DOI: https://doi.org/10.36094/sc.v91.2025.Extraction_Characterization_and_Evaluation.Maity.191

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*Sci. and Cult.* 91 (3–4) : 191-200 (2025)

# EXTRACTION, CHARACTERIZATION AND EVALUATION OF ACID-SOLUBLE COLLAGEN FROM HILSA (*Tenualosa ilisha)* SCALES: AN APPROACH TOWARDS BIORESOURCE VALORIZATION

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*This study investigates acid-soluble collagen (ASC) extraction from Hilsa fish (Tenualosa ilisha) scales, an underutilized bioresource in South Asia. Scales showed high protein (24.84%) and ash (21.43%) content, suitable for collagen extraction. Characterized as type I, the collagen exhibited α1, α2, β, and γ chains (50-250 kDa) with glycine, proline, and lysine dominance. Ultraviolet spectrophotometer (UV) and Fourier Transform Infrared spectroscopy (FTIR) confirmed structural integrity, while antioxidant activity was demonstrated through 2,2-diphenyl-1-picryl-hydrazyl (DPPH) and 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS) assays. These findings highlight innovative waste valorization for fishery by-products.*

***Keywords:*** *Collagen Extraction, Tenualosa ilisha, Amino Acid, FTIR Spectroscopy, Biomedical Applications*

## *Introduction*

Collagen, an essential structural protein, has a crucial role in maintaining the integrity and functionality of connective tissues, including cartilage, bones, tendons, and skin[1,2]. Constituting 25–35% of an animal's total protein content[3], collagen's unique composition of amino acids forms robust, elastic triple-helical strands composed of three left-handed á-polypeptide chains[4,5]. Of the 28 identified collagen types, type I predominates and serves diverse applications in the food, biomedical, cosmetic, and pharmaceutical industries[6]. Its exceptional properties, including biocompatibility, bioactivity, and structural versatility, have made collagen a cornerstone material in tissue engineering, drug delivery systems, and regenerative medicine[7,8]. Despite its versatility, mammalian-derived collagen faces ethical and safety concerns, including the risks associated with zoonotic diseases like bovine spongiform encephalopathy (BSE) and avian influenza. As a safer and more sustainable alternative, fish-derived collagen has gained prominence, offering higher bioavailability, faster absorption, and fewer ethical constraints[9, 10]. Collagen can be found in abundance and in an environmentally acceptable manner in marine byproducts such fish bones, scales, and skin, aligning with sustainability initiatives like the European Commission's "Blue Growth" strategy[11]. The global seafood industry, generating over 20 million tons of by-products annually, provides an untapped resource for high-quality collagen extraction[12]. Fish collagen also exhibits unique biofunctional properties, including antioxidant and antimicrobial activities, which enhance its utility in wound healing and food preservation[13,14]. Furthermore, its

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application extends to antifungal drug development, demonstrating enhanced therapeutic potential[15]. With global fish production reaching 176 million tons in 2020, fish by-products— making up as much as 70% of the weight of the fish—offer a sustainable, high-value alternative to traditional feed and low-value uses[16,17]. Utilizing these by-products mitigates environmental impact and supports producing high-quality, pathogen-free collagen for various applications, marking a significant advancement in sustainable biomaterial development[18, 19].

The collagen monomer is a long, cylindrical protein measuring 2800 Å in length and approximately 14–15 Å in diameter. Its fundamental unit, tropocollagen, comprises three polypeptide chains coiled in a triple-helical configuration stabilized by interchain hydrogen bonds between-CO and-NH groups. This triple-helical architecture, characterized by one α2(I)-chain and two α1(II)-chains in type I collagen, exhibits distinct amino acid compositions and molecular masses of approximately 300,000 Da for the complete molecule and 100,000 Da for each chain[20]. Over 19 collagen types have been identified to date, with structural and functional variations contributing to their categorization. Collagens are broadly classified into fibril-forming (types I, II, and III), basement membrane-forming (type IV), and microfibrillar (types VI and VII) groups. Collagen type I, the most prevalent, forms strong fibrils and is predominant in connective tissues such as bones, tendons, and skin. On the other hand, type IV collagen disrupts the triple-helical conformation due to non-helical domains, making it integral to the basement membrane[21]. Cross-linking during collagen maturation enhances its tensile strength, a property critical for biological functions and industrial applications. Interestingly, fish collagen exhibits fewer cross-links than terrestrial vertebrates, resulting in unique solubility and mechanical properties suitable for diverse applications[22]. Its unique properties, such as forming fibroblast networks and protecting against pathogens, environmental pollutants, and malignant cells, make it an attractive candidate for regenerative medicine and cosmetics[23]. However, marine collagen's low denaturation temperature restricts its usage in biomedicine because of its lower proline and hydroxyproline concentration[24]. The scales of fish, often discarded as waste, are a rich source of type I collagen with unique structural and thermal properties. In experiments conducted on tilapia (*Oreochromis sp.*), collagen with high denaturation temperatures (57.9–79.0°C) has been associated to strong intra- and interchain linkages such hydrogen bonds and Van der Waals interactions. However, the fact that the collagen from carp (*Cyprinus carpio*) scales has a lower denaturation temperature (32°C) indicates that the stability of collagen varies by species. Additionally, collagen derived from fish scales has outstanding water absorption (13.3%) and retention qualities (15%), which makes it appropriate for use in medical procedures including tissue engineering and wound healing[25].

