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Extraction of acid soluble collagen from *Coryphaenahippurus*(Mahimahi) and its application in tissue engineering

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ABSTRACT

Collagen is a structural protein in the extracellular matrix which provides mechanical support and rigidity to the tissues in the skin. Many extraction techniquesprevail for the extraction of collagen of from Fish skin having a wide availability and largely discarded as waste byproduct. Also, skin are the major sites of fibroblasts cells that aid in tissue injury and they have excellent wound healing capacities. Therefore, Current study focuses on extraction of collagen from the skin of *Coryphaenahippurus*(Mahimahi) fish by acid assisted method. After extraction, the sample was characterized using different spectral methods such as UV, FTIR, SEM, TGA, DSC, MALDI and SDS-PAGE. UV peaks at 270 – 280 nm confirmed the protein peaks of collagen in the sample. TGA analysis showed the poor thermal stability of the collagen. ATR analysis confirmed the presence of functional groups of amide present in the sample. MALDI TOF protein sequencing showed the presence of collagen in the sample. SDS PAGE analysis showed the presence of bands of type I α1, α2 and β chains. Fibril assay and staining using safranin and van gieson dyes showed the fibrillar structure of the collagen under microscope. Hydroxyproline analysis was done to detect the presence of hydroxyproline, the major amino acid in collagen and estimated to be 0.62 µg/mg of sample. After conformation of presence of collagen, wound scratch assays was carried for checking its wound healing applications as well as MTT assays was carried out to estimate its cytotoxicity level. At a higher concentration of 100 µg/ml, the sample showed 88.36% of migration rate and93.2% of cell viability against S3T6 cell line. In conclusion, we extracted thermostable, bioactive, non-toxic variety of collagen from the *Coryphaenahippurus*(Mahimahi) fish.

KEY WORDS:Wound healing, Mahi-Mahi, Scaffold, Acid soluble collagen (ASC), Hydroxyproline(HP).

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I. INTRODUCTION

The solid waste management of marine and aquatic species is a big challenge due to their eco-balance threats.[1-2] This is due to the fact that these solid wastes especially from those of fish farms affects different ecosystem and can result in the modification of food web chains.[3]These wastes can be developed into useful products of therapeutic importance[4]. Some of the examples includes the processing of fish skins and scales into animal feed and extraction of oil from fish skins,[5] development of biodegradable ink from squid wastes, development of chitosan as a result of shrimp waste processing.[6]

Similar to the development of therapeutically important products from that of the solid wastes of marine and aquatic species, one which is very much important and also critical in the field of medicine due to its varied applications is the extraction of collagen from fish waste.[7]Collagen finds application in various industries such as pharmaceutical, medical, cosmetic and food.[8]Pharmaceutical industries develop collagen in the form of biomaterials such as pills due to its bioavailability and weak antigenicity properties.[9]Medical industries make use of collagen in tissue engineering due to its wound healing properties.[10]There have been increased studies on biodegradable elastomers with its application in the medical industry for tissue engineering, bio-imaging and drug delivery. Biodegradable elastomers can be efficient in treating wounds and burn in patients and can also be used in invasive treatments as they are biocompatible.[11] Collagen's anti-ageing properties paves its applications towards the cosmetic industries[12] and also in the food industry as food additives.[13-14]

Collagen is the principal structural protein in animal bodies.[15] The long chains of amino acids wound to form a triple helix structure which provides mechanical strength and elasticity. Hydroxyproline is the major amino acid present in the protein structure of collagen. The abundance of hydroxyproline is comparatively lesser in fish sources than that of bovine and other sources. This is due to the decreased availability of nascent oxygen in seawater.

