Research Article

Isolation and characterization of acid-soluble Piscean collagen from sea fish *Morone americana*

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(Received: 26-11-2024 Revised: 23-01-2025 Accepted: 29-01-2025)

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ABSTRACT

Aim: We used the Indian white perch tiger, one of the common fish found in the fish waste, to extract acid soluble collagen (ASC), to increase the utilization of fish waste from the fish industries; and to focus on the natural raw materials in cosmetics, as they are less harmful to the skin, and investigated some biochemical properties.

Result: The yield of isolated ASC was found to be 0.521%. The sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) study showed that the isolated collagen was Type-I and consisted of two α subunits1 and 2 respectively, with a molecular weight of approximately 148 kDa. The ultraviolet (UV) absorption spectrum of collagen showed absorption at 226 nm. The Fourier transform infrared spectroscopy (FT-IR) spectrum of ASC showed the peaks for Amide-I, II, and III corresponding to functional groups of the protein. The high absorption peak was observed at 226 nm, corresponding to C=O, COOH, and $CONH_2$ from the Ultraviolet spectrometer. Through all these confirmatory studies, it was concluded that the extracted collagen was found to be Type-1 and it was found to be linear with the similar studies carried out for different marine sources. Collagen's ability to absorb and hold moisture was examined in this study, and it was discovered to be superior to the control substance, glycerol. Because of this, the collagen from Indian white perch tigers may be used as a moisturizer and may have applications in the cosmetic industry.

Keywords: Indian white perch tiger, Acid soluble collagen (ASC), Water absorption and retention, Moisture absorption.

1. INTRODUCTION

Collagen is the most predominant content of tissues, bones, tendons, and skin. The primary structure of the collagen consists of Glycine [1]. The other two chains are proline and hydroxyproline. Glycine helps the chain withstand stress and form a tight configuration. In vertebrates, collagen makes up 25% of all proteins [2]. Collagen has been categorized into the following groups: fibril-associated collagen, network-forming collagen, anchoring collagen, Transmembrane collagen, and basement membrane collagen. The most prevalent of them is collagen which forms fibrils [1]. Despite coming in various forms, they all share the right-handed triple helix structure comprising three chains. Currently, 28 different types of collagens are known, but Types I, II, III, and IV are the most prevalent [2]. These could have been created by combining three different chains, as in Type I, II, V, or VI, or three identical chains, as in Type II, III, VII, VIII, or X. The left-handed helix formed by each of the three -chains has 18 amino acids per turn. Every third position of the polypeptide chains must contain a glycine residue for the triple helix to form, resulting in (Gly-X-Y) repeated units. The glycine residue is placed in the centre of α -chains, while the other amino acids occupy the outer space. Proline and hydroxyproline frequently occupy the X and Y positions [3]. The ability of 4-hydroxyproline to form H-bonding is what gives the triple helix its

stability [4]. The core of the collagen that forms fibrils is the triple helix [5].

Up until this point, collagen was obtained from cow and pig skins, but this had the drawback of potentially transmitting diseases like bovine spongiform encephalopathy (BSE) and foot-and-mouth diseases, which has led to a decline in its use. So, there was an urge to find another alternative for the collagen source, which led to the research of extracting collagen from marine sources. The collagen from the marine source was found to have high availability and lack of risk of disease transmission. However, there are a lot of tasks that are accomplished in the extraction field [5]. In 1943, marine collagen was first taken out of gemmules of *Spongilla* [6]. Even Atlantic salmon showed the presence of Type-1 collagen, upon evaluation of collagen obtained from Tilapia fish [7]. Acid-soluble and pepsin-soluble collagen were also present. The collagen obtained from jellyfish was found to be Type-1 with a higher moisture absorption rate [8]. The skin and bones of marine sources typically contain Type-I fibrillar collagen. The types of genes involved in protein biosynthesis are what give rise to this diversity. Because of its high biocompatibility, non-toxicity, and biodegradability, collagen has a wide range of uses in the cosmetic, pharmaceutical, medical, and food industries. Due to its excellent bioactivity toward skin repair and regeneration, it has been widely used in the development of cosmeceutical products [2]. Due to its numerous bioactive qualities, collagen derived from marine sources has also been utilized extensively as a scaffold for tissue engineering [9]. It has a greater capacity for absorption than collagen derived from animal sources. It also has improved mechanical strength and a milder smell.