Mammalian collagen is extensively utilized, concerns related to disease transmission, religious restrictions, and ethical considerations have spurred the search for alternative sources[26]. Fish-derived collagen offers a sustainable and pathogen-free alternative, particularly from underutilized fishery by-products such as scales, skin, and bones. Gangetic fish species, including the commercially and culturally significant hilsa (*Tenualosa ilisha*), present an untapped potential for collagen extraction. Hilsa, renowned for its ecological and nutritional importance in India, particularly in states like West Bengal and Assam, generates considerable processing waste, with scales comprising 30–40% of the fish's body weight[27, 28]. Despite this abundance, hilsa scales remain underexplored as a collagen source, especially for high-value biomedical applications. The extraction process for fish collagen typically involves preparation, extraction, and recovery. Pre-treatment steps, including cleaning and chemical treatment with agents like sodium chloride (NaCl) and hydrogen peroxide ($H_2O_2$), effectively remove impurities and enhance collagen purity[26]. Extraction at low temperatures (around 4°C) is critical to prevent thermal denaturation and maintain structural integrity. Recovery steps, including salt precipitation, dialysis, and freeze-drying, yield collagen in a powder form suitable for various applications[29]. The solubility and functional properties of collagen are influenced by pH, salt concentration, and processing conditions, with optimal solubility observed in acidic environment[30].

This study investigates the extraction and characterization of collagen derived from hilsa scales, emphasizing its physicochemical and biofunctional properties. Given the increasing global demand for sustainable biomaterials, the findings aim to valorize hilsa processing waste, contributing to waste management and high-value material production. By exploring the bioactivity of hilsa collagen, this study highlights its potential for innovative applications in biomedicine, food technology, and environmental management, positioning *T. ilisha* as a model species for fish collagen research and sustainable biomaterial development.

## Materials and Methods

***Preparing Fish Scales:*** Scales from Hilsa fish were collected from the Ganga River and carefully cleansed with cold distilled water to remove any dirt or contaminants. The cleaned scales were then cut into small pieces using sterile scissors, placed in polyethylene bags, and stored at -20°C to maintain their biochemical integrity. The scales were kept in storage for no more than three months in order to avoid deterioration. The collagen content of the scales was retained for later extraction and examination thanks to this preservation method.

***Extracting Acid-Soluble Collagen (ASC) from Hilsa Scales:*** Demineralization and defatting of the Hilsa scales with a modified procedure based on Carolina et al.[31] was used to extract acid-soluble collagen (ASC). 200g of dried fish scale sample was dissolved in 0.1M NaOH solution at a 1:10(W/V) for 6 hours at room temperature. The supernatant was discarder and remaining was rinsed with detailed water until the pH reaches 7. This was exposed for three days at room temperature in a 0.5 M acetic acid ($CH_3COOH$) solution at a 1:10 ratio. Distilled water was used to wash the scales until the pH reached 4.6. The solution was filtered to separate the scales from the solution, which were later lyophilized. The dialyzed collagen solution was lyophilized using a freeze-dryer (Heto Maxi Dry Lyo Freeze-Dryer, Chennai, India) to obtain collagen in powdered form[32]. The lyophilized ASC was stored at –20°C for subsequent analyses.

***Acid-soluble Collagen (ASC) Yield After Extraction:*** The collagen yield was calculated using the methodology of Chauychyan et al.[18] and Alhana et al.[33]. Additionally, the yield was calculated using the formula below:

$$\text{Yield} = \frac{\text{Dry collagen weight}}{\text{Wet scale weight}} \times 100\%$$

***Estimated Scale Composition:*** Scale proximate analysis was carried out in accordance with accepted procedures[34]. Samples were dried at 102±2°C until their weight remained constant in order to measure the moisture content[35]. A LABQUEST BOROSIL KDL040 equipment was used to quantify the crude protein content, and a muffle furnace set to 600°C for four hours was used to measure the total amount of ash. A gadget called SCIENCE EQUIP CALCUTTA was used to measure the lipid content.

***Analysis of the Extracted ASC's pH***: The pH of ASC samples was measured with changes using a pH meter (Eutech pH 700), as suggested by Martínez-Ortíz et al.[36]. In 10.0 mL of distilled water, about 1.0 mg of samples were dissolved. Previously, the pH meter was calibrated using prepared buffer solutions at pH 4.0 and pH 7.0. Once calibrated, dissolved samples were measured and documented.