Poultry sources from the bovine tail and porcine skins are widely used for the extraction of collagen. Tendons from tails of mammals such as rat[16] are also other sources. Marine sources of collagen are mostly from fishes such as sharks, leather jacket, catfish, etc., and are extracted from bones, fins, tails, scales and skins. Sources from poultry animals can pose a threat after the outbreak ofBovine spongiform encephalopathy (BSE) and hence are made to choose alternative sources.[17]

Marine collagen has unique applications on wound healing[18] due to their properties such as moisture prevention and loss of heat from wounded tissue as well as providing microbial infiltration barrier.[19]Collagen scaffolds are also developed for the treatment of diabetic foot ulcer. A polycaprolactone-collagen scaffold loaded with simvastatin is used in treating diabetic foot ulcers as they are potential candidates in skin regeneration and also an efficient drug

delivery system.[20] Sources of collagen from cartilaginous fishes produce significant collagen yields than those of Chondrichthyes—which includes sharks, skates, and rays[21]. Skin from fish is chosen, owing to their availability since it contains elastin and is thicker than non-cartilaginous types[22]. In the present study, the fish species *Coryphaenahippurus* has been chosen for the extraction of collagen from its skin.

II. MATERIALS AND METHODS

Raw materials and Reagents

Fresh skin and scales from the fish species *Coryphaenahippurus(MahiMahi)* was purchased from the local market located at the coastal areas of KovalamBeach, Chennai. Sodium hydroxide, Sodium Lauryl Sulphate, Acrylamide, Coomassie Brilliant Blue G 250, Bis-Acrylamide, HCl, Glycine, Sucrose, Bromophenol Blue, Methanol, Methyl Cellosolve, Sodium Phosphate Monobasic Anhydrous, Disodium Phosphate, Hydrochloric Acid were purchased from SRL chemicals, India. Acetic acid was procuredfrom Rankem chemicals, India. Sodium chloride, TEMED, Citric Acid Monohydrate, Sodium Acetate Trihydrate, PDAB, Chloramine T trihydride (AR), Safranin was obtained from HIMEDIA chemicals, India. Tris(hydroxymethyl) amino methane(AR) was gained from SDFine Chem Limited, India.Calf Skin Collagen (C3511), L – Hydroxyproline, Ammonium Per sulphate and Van Gieson were bought from SIGMA-ALDRICH, USA.

Pretreatment and Extraction of collagen

Extraction of collagen from fish was carried out as reported by Muralidharan *et al*, 2013.[23] Briefly, skin was peeled and washed using deionized water and cut into small pieces. Fish samples were washed using 0.8 M saline solution for 10 min and samples were incubated in an alkali solution (Sodium hydroxide) for a period 4 days. After alkali treatment, samples are thoroughly washed and homogenized at 2000 RPM at 4°C. After homogenization, acetic acid extraction method was used to extract Type I Collagen. The sample was subjected to acid digestion for about 3 days and then centrifuged. The supernatant was collected and it was precipitated by salting out technique. The precipitate was collected and centrifuged after 24 hours. After centrifugation, the pellet containing collagen was purified by dialysis with an acid (acetic acid) and stored at 4°C.

Fibril assay - Staining and Visualization by Van Gieson and Safranin

Purified acid-soluble collagen (ASC) was air dried and dyed with Van Gieson and Safranin stains respectively. Under the compound microscope with 40X magnification, the stained samples were observed and the collagen structure was examined.

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Characterization Methods:

Using UV spectroscopy, the diluted samples from dialysis were subjected to absorption spectra for identification of protein peaks (Shimadzu UV-2401).To evaluate molecular weight of ASC, the resulting bands have been compared with normal bands. Maximum reflectance Attenuated Total Reflection - Fourier Transform Infrared (ATR-FTIR) spectroscopy analysis (Thermo-electron scientific, Madison, USA) within 400-4000cm-1 range was used to obtain (Fourier Transform Infrared) FTIR spectra of purified ASC.The processed ASC sample was air-dried and observed under Scanning Electron Microscopy (SEM - TESCAN, Czech Republic). Under SEM, the morphology of the collagen fibrous structures has also been observed.The characterization approach offers important structural insights into the origin of collagen fibrils and their organization. Purified ASC was thoroughly dried on the (Matrix-Assisted Laser DesorptionIonization)MALDI plate in ambient condition. Once the matrix had cooled, the (Matrix-Assisted Laser DesorptionIonizationTime of Flight Mass Spectrometry) MALDI TOF MS analysis was carried out in linear positive mode on a Micro Flex LRF Mass Spectrometer (Bruker). SDS-PAGE was performed on the extracted ASC. Test Sample (100 µl) was mounted on polyacrylamide gel (Sodium dodecyl sulphate–polyacrylamide gel electrophoresis)with 8% gel-resolving and 5% stacking gel. Upon electrophoresis, staining and de-staining were performed using Coomassie Brilliant Blue R-250 and a de-staining reagent.