In many scientific fields, including pharmaceuticals, tissue engineering, food additives, and packaging, collagen-based materials have been acknowledged as the most alluring ones. It has several uses in the beauty industry because of its special qualities, which include being natural, humectant, anti-aging, anti-wrinkling, UV-photo protective, and antioxidant [6].

Both *in-vivo* and *in-vitro,* collagen-based biomaterials have a wide range of applications. The most typical collagens employed in the creation of collagen-based biomaterials are those that produce fibrils. Drug administration Collagen application is typically designed as a membrane [6]. The development of new bio-based topical formulations made use of the collagen skeleton of marine sponges [10], [11]. The human body produces less collagen with advancing age and an unhealthy diet. As a result, collagen has been added to numerous foods. Collagens are added to food as food additives to improve rheological properties, cut back on fat intake, and make sure there is an adequate supply of animal nutritive fibers. The shelf life of various food products is still extended by the use of edible films and coatings, which give the food product structural integrity and vapour permeability. Sausage casings can also be made from collagen. The films have excellent oxygen barrier properties, but these are severely constrained by their high moisture sensitivity, which compromises their thermo-mechanical properties. Lamination could be increased to make this better [12]. Antioxidant properties of marine collagen increase its use in skincare products to repair/prevent damage brought on by various factors. Several hydrogels that support anti-aging performance also require collagen. Additionally, collagen-derived short polypeptides and tiny peptides can be used in cosmetic formulations. Collagen exhibits a variety of properties in its various forms, which has given scientists new directions to explore [13]. The major objective of the study was to use the skin, fin, and scales of the sea fish Indian white perch tiger (*Morone americana*) of the Actinopterygii family, to isolate, characterize, and study the biochemical properties used as excellent material for cosmetic applications. The choice of fish was based on availability, cheaper prices, and the fact that it is a major component of fish waste. Common fishes such as Indian mackerel, Indian oil sardine, Tilapia, Catla fish, Rohu, and Pufferfish, etc., have already been studied for the same purpose. This study focuses on the isolation and characterization of collagen type-I from the Indian white perch tiger *(Morone*

americana) of the Actinopterygii family, which is widely available at low prices in Mangalore, Karnataka.

2. MATERIALS AND METHODS

2.1. Fish samples

Scales, fins, and skin of *Morone americana* were collected from fishermen; it was cleaned with distilled water and stored at -20℃ till the completion of isolation.

2.2. Chemical reagents

Sodium Hydroxide (0.1M) (1:10 w/v), Sodium Hydroxide (6N), Ethylenediaminetetraacetic acid disodium salt (EDTA)(0.5M), Butyl Alcohol (10%)(1:10 w/v), Acetic acid (0.5M)(1:4 w/v), Sodium Chloride, Hydrochloric acid, Tris HCl (0.05M), Acetic acid (0.1M), (Bovine serum albumin, Calfskin type-I collagen, Copper Sulphate (0.5%), Sodium potassium tartrate (1%), Sodium Carbonate (2%), Sodium hydroxide (0.1N), Folin-Ciocalteu reagent.

2.3 Instrumental Analysis

With the help of an FT-IR Spectrophotometer (IR Prestige-21, Shimadzu Corporation, Japan), the structural characteristics of the isolated ASCs were determined. The frequency range used for the analysis was 4000-400 cm-1. Field Emission Scanning Electron Micrograph (Carl Zeiss Microscopy Ltd.) was used to examine the microstructure of ASC. Thermo gravimetric analysis (TGA) was performed (Model: SDTQ 600, TA Instruments, UK) to ascertain the thermal stability. The UV-Vis spectrophotometer (Spectroquantpharo 300) was used to analyze the isolated ASC in the UV-visible range between 200 and 800 nm.