***Collagen Solubility at Different pH:*** The solubility of collagen was measured at different pH values using 0.1 M acetic acid. To get the final concentrations of mg/ml, collagen samples were dissolved in 0.1 M acetic acid and gently swirled at 4°C. 8 ml of collagen solution (3 mg/ml) was adjusted with either 6 M HCl or 6 M NaOH to achieve a final pH range of 1–10 in order to ascertain the impact of pH on collagen solubility. The final volume of the solution was diluted to 10 milliliters using distilled water. After 30 minutes of gentle stirring, the mixtures were centrifuged at 20,000g for 30 minutes at 4°C. The Bradford technique was used to measure the protein content of the supernatants using bovine serum albumin as the protein standard.

***The Amino Acid Composition of ASC:*** Collagen extracted from Hilsa fish scale was tested for amino acid content using the procedure described by Ishida et al.[37]. In conclusion, collagen samples were hydrolyzed for 24 hours at 110°C under anaerobic conditions using 6 M hydrochloric acid. Ultra-performance liquid chromatography (UPLC) was used to analyze the derivatives after the samples were hydrolyzed, neutralized with 6 N sodium hydroxide, filtered through a 0.22 µm nitrocellulose membrane, and then derivatized using an AccQ-Fluor Reagent kit (WAT052880, Waters). Using the FAO/WHO standard for essential amino acids in proteins, the essential amino acid (EAA) content of the collagen was determined in line with Pal et al.[38]. A thorough summary of the amino acid composition was given by the results, which were reported as grams of amino acids per 100 g of protein.

***The ASC Collagen Protein Pattern of Hilsa Scale is Analyzed Using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE):*** The collagen protein pattern that was extracted from the scales of Hilsa fish (*T. ilisha*) was examined using the methodology described by Laemmli[39] and Pal et al.[40]. The collagen samples were first dissolved in 0.1 mol/L acetic acid to reach the final concentration of 3 mg/mL. An equal volume of sample loading buffer (0.65 mmol/L Tris-HCl, pH 6.8, 2% SDS, 10% sucrose, and 5% α-mercaptoethanol) was added to the solubilized collagen. To ensure complete denaturation, the samples were heated to 95°C for three minutes. Further, the samples were resolved in SDS-PAGE as described by Laemmli[39].

***Analysis of ASC Collagen's UV Absorption Spectrum Using Hilsa Scales:*** In accordance with a modified procedure described by Duan et al.[2]. In summary, a UV-Vis spectrophotometer (CLARIOstar Plus multi-plate reader) was used to evaluate the ultraviolet absorption spectra of acid-soluble collagen (ASC). For spectral analysis, 10 mL of 0.5 M acetic acid was used to dissolve about 10 mg of collagen sample at a 1:1 (w/v) ratio. The resultant solution was then put in a Costar 96-well plate. Measurements were made using the 0.5 M acetic acid solution as a reference, and the spectrum was recorded throughout a wavelength range of 200–400 nm.

***FT-IR:*** Fourier Transform Infrared Spectroscopy (FT-IR) was used to study the secondary structure of the collagen isolated from the hilsa scales. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) (PerkinElmer, Spotlight200i) was used to record the FT-IR spectra of acid-soluble collagens (ASCs)[41]. About 1.0 mg of ASC samples were used for the analytical process. Spectral measurements were carried out in the range of 4000 to 600 $cm^{-1}$ with a resolution of 4 $cm^{-1}$ for 32 scans at 250°C. The spectrum obtained was analyzed using Spectrum Quant software.

***Assay for Antioxidant Activity:*** Antioxidant activity was measured using both the DPPH and ABTS assays[42]. For the ABTS test, 200 µL of ABTS solution and 100 µL of sample solution were added to the microplate wells. The microplates were then allowed to sit at room temperature for ten minutes. At 405 nm, the absorbance was measured. For the DPPH experiment, 96% ethanol was mixed with a 0.2 mM radical DPPH solution. At a wavelength of 540 nm, the absorbance of the DPPH solution was measured. After mixing 100 µL of the sample solution with 200 µL of DPPH, the mixture was allowed to stand for half an hour. At a wavelength of 540 nm, the mixture's absorbance was measured.

***Analysis of Statistics:*** Each experiment was conducted in triplicate using the same batch of samples, unless otherwise noted. For the results, the mean ± standard deviation (SD) was employed. To compare means, Tukey's Test and Analysis of Variance (ANOVA) were employed. If the p-value was less than 0.05, it was considered statistically significant.