Thermal Stability of Collagen

TGA Analysis

Thermogravimetric Analysis (TGA) is used to analyze the change in weight of a sample due to temperature or heating time. Collagen usually has a very poor thermal stability and is subjective to protein denaturation. Hence the protein sample extracted from *Coryphaenahippurus* (Mahimahi) was subjected to Thermogravimetric Analysis (Model Q50V20.6, USA) to evaluate the thermal stability of the sample.

Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) was performed to check the thermal stability of collagen. The collagen samples were blotted into aluminum pans and subjected to thermal denaturation. The investigation was done with a DSC instrument of model no Q200V23.10, USA and the resulting peaks will provide the optimum denaturation temperature of the collagen sample extracted from *Coryphaenahippurus* (Mahimahi).

Estimation of Hydroxyproline

Hydroxyproline is the major amino acid present in collagen and can be quantified using colorimetric assays. The procedure was carried according to the modified method of Woessner (1961)[24]. To a 1 ml of sample, 1 ml of cupric sulphate (0.05M), 1 ml of NaOH (2N) and finally 1 ml of hydrogen peroxide (6%) were added. The resulting reaction mixture was vigorously shaken and was incubated in a water bath at 80°C for 5 minutes. The solution was then cooled to room

temperature and added with 4 ml of sulphuric acid (1.5N) followed by 2 ml of propanol containing 5 % of p-dimethylaminobenzaldehyde. The final solution was vigorously shaken and kept in water bath at 80°C for 30 minutes. Absorbance reading was measured at 560 nm and reaction mixture with water in place of the sample was taken as blank. L – Hydroxyproline was used as the standard reference.

Turbidity Assay

IST based standard turbidity assays protocol was used to measure the turbidity of the free collagen present in the sample. It was stated that collagen possess self-assembly mechanism which can be proven by directly measuring the turbidity of the sample. 0.2 N of NaOH and phosphate buffer at pH 7.2 are mixed at a ratio of 2:1 and equal amounts of collagen sample (1mg/ml) are mixed along. Relative Turbidity of the sample was constantly monitored at 313 nm at rate of 6 sec/reading. The entire reaction was carried out in a controlled environment with an average temperature of 37°C±2°C (Optimum temperature)

Wound Scratch Assay

Invitro studies of a wound healing is assessed by performing a Scratch assay**.** Fibroblast cell lines were cultured on a 96 well plate and subjected to a scratch or an opening to create a wound on its monolayer.Varying concentration of collagen (say 10 µg, 25 µg, 50 µg and 100 µg) were added to the monolayer and were incubated for 24 hrs at 37 °C with 5% humid CO_2 Incubator. Images were captured at the beginning and at regular intervals during cell migration and time taken to close the wound. The images were compared and the percentage of migration were quantified.

Cell viability

Cell viability and cell cytotoxicity studies were performed using MTT assay. MTT assay was performed on the S3T6 cell lines using collagen samples. The S3T6 cells lines were assayed over a period of 14 days. The cell viabilitywas estimated at 540 nm.

III. RESULTS AND DISCUSSION

Staining and Visualization by Van Gieson and Safranin

The structure of acid soluble collagen fibers was analyzed using Van Gieson and Safranin stains. Van Gieson staining is usually used to stain connective tissues especially by differentiating collagen from that of other connective tissues. Once the staining was proceeded and visualized under a microscope, the elastic fibers of the collagen and its fibrillar protein structure were visualized and is highlighted as shown in the Figure 1A & 1B. The type-II collagen fibers were visualized using Safranin stain as it collectively stains thefibers of type-II collagen. Safranin stain being a cationic dye visualizes the proteoglycan and glycosaminoglycans which plays a major part in the matrix of the collagen fiber. The protein fibrillar structure of type-II collagen were visualized and highlighted as shown in the Figure 1A, 1B. Thus, both the Van Gieson and Safranin staining were useful in preliminary analysis by visualization of collagen fibers due to their selectivity towards the elastic fibers of collagen.[26]