2.4 Extraction of Acid Soluble Collagen (ASC)

Acid-soluble collagen was extracted from the skin, fins, bones, and scales of the Indian white perch tiger using the Nagai Suzuki method [14], with a small variation. Weighed fish samples were thoroughly washed with ice-cold distilled water and ground. The ground mixture was added to 0.1M NaOH (1:10 w/v) and allowed to stir for 48 hours. This process was repeated for an additional 5–10 hours to completely remove the impurities. By washing the residue with ice-cold distilled water, the pH of the residue was kept at a neutral level. The residue was blended with a 0.5M EDTA solution for 5 days to decalcify it. Following a cold distilled water wash and a 10% butyl alcohol wash to remove fat, the samples were extracted for three days with 0.5 M acetic acid. A fresh cotton cloth was used to collect the extracted sample, which was then centrifuged for 30 minutes at 1000 rpm. The resulting supernatant was salted by adding NaCl while still containing Tris-HCl to a final concentration of 2.5M. This process was allowed to stir for an entire night before centrifugation produced the pellet. The obtained pellet was dissolved in 0.5M acetic acid. The solution was dialyzed both against distilled water and 0.1M acetic acid. The Amicon Ultra centrifugal tube with 100 kDa filters was used to centrifuge the collagen for 30 minutes at 2°C and 4000 rpm. To obtain Acid Soluble Collagen, the filtrate was then lyophilized.

$$\text{Yield}(\%)=\frac{(\text{Weight of the lyophilized collagen (g)})}{(\text{Weight of the sample taken(g)})}\text{X100} \qquad [1]$$

2.5 Protein Estimation

Using Lowry's method, the protein content of the ASC was estimated. The solution "A" was prepared by combining 0.1N sodium hydroxide with 2% sodium carbonate, Solution B by combining 1% Sodium Potassium tartrate solution with 0.5% Copper Sulphate. Bovine serum albumin standard was made by dissolving 10 mg of the protein in 100 mL of distilled water in a volumetric flask. 0.2, 0.4, 0.6, 0.8, and 1.0 mL were pipetted into a 10 mL tube, then made up to the mark with distilled water, with the same solution used to prepare the sample solutions. 4 mL of an alkaline copper sulfate solution was added to each flask, thoroughly mixed, & allowed to stand at room temperature for 10 minutes. After adding 0.5mL of the FC reagent to each tube, it was mixed right away. The solutions were then left to stand for 30 minutes, and the absorbance of the resulting purple-coloured solution was measured at 660 nm. A blank was created by combining 2 mL of distilled water with 4 mL of alkaline copper sulfate and then allowing it to sit at room temperature for 10 minutes. The previously incubated sample

received 0.5mL of FC reagent, which was then added & further incubated at room temperature [15].

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The Sodium Dodecyl Sulphate Poly-acrylamide Gel Electrophoresis was used to determine the protein sequence in the isolated collagen. The protein that was extracted from the collagen was examined, according to *Tang et al.*, (2015).

The sample was initially dissolved in 0.1M acetic acid, then in 2% SDS and 20mM Tris HCl (pH 8.8). Following this step, the samples were examined with a 13% separating gel and a 10% stacking gel. To perform electrophoresis, the gel was stained with acetic acid, methanol, and 0.025% Coomassie brilliant blue-R. The extra-pure collagen from marine fish was used as the benchmark [16].

2.7 Water absorption and retention analysis

To maintain humidity levels of 43% and 83%, respectively, 100mg of freeze-dried collagen sample was dissolved in 10µL deionized water and kept in a desiccator with saturated ammonium sulfate and sodium carbonate solution solutions. Every 6 hours, weight change was recorded, and the percentage of water absorbed and retained was calculated using the equation below [17].