## *Results and Discussion*

***Extraction Yield of Collagens from the Scales of Tenualosa ilisha:*** Based on the dry weight of the scales, the collagen yield from *Tenualosa ilisha* scales was determined to be 18% (w/w). Weight of freeze-dried collagen (g)/ weight of original dry scales (g) × 100 was the calculation used to calculate yield (%). This value highlights the efficiency of acid-soluble collagen extraction

**Table 1. Proximate composition of Hilsa Scale**

| Scintific name | Location/ River name | Parameters (% wet weight) | | | | |
|---|---|---|---|---|---|---|
| | | Moisture prtein | Crude | Crude Lipid | Total Ash | Total Carbohydrate |
| Hilsa (*Tenualosa Ilisha)* Scale | Farakka (Lower stretch of river Ganga) | 49.98±1.19 | 24.84±0.47 | 0.45±0.08 | 21.43±0.50 | 3.30±0.48 |

Note: All values are presented as mean ± standard deviation (N=3)

**Table 2. Amino acid composition of acid-soluble collagen extracted from *Tenualosa ilisha* scales (expressed in g/ 100 g).**

| Amino acid (g\100gsample) | Acid soluble collagen |
|---|---|
| **Essential** | |
| His | 0.059±0.04 |
| ILE | 0.057±0.05 |
| LEU | 0.126±0.01 |
| MET | 0.014±0.03 |
| PHE | 0.041±0.04 |
| THR | 0.379±0.05 |
| TYR | 0.007±0.02 |
| LYS | 0.737±0.03 |
| VAL | 0.184±0.01 |
| ARG | 0.030±0.01 |
| GLY | 0.297±0.02 |
| GLU | 0.257±0.01 |
| PRO | 0.061±0.04 |
| CYS | 0.011±0.02 |
| **Non-essential** | |
| SER | 0.034±0.03 |
| ASP | 0.150±0.01 |
| ALA | 0.139±0.05 |

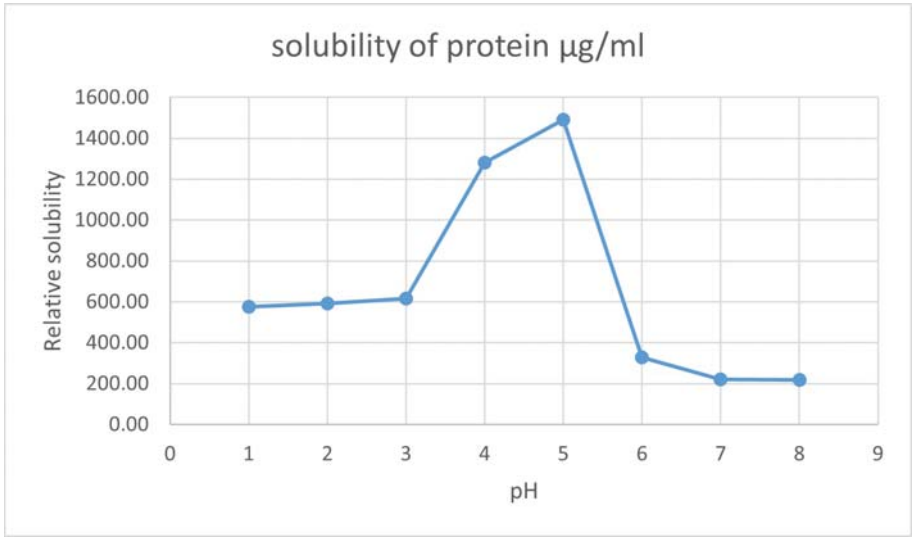


**Fig. 1:** pH-Dependent Solubility Profile of Acid-Soluble Collagen (ASC) Extracted from Hilsa (*Tenualosa ilisha)*

from hilsa scales compared to Indian carps like catla (*Catla catla*) and rohu (*Labeo rohita*), which exhibited lower yields when extracted with 0.5 mol $L^{-1}$ acetic acid alone[40]. Like other fish species, the yield of ASC from hilsa scales is influenced by various factors, including species-specific biological conditions, collagen structure, and extraction methodologies[40, 7]. The results emphasize the critical role of enzymatic digestion in improving collagen recovery, highlighting its potential for sustainable collagen production and its suitability for biomedical and industrial applications.

***Proximate Composition of Tenualosa ilisha Scales:*** The proximate composition of the Hilsa scales revealed a high moisture content (49.98 ± 1.19%), substantial crude protein (24.84 ± 0.47%), moderate total ash (21.43 ± 0.50%), low crude lipid (0.45 ± 0.08%), and total carbohydrate (3.30 ± 0.48%) (Table 1). These findings suggest the potential of Hilsa scales as a collagen source due to their high protein content, a key determinant for efficient collagen extraction and its biomedical and industrial applications. The significant protein content aligns with earlier studies on fish scales, including those of silver carp (*Hypophthalmichthys molitrix*)[43] and spotted golden goatfish (*Parupeneus heptacanthus*)[44], where scales demonstrated high protein