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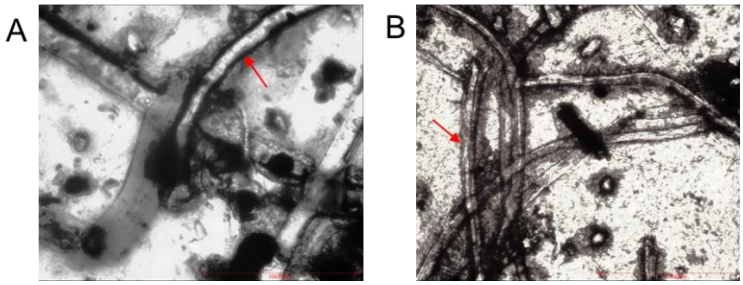

Fig 1A Van Gieson staining

Fig 1B Safranin staining

UV Visible Spectroscopy

The UV-Vis spectral absorption of protein is generally seen at 280nm.[27] According to the spectral results of the acid soluble collagen extracted from *Coryphaenahippurus*, peaks are seen at the range of 270nm to 280nm. Thus, in accordance with the protein spectral analysis, presence of collagen in the sample is confirmed with its peak at 277nm. A shoulder peak nearly at 279nm and 280nm shows that the collagen present in the sample is pure and does not contain any other moieties (Figure 2).

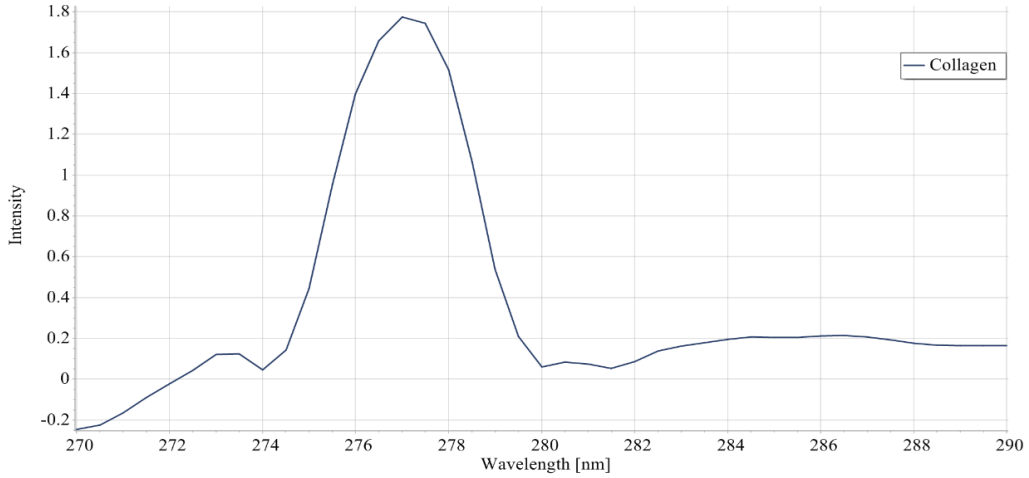

Fig 2UV Visible absorption spectrum of the ASC extracted from *Coryphaenahippurus*

FT-IR Spectroscopy: Attenuated Total Reflection (ATR) Mode

FT-IR Spectral analysis by ATR mode will assist in identification of the functional groups present in the sample. Based on the range of absorbance, ATR is used to detect the functional group present in the sample. Since collagen is a protein sample, amide is the major functional group present. Various types of amide groups are classified according to its range of absorbance (Figure 3). Both peaks 1 and 2 present at range of 2900 cm^{-1} and 2800 cm^{-1} are mainly due to NH and CH_2 stretching of the type A amide bonds present in the sample. Similarly peaks 3 and 4 at the range of 2300 cm^{-1} and 1650 cm^{-1} are due to the C-C bond and NH bending coupled with CN stretch of the type I amide bond present in the Collagen sample.

