of gelatin production in industries. Gelatin can be extracted from fish skin, which is a major waste produced by fish processing industries. The present study, gelatin was extracted from the two fish species shark and sheela. The gelatin was produced by means of extraction process using alkaline and acid pre-treatment followed by extraction with distilled water and drying process. The extracted gelatin exhibited different physicochemical characteristics. The presence of extracted gelatin was confirmed by the gelatinase test and FT-IR analysis. The extracted gelatin was purified by dialysis method. Certain characteristics of extracted gelatin from two different fish skin were evaluated. The protein content of extracted gelatin was found to be 55mgfor *Carcharhinus amblyrhyncho* and 50mg for *Sphyraena barracuda* estimated by Lowry's method. The turbidity of the gelatin gradually increased depending on the pH range. The extracted gelatin colour appeared white in *Carcharhinus amblyrhyncho* and *Sphyraena barracuda* was yellow in colour. pH of the extracted gelatin was similar to the pH of commercial gelatin. The gelling temperature of the *Carcharhinus amblyrhyncho* was rapid compared to that of *Sphyraena barracuda*. The extracted gelatin from both the species were found to exhibit similar melting temperature, Emulsifying stability index of the extracted gelatin increased with increasing concentration. Foaming properties of the extracted gelatin for *Carcharhinus amblyrhyncho* was higher than the *Sphyraena barracuda*. It was used in the preparation of jelly and thus its application was determined.

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