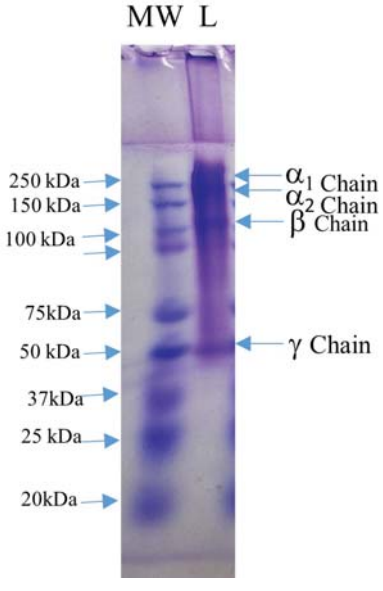


**Fig. 2:** SDS-PAGE Analysis of Collagen Extracted from Hilsa (*Tenualosa ilisha)* Scales

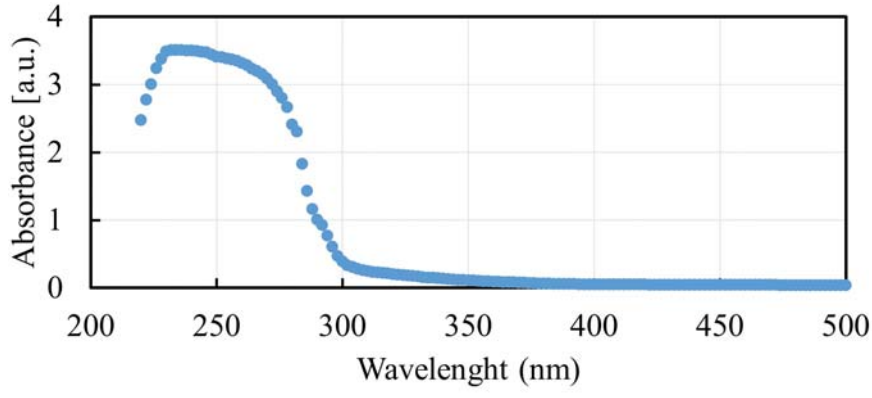


**Fig. 3:** UV-Visible Absorption Spectrum of Acid-Soluble Collagen Extracted from Hilsa Scales showed a distinct peak at 232 nm, corresponding to the triple-helical structure and functional groups of collagen, and a secondary peak at 280 nm, to aromatic amino acids.

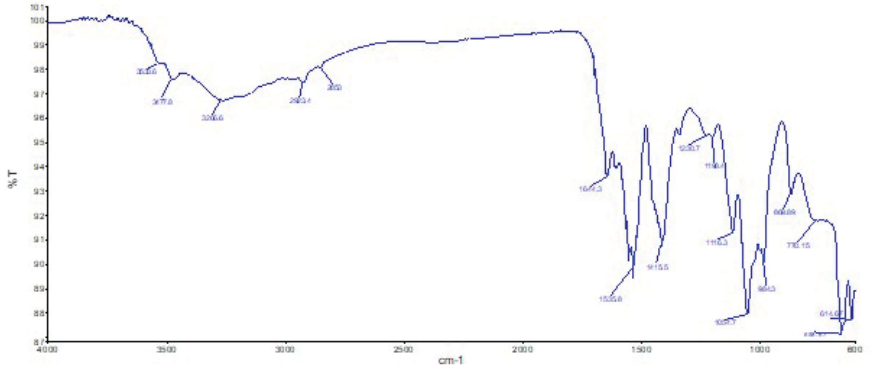


**Fig. 4:** FTIR Spectra of Acid-Soluble Collagen (ASC) from Gangetic Hilsa Scales

**Table 3. Peak Locations and Functional Group Assignments in the Fourier-Transform Infrared (FT-IR) Spectra of Collagen Extracted from *Tenualosa ilisha* Scales**

| Peak Assignment | Wavenumber ($cm^{-1}$) | Functional Group/ Structural Insight | Comments |
|---|---|---|---|
| **Amide A** | 3477.8, 3266.6 | N-H stretching frequency | Indicates the presence of protein compounds in collagen. |
| **Amide B** | 2923.4 | Asymmetric stretching of CH, vibration | Confirms hydrocarbon structure integrity in the collagen molecule. |
| **Amide I** | 1644.3 | C=O stretching, associated with the secondary structure of peptides and hydrogen bonding within the triple helix. | Sensitive marker of peptide secondary structure; confirms the presence of collagen triple helix. |
| **Amide II** | 1535.8 | N-H bending and C-N stretching | Supports the triple-helical conformation of collagen molecules. |
| **Amide III** | 1230.7, 1198.4 | Combination of C-N stretching and N-H bending | Identifies the triple-helical structure of collagen, characteristic of type I collagen integrity. |