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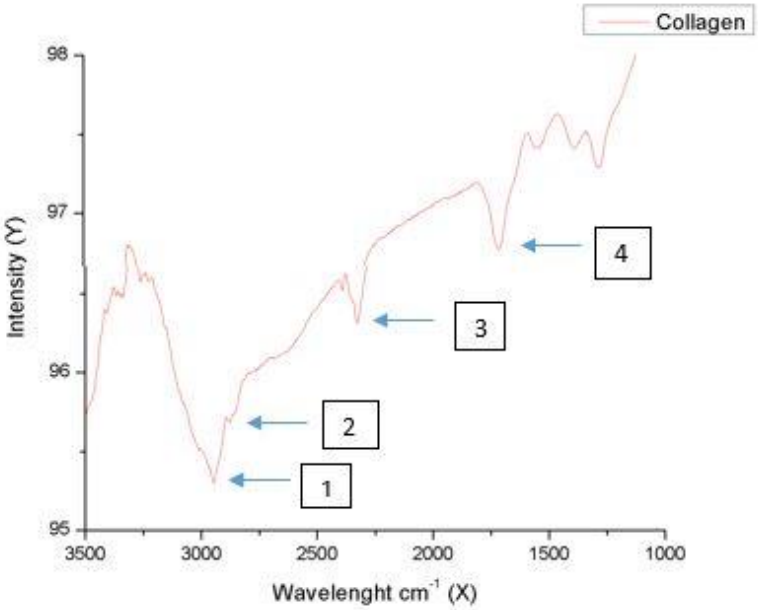

Fig 3 FTIR - ATR spectrum of the ASC extracted from *Coryphaenahippurus*

Scanning Electron Microscopy

The acid soluble collagen when visualized in Scanning Electron Microscopy revealed collagen fibrils in tubular structure and is complex and multi-layered. However, some of the structures had mild irregularity in their geometry. The porosity and tubular fibrous structure revealed by SEM proves the structural integrity of the collagen sample.Similar fibrous, tubular and porous structures were also seen in *Sphyrnalewini*[28] and *Evenchelysmacrura*[29], on the contrary, *Ctenopharyngodonidella*[30] and *Scallop mantle*[31] revealed regular uniform network.

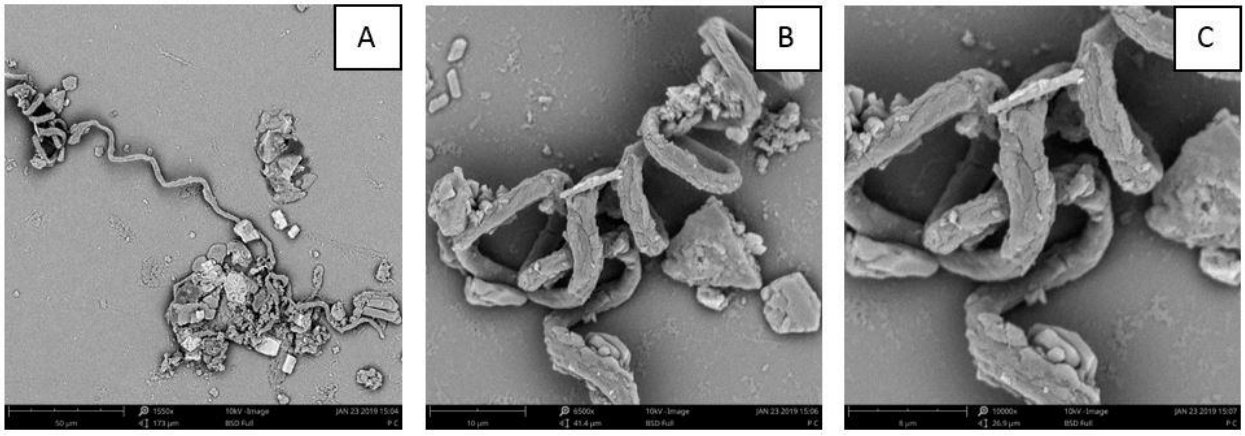

Fig 4 SEM images of the ASC extracted from *Coryphaenahippurus*Fig 4A – 1550X, Fig 4B – 6500X, Fig 4C – 10000X

MALDI – TOF Mass Spectroscopy

Acid soluble Collagen sample extracted from *Coryphaenahippurus*was subjected to MALDI – TOF Mass spectroscopy and the result was depicted in the figure 5. From the mass spectrum, distinctive monoisotropic peaks of the amino acid peptides were clearly visible. 441.283,

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482.362, 656.109, 688.075, 861.164 and 893.113 were the major m/z peaks present in the sample. Hence the overall molecular weight of the sample was found out to be 89.3 kDa. The three intense peaks 441.283, 656.109 and 861.164 can be due to the α1, α2 and β sub units of the sample.