Moisture absorption (%) =

$$\frac{\text{The weight of the sample under water saturation}}{\text{The weight of the sample in the dry state}} \times 100 \quad [2]$$

Moisture retention (%)

$$\frac{\text{The weight of the sample under water saturation} - \text{the weight of the sample in the dry state}}{\text{The weight of the sample in the dry state}} \times 100 \quad [3]$$

2.8 Effect of pH on collagen solubility

The isolated collagen was prepared in 3mL of 0.5M acetic acid (3mg/mL), and the mixture was stirred at 4°C to completely solubilize the collagen. An amount of 3mL of each collagen sample of pH 2,4,6,8 & 10 was taken in centrifuge tubes and made up to a final volume of 5mL with distilled water. The solutions were centrifuged at 10,000 rpm after being gently stirred for 30 minutes at 4°C [18].

3. RESULTS AND DISCUSSION

3.1 Preparation of acid-soluble collagen

The amorphous and white-in-colour collagen isolated from the Indian white perch tiger had a yield of 0.512%. The collagen obtained was found to be different as compared with other marine sources and is tabulated in Table 1.

Table 1: Comparison of yields of collagen obtained for various species.

Source	Species	Yield (%)	References
Scale	*Hypophthalmichthys*	PSC-2.7	(14)
Scale	*Ctenopharyngodon idellus*	ACS – 16.1	(15)
Scale	*Oreochromis niloticus*	PSC – 14.9	(16)
Bone	*Magalaspis Cordyla*	ACS – 30.5	(17)
Bone	*Otolithes ruber*	PSC – 27.6 ACS – 45.1	(17)
Skin	*Labeo rohita*	PSC – 48.6 ACS – 46.13	(18)
Skin	*Oreochromis niloticus*	ACS – 39.4	(14)
Skin	*Misgurnusanguilli Caudatus*	ACS – 22.42	(19)
Skin	*Oreochromis niloticus*	PSC – 27.32 ACS – 27.2	(20)
Skin, Bone, Fin	*Morone Americana*	ACS-0.512	Current study

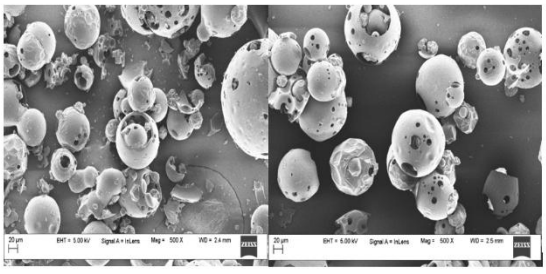
The type of fish, age, physical characteristics, laboratory temperature, and pH of the solutions could all play a role in this variation, as well as

differences in the collagen molecules' ability to cross-link. When compared to other data, the obtained yield is found to be lower [19]. This might be caused by the collagen molecules' telopeptide regions being strongly crosslinked. Protein content was found to be 28.9 mg/L overall. The intensity of the color change after adding the FC reagent is directly proportional to the protein concentration. Due to the absence of tryptophan amino acid and the oxidation of other amino acids like tyrosine, cysteine, histidine, and asparagine, the color change is typically caused by proline binding with FC reagent [5, 8, 21].

4. CHARACTERIZATION

4.1 FESEM Analysis

According to Tamilmozhi, Veeruraj, and Arumugam [28], the collagen isolated from marine sources typically takes the form of fibrils. There are typically two types of collagens: fibrillar and non-fibrillar. The collagen isolated from Indian white perch tigers is non-fibrillar as opposed to the fibrillar form that marine collagens frequently take. The collagen surface was textured and uneven, as evidenced by the micrographic structure. Hydrophobic interactions, electrostatic interactions, and hydrogen bonding may all be contributing factors to the crosslinking between the networks. However, it was discovered that the isolated collagen was pure and could be used immediately for more research **(Fig.1a, 1b)**.



(a) (b)

Fig.1(a) represents isolated collagen and (b) represents standard collagen

4.2 Thermogravimetric Analysis

TGA was used to evaluate the thermal characteristics of collagen because it gives quantitative data on mass change related to dehydration, breakdown, and oxidation as a function of time and temperature. The collagen molecule shows a major loss in mass percentage in the range of room temperature to 200°C, 200°C-300°C and at 450°C. (Fig. 2)

The standard collagen and isolated collagen showed similar TGA patterns with loss in mass from room temperature to 200°C due to the loss of water molecules. The loss of around 200°C-300°C is due to the decomposition of collagen molecules. The third drop was observed at 450°C, due to the complete degradation of the collagen [3, 22]. The obtained result was found to be similar to the collagen isolated from scales of Mediterranean Fish [23].