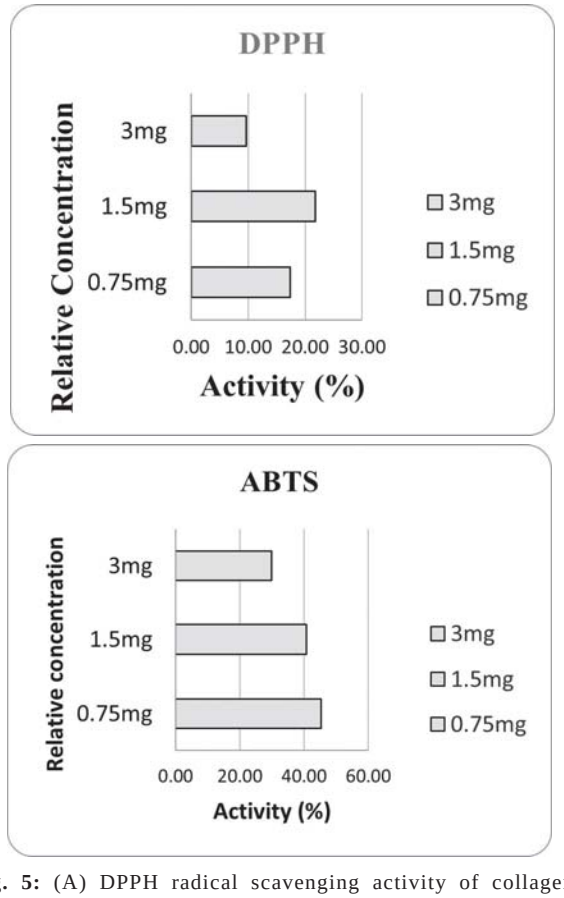


**Fig. 5:** (A) DPPH radical scavenging activity of collagen at concentrations of 0.75, 1.5, and 3 mg, showing dose-dependent activity with a peak of 21.80% at 1.5 mg. (B) ABTS radical scavenging activity, demonstrating superior activity at lower concentrations, with a maximum of 45.37% at 0.75 mg.

concentrations suitable for collagen isolation. The low lipid and carbohydrate contents further favour the extraction process by minimizing lipid oxidation and non-collagenous interference, as supported by author[44]. Ash content (21.43 ± 0.50%) indicates the presence of minerals that might influence collagen extraction. The results resonate with Mahboob's et al.[45] findings, highlighting that the mineral matrix of fish scales contributes to the structural integrity of collagen fibrils.

***Collagen Solubility at Different pH:*** The solubility profile of acid-soluble collagen extracted from Hilsa demonstrated a pronounced dependence on pH, with the highest solubility observed in the acidic range. ASC exhibited peak solubility values at pH 1 (576.2 mg/ml), pH 2 (590.73 mg/ml), and pH 3 (617.50 mg/ml). Solubility significantly declined at neutral and alkaline pH, reaching minimal values at pH 7 (219.69 mg/ml) and pH 8 (216.23 mg/ml) (Fig. 1). These findings align with earlier reports[46], who observed optimal solubility of collagen at acidic pH (1–4) and diminished solubility near the isoelectric point (pI). The high solubility of ASC in acidic conditions can be attributed to increased electrostatic repulsion between positively charged collagen molecules, which disrupts intrinsic molecular stability and prevents aggregation[46]. This behaviour is consistent with findings[47], who also noted enhanced solubility of collagen extracted using acidic methods from various fish species. In contrast, the reduced solubility at neutral and alkaline pH is linked to the net neutral charge at the pI, leading to diminished electrostatic repulsion. Consequently, hydrophobic interactions and van der Waals forces dominate, promoting molecular aggregation and reducing solubility, as reported by Mezzenga and Fischer[48]. The distinct solubility behaviour of ASC further highlights the efficiency of the acid extraction method, which preserves high molecular weight proteins and promotes enhanced solubility compared to enzymatically extracted collagen[47]. These findings corroborate earlier studies, including Singh et al.[49], who observed similar solubility patterns in collagen derived from striped catfish (*Pangasianodon*

*hypophthalmus*). Our study underscores the pH-sensitive nature of collagen solubility and reinforces the applicability of ASC from Hilsa as a promising biomaterial for various biochemical and pharmaceutical applications.

The solubility of ASC demonstrated a pronounced dependence on pH, with peak values observed in the acidic range: pH 1 (576.2 mg/ml), pH 2 (590.73 mg/ml), and pH 3 (617.50 mg/ml). Solubility declined sharply at neutral and alkaline pH, reaching minimal values at pH 7 (219.69 mg/ml) and pH 8 (216.23 mg/ml). Acidic conditions enhanced electrostatic repulsion, disrupting molecular aggregation, while neutral pH promoted hydrophobic interactions, reducing solubility.