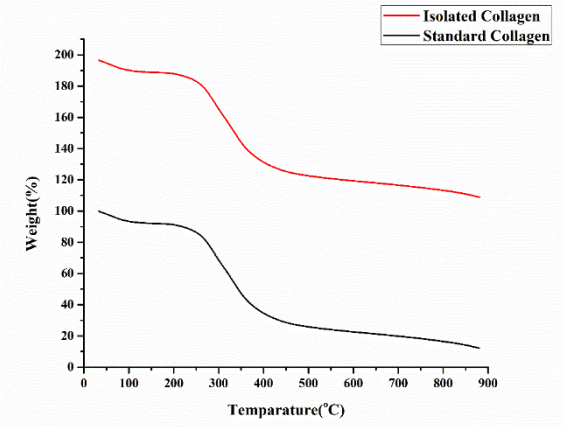
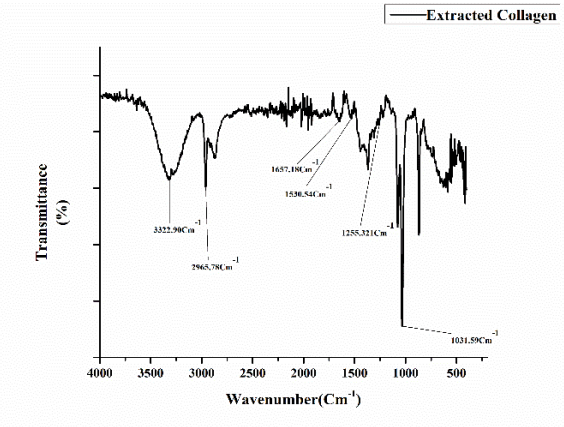


Fig.2 Thermogram of isolated collagen and standard collagen

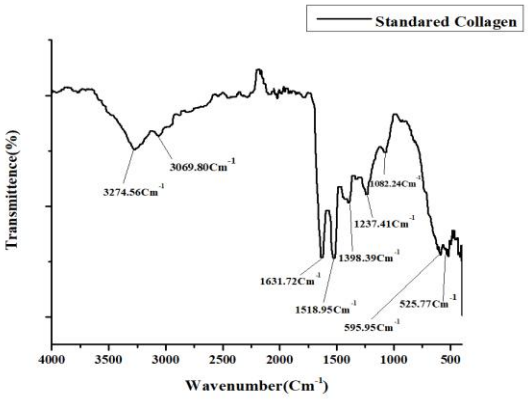
4.3 Fourier Transform Infrared Spectroscopy

Generally, the FT-IR spectrum of collagen exhibits five major amide bands namely Amide A, Amide B, Amide I, Amide II, and Amide III, which indicates the existence of a triple helical structure. The Amide A band lies in the region of 3322.90 cm^{-1} followed by the Amide B band at 2965.78 cm^{-1}. Amide I, Amide II, and Amide III show absorption bands at 1657.18 cm^{-1}, 1530.54 cm^{-1}, and 1255.32 cm^{-1} respectively, which resembles the characteristics of Type-1 collagen concerning the standard collagen, which showed absorption bad for Amide A, was observed at 3320.56 cm^{-1}. Due to C-N stretching, N-H bending vibration, and wagging vibration of CH_2 groups of the glycine backbone and proline side chains, Amide B is at 3069.80 cm^{-1}; Amide I is at 1631.72 cm^{-1}; Amide II is at 1518 cm^{-1}; and Amide III is at 1237 cm^{-1}. The regions that Amide I, Amide II, and Amide III display suggest that the polypeptide chain is triple-helical in shape. Due to the peptide's role in H-bonding, the band seen at 3322.90 cm^{-1}

corresponds to -NH stretching vibration. The pyrrolidone ring of hydroxyproline vibrates at 1366.76 cm^{-1}, which corresponds to the absorption band at that wavelength. The stretching of the CH_2 atom is what causes the absorption band at the bond at 2965.78 cm^{-1}. It was assumed that the stretching vibration would be in the range of 1657 cm^{-1}, which can be used to examine the protein's secondary structure. The observed Amide II band at 1530 cm^{-1} is associated with -NH bending vibration. The Fig-3 provides the results of the FTIR analysis of the extracted collagen [22, 23]. **(Fig.3, 3a, 3b)**