***Collagens from T. ilisha Scales were Analyzed Using SDS-PAGE:*** SDS-PAGE analysis of collagen extracted from the scales of Hilsa revealed distinct electrophoretic patterns indicative of type I collagen. Consistent with the structural makeup of type I collagen, the study found two primary α components (α1 and α2) as well as β and γ chains. The α1 band exhibited greater density than the α2 band, a characteristic also reported in other fish species, such as spotted golden goatfish (*Parupeneus heptacanthus*) and Indian carps[44, 45]. The molecular weight of Hilsa collagen ranged from 50 to 220 kDa, aligning with the findings for other fish-derived collagens (Figure 2). This range reflects the integrity of the collagen and correlates with its high thermal stability, as Lin and Liu[50] supported. The observed patterns reaffirm the structural fidelity of Hilsa collagen and its suitability for applications requiring robust thermal stability and mechanical properties. These findings align with previous studies on fish-derived collagens[44,45], emphasizing the robustness, thermal stability, and suitability of Hilsa collagen as a sustainable biomaterial.

***Composition of Extracted Collagen's Amino Acids:*** The amino acid makeup of Hilsa scale-derived ASC revealed a profile indicative of type I collagen, with significant implications for its biomedical and industrial applications. For the first time, the amino acid contents of Hilsa collagen have been disclosed, underscoring its uniqueness and potential. Among essential amino acids, lysine (0.737±0.03 g/100g) and threonine (0.379±0.05 g/100g) were prominent, contributing to collagen's structural integrity and cross-linking abilities (Table 2). Glycine (0.297±0.02 g/100g) was the most abundant amino acid, a hallmark of collagen, as previously reported for carp collagens by Duan et al.[7]. Glycine's repetitive occurrence facilitates the triple-helix structure, crucial for collagen's mechanical stability. Proline (0.061±0.04 g/100g), along with glycine, plays a critical role in collagen's thermal stability by forming pyrrolidine rings that enhance the rigidity of the helix structure, consistent with findings by Sinthusamran et al.[51] and Pal et al.[40]. A minor amount of cysteine (0.011±0.02 g/100g) was detected, further confirming the type I collagen nature of the extracted protein, as observed in catla and rohu scales by Pati et al.[52]. Non-essential amino acids, including alanine (0.139±0.05 g/100g) and aspartic acid (0.150±0.01 g/100g), were also notable, contributing to the overall stability and functionality of the collagen. This comprehensive amino acid profiling highlights the distinctive properties of Hilsa collagen, marking it as a sustainable and valuable resource for biomedical and industrial applications while complementing findings on other fish species[52, 53]. These findings confirm the high quality and functional potential of Hilsa collagen as a biomaterial, further reinforcing its utility in diverse applications.

***UV Absorption Spectrum:*** In this work, Collagen extracted from Hilsa fish was tested for absorption characteristics using UV-visible spectrophotometry. One of the primary and easiest ways to analyze collagen is to scan the sample from 210 to 400 nm since the triple-helical structure of collagen has a maximum absorbance peak at roughly 230 nm[40]. The present study's extracted collagen's UV absorption spectra confirmed the presence of collagen's distinctive structural properties with a significant absorbance peak at 232 nm and a smaller secondary peak at 280 nm (Figure 3). Collagen's distinct molecular structure includes C=O, COOH, and CONH2 groups in its polypeptide chains, which are responsible for the absorbance at 220–240 nm[47]. Tryptophan, tyrosine, and phenylalanine are aromatic amino acids found in collagen molecules, which are probably the cause of the absorbance peak at 280 nm[54]. With a maximal absorption near 220 nm, acetic acid had no effect on the collagen's spectral characteristics. The absorbance of 0.5 mol per liter acetic acid, which was used as a baseline correction in this study, had no effect on the spectra of collagen. These findings are consistent with previous studies, where collagen showed a maximum absorbance near 230 nm, indicating the robustness of the extraction and characterization methods[40]. Overall, the UV-Visible spectroscopic analysis confirmed the successful collagen extraction from Gangetic Hilsa fish, with spectral features characteristic of native collagen, demonstrating its potential for various biomedical and industrial applications.

***FTIR Spectroscopy Analysis of Acid-Soluble Collagen (ASC):*** The FTIR spectra of ASC extracted from Hilsa scales revealed characteristic absorption peaks indicative of type I collagen, underscoring the structural integrity and functional groups essential for biomedical