(a)



(b)

Fig.3 (a) FT-IR spectrum of isolated collagen (b) FT-IR spectrum of standard collagen

Similar results were obtained for the work done by Niveditha *et.al.*, [26], on Indian oil sardines. The amide A and B corresponding to NH stretching were found at 3000-3400 cm^{-1}. The amide-I band corresponding to -C=O stretching vibration was at 1600-1700 cm^{-1}, amide-II at 1550-1600 cm^{-1}, and amide-III at 1200-1350 cm^{-1} was observed [24].

Table. 1 Comparison of the IR frequencies of standard collagen v/s extracted collagen

	Standard Collagen	Extracted Collagen
Amide A (3000 cm^{-1} to 3400 cm^{-1})	3322.90 cm^{-1}	3320.56 cm^{-1}
Amide B (2900 cm^{-1})	2965.78 cm^{-1}	3069.80 cm^{-1}
Amide I (1600 cm^{-1} to 1700 cm^{-1})	1657.18 cm^{-1}	1631.72 cm^{-1}
Amide II (1550 cm^{-1} to 1600 cm^{-1})	1530.18 cm^{-1}	1518.95 cm^{-1}
Amide III (1200 cm^{-1} to 1350 cm^{-1})	1255.32 cm^{-1}	1237.41 cm^{-1}

4.4 UV-Visible spectroscopy (Fig.4)

The wavelength scanning between 200 nm to 400 nm shows a maximum peak at 230 nm and a negative peak at 204 nm for the triple helical collagen [25] .

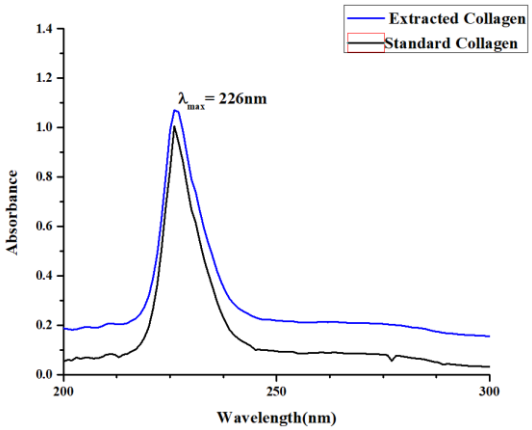


Fig.4 UV-Vis spectrum of isolated and standard collagen

Since tryptophan was not present in the collagen obtained from the Indian white perch tiger, it did not exhibit maximum protein absorption at 280 nm as is typical, but rather at 226 nm. It is mainly attributed to peptide bond absorptions by n-π^*

transitions f groups of C=O, COOH, and $CONH_2$ in the polypeptide chains of collagen. The results obtained are in comparison with the results obtained for Indian mackerel. It was observed that the collagen obtained from Indian mackerel exhibited a major band at 225 nm and also a small band at 205 nm. Similarly, the collagen obtained from carp also showed a major absorption band at 223 nm [26, 23, 24].

4.5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

In Fig. 5, ASC protein patterns from an SDS-PAGE study are displayed. There were no variations in patterns between the four species, and the SDS-PAGE pattern revealed that all ASCs were made up of two distinct chains, α1 and α2, as well as a β component. It was hypothesized that all ASCs were Type I collagens (consisting of two separate chains, a1 and a2, and the density of α1 is higher than α2) based on the electrophoretic pattern and mobility of collagens.

The extracted collagen in this investigation showed bands at 160.1 kD, 145.6 kD, and 140.3 kD, while the conventional collagen showed bands at 163.5 kD, 149.8 kD, and 132.6 kD. Similarly, the collagen obtained from Silver carp and shark skin also showed similar patterns and those following the rat tail tendon collagen (RTTC). A smeared banding pattern was observed for shark skin collagen in comparison with silver carp collagen, probably due to slight degradation of the protein caused due to multiple rounds of concentration from thickened shark skin with placoid scales. The banding pattern obtained for silver carp had β- chain, due to the inter and intra collagen molecular crosslinks that were left intact since the extraction protocol was acid soluble. Marine fishes have been reported to contain Type-I Collagen in Sea cucumber [27]. Indian Sardinella [26], Sailfish, [28], balloon fish [29], Catfish [30] and Catlafish [31].

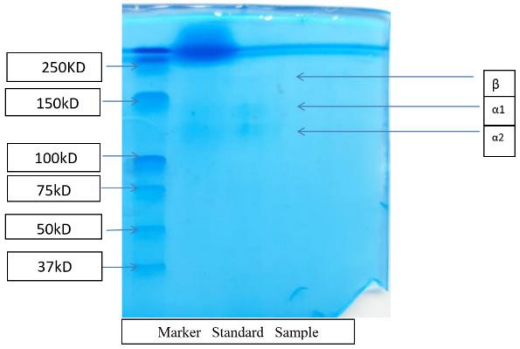


Fig.5 SDS–PAGE pattern of acid-soluble collagen from Indian white perch tiger

4.6 Effect of pH

The collagen extracted from the Indian white perch tiger was soluble in an acidic pH range from 3.0 to 5.0 with the highest solubility at pH 4.0, as compared to the results obtained for collagen from Tilapia fish and scales of Grey mullet. The decrease in solubility was observed at pH 7.0 (Fig. 6). This variation in the solubility of collagen is caused by the difference in the traits of the molecules and the conformations [3, 30].

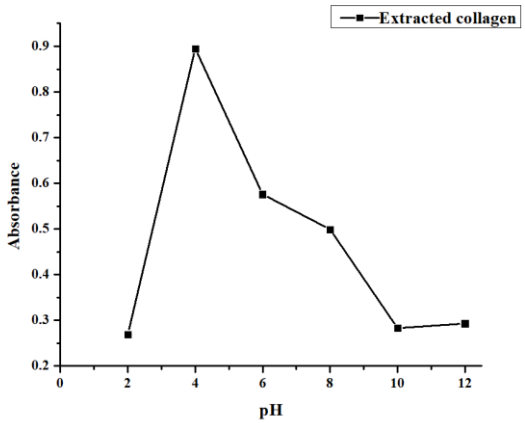
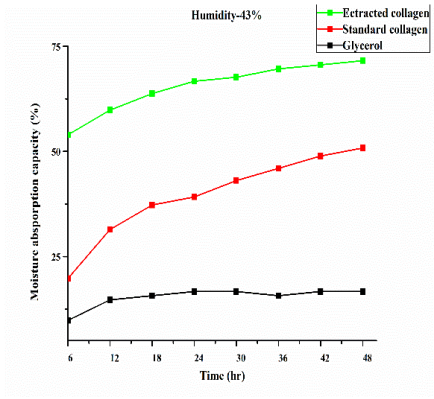


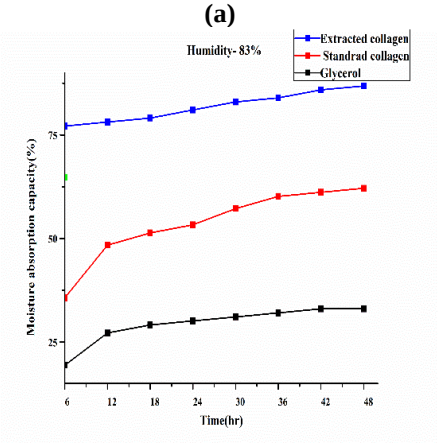
Fig.6 Graph showing the solubility of the collagen with variable pH

4.7 Moisture absorption and retention analysis for cosmetic application

The extracted collage along with standard marine collagen was compared with the reference glycerol for water absorption and water retention study.