applications. The prominent amide A peak at 3477.8 $cm^{-1}$ and 3266.6 $cm^{-1}$, corresponding to N-H stretching vibrations, confirmed the presence of protein compounds, aligning with findings by Chen et al.[55] and Matmaroh et al.[44] (Fig. 4). This peak shift towards higher wavenumbers indicates significant hydrogen bonding, an essential collagen molecular structure attribute. The amide B peak observed at 2923.4 $cm^{-1}$ represents asymmetric CH, stretching, which reflects the presence of collagen's molecular backbone, corroborating the structural insights reported by Pal and Suresh[56]. The amide I peak at 1644.3 $cm^{-1}$, attributed to C=O stretching, highlighted the preservation of secondary structure and triple-helix stability, which are essential for the functional applications of collagen, as noted by Abedin et al.[57]. Amide II and amide III peaks at 1535.8 $cm^{-1}$ and 1230.7 $cm^{-1}$, respectively, provide further confirmation of collagen's structural fidelity, particularly its intermolecular hydrogen bonding and triple-helical integrity, as similarly reported by Matmaroh et al.[44] (Fig. 4, Table 3). These findings confirm that ASC from Hilsa possesses a stable triple-helical structure, akin to commercial collagen, with significant biomedical implications. Compared to other aquatic sources, the slightly higher wavenumbers of amide II and III in ASC suggest enhanced molecular stability and structural robustness, which are advantageous for tissue engineering applications[56]. This study marks the first comprehensive FTIR characterization of ASC from Gangetic Hilsa, complementing earlier investigations on fish-derived collagen[44,56]. These results underscore the potential of Hilsa collagen as a sustainable and high-quality source for biomedical and industrial applications, particularly in tissue scaffolding and drug delivery systems.

The FTIR spectra of ASC extracted from Gangetic Hilsa scales exhibit characteristic peaks indicative of type I collagen: amide A (3477.8 $cm^{-1}$, 3266.6 $cm^{-1}$), confirming N-H stretching vibrations and hydrogen bonding; amide B (2923.4 $cm^{-1}$), reflecting CH, asymmetric stretching; amide I (1644.3 $cm^{-1}$), attributed to C=O stretching; and amide II (1535.8 $cm^{-1}$) and III(1230.7 $cm^{-1}$), confirming triple-helical integrity.

***Antioxidant Activity:*** The antioxidant activity of collagen extracted from Hilsa scales was assessed using DPPH (2,2-diphenyl-1-picryl-hydrazyl) and ABTS (2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate) radical scavenging assays. These assays demonstrated the potential of collagen as a natural antioxidant, aligning with prior studies on marine-derived collagen[53, 58]. In the DPPH assay, the radical scavenging activity increased with collagen concentration, reaching 21.8% at 1.5 mg and 17.41% at 0.75 mg, indicating dose-dependent activity. The lowest antioxidant activity (9.63%) was observed at 3 mg (Fig. 5A). The result comparable to the reported on collagen derived from silvertip shark cartilage (*Carcharhinus albimarginatus*)[53]. This demonstrates the ability of Hilsa collagen to stabilize free radicals, reducing oxidative stress. The ABTS assay further corroborated these findings, with scavenging activity significantly enhanced at lower concentrations. At 0.75 mg, the activity peaked at 45.37%, followed by 40.67% at 1.5 mg and 29.93% at 3 mg (Fig. 5B) These results surpass previously reported values for pepsin-solubilized collagen from *Stichopus japonicus*[58], highlighting the high radical scavenging potential of Hilsa collagen. The data validate the efficacy of Hilsa collagen as a functional biomaterial with robust antioxidant properties. These properties likely stem from the presence of bioactive peptides generated during extraction, which enhance free radical scavenging, as suggested[59]. These results validate the collagen's robust antioxidant potential, comparable to or exceeding marine-derived collagens[53, 58]. Thus, Hilsa collagen emerges as a promising candidate for biomedical and industrial applications, contributing to sustainable and eco-friendly resource utilization.

## *Conclusion*

This study successfully extracted and characterized acid-soluble collagen (ASC) from the scales of Hilsa, providing a sustainable solution for bioresource valorization. The extraction process yielded a significant collagen content (18% w/w), demonstrating high efficiency and showcasing the potential of hilsa scales as an underutilized yet valuable resource. Characterization studies confirmed the collagen as type I, evidenced by SDS-PAGE, UV-Vis spectrophotometry, and FTIR spectroscopy, which highlighted its structural integrity, functional groups, and secondary structure. The collagen displayed optimal solubility at acidic pH and retained excellent purity, stability, and antioxidant properties, as demonstrated by its free radical scavenging activity in DPPH and ABTS assays. These findings underscore the biomedical potential of hilsa-derived collagen, particularly in tissue engineering, wound healing, and cosmetic applications, while also revealing promising avenues for its integration into food, packaging, and other industrial sectors. By valorizing fishery by-products, this research not only contributes to sustainable development goals but also promotes eco-friendly waste management, emphasizing the broader significance of aquatic bioresources in advancing scientific and industrial innovation.

## Acknowledgments

The authors express their sincere gratitude to the Ministry of Jal Shakti, Government of India, for funding this research under the National mission for clean Ganga initiative, specifically the "Fish Conservation and Stock Enhancement of Fishery of Ganga River Basin" project (Project Code: Ad-35012/1/2023-NMCG-NMCG). ❐

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