(a)



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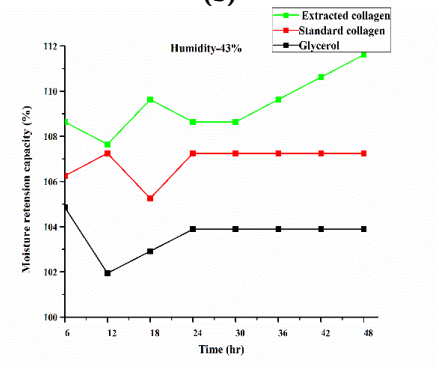
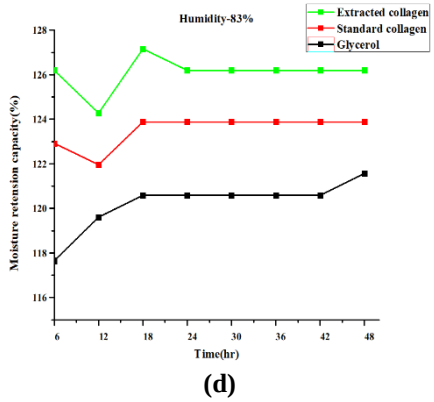


(c)



(d)

Fig.7(a) and (b) depict the water absorption capacities of collagens in comparison to glycerol at 43% and 83% relative humidity; Figures (c) and (d) depict the moisture retention capacity of collagens and glycerol

From the graph (Fig. 7a, b, c, d), it can be deduced that within 12 hours the water absorption of the collagen and glycerin increased sharply, and after 24 hours the increase in absorption was gradual. After 24 hours at 43% humidity, the moisture retention for glycerin remained constant, whereas extracted collagen gradually increased under the same circumstances. When compared to glycerin, the extracted collagen did not exhibit much difference in moisture retention at 83% humidity. According to the above findings. The presence of polar functional groups like carboxylic, hydroxyl, and guanamine groups and their capacity to form H-bonds with water may be responsible for collagen's improved results for moisture absorption and retention. According to Swatschek *et.al.*, [33] collagen cannot be absorbed by the skin and instead only remains on the skin's surface despite changes in the skin's moisture content. Therefore, collagen can be used as a functional ingredient in cosmetics [32, 33].

4.8. Limitations of the study

The main objective of the present study was to isolate and extract type-I collagen from the Indian white perch tiger, a marine fish species. However, the results obtained were not as anticipated. Instead of obtaining fibrillar collagen, the extracted collagen was found to be non-fibrillar. This was surprising considering that the source of collagen was a marine fish.

5. CONCLUSION

The collagen extracted from the Indian white perch tiger was found to be Type-I collagen with a yield of 0.52%. The FT-IR spectroscopy confirmed the presence of three amide bands A, B, and C. The surface morphology of the collagen was studied through a Scanning electron microscope. Through thermo gravimetric analysis, the thermal stability of the extracted collagen was studied and the SDS-PAGE analysis revealed the type of collagen present. Through all these confirmatory studies, it was concluded that the extracted collagen was found to be Type-1 and it was found to be linear with the similar studies carried out for different marine sources. Collagen's ability to absorb and

hold moisture was examined in this study, and it was discovered to be superior to the control substance, glycerol. Because of this, the collagen from Indian white perch tigers may be used as a moisturizer and may have applications in the cosmetic industry.

ACKNOWLEDGMENT

The authors acknowledge the University Instrumentation Centre, Mangalore University, as well as DST-PURSE, for providing the facilities for SEM, TGA, UV spectral analysis, and FT-IR analyses that were carried out in this study.

CONFLICT OF INTEREST

We do not have a direct financial relationship with the commercial identities mentioned in the manuscript which might lead to a conflict of interest.

FUNDING INFORMATION

No funding was received for this research work.

ETHICAL INFORMATION

This research does not involve studies on human subjects, human data, or tissues.

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