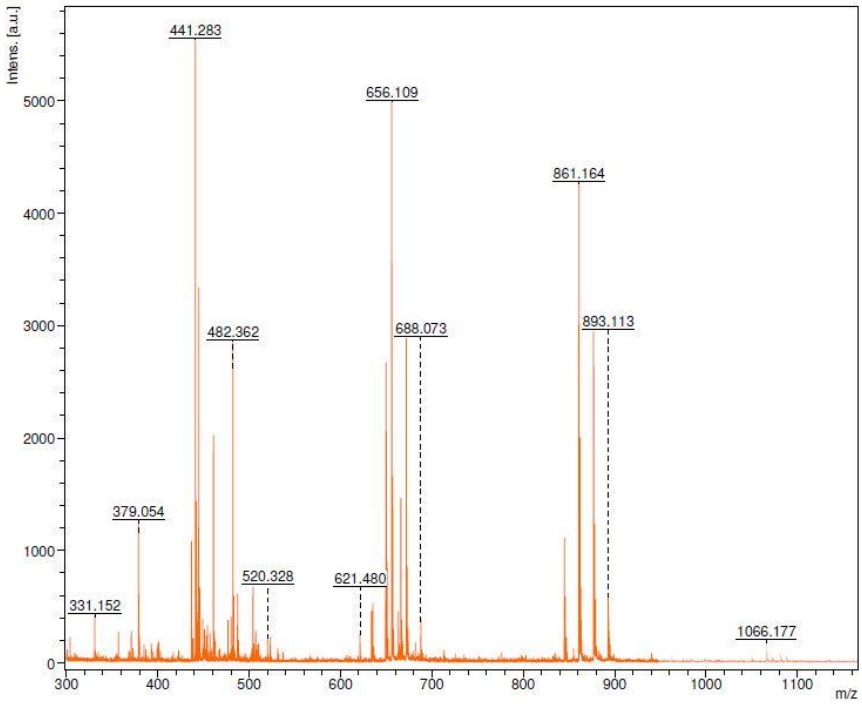

Fig 5 Mass spectrum of the ASC extracted from *Coryphaenahippurus*

TGAAnalysis

Collagen has a very poor thermal stability. It denatures even at room temperature. Hence from the graph it was inferred that denaturation starts much before 81.21° C which confers to its poor thermal stability. According to fig 6, the denaturation temperatures of the sample started at 81.21° C where 90.53% of the sample started to gradually degrade. A steep deflation was seen at the curve stating the poor thermal stability of the sample. After a while, only 3.499% of the sample remains depicting that 97% of the protein sample got completely denatured at 182.80° C and leaving behind 3.011% of residue which is approximately 1.22 mg by weight. The graph usually takes a bend at the end if there is presence of any aromatic compounds. But it remains linear till the end showing there was no aromatic impurities present in the sample.

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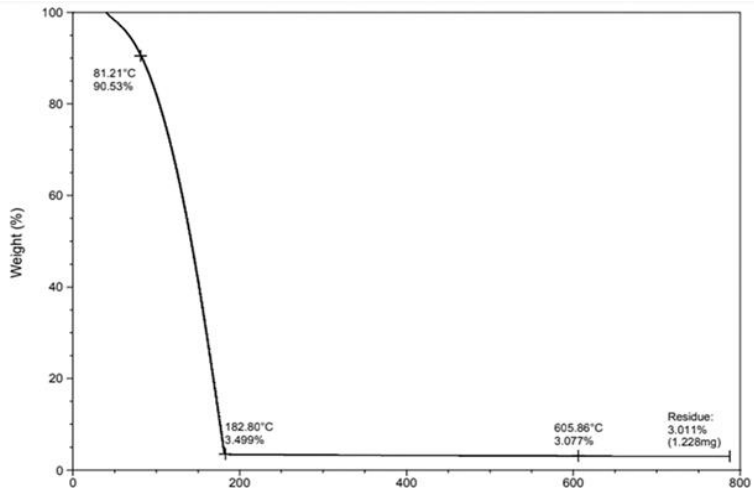

Fig 6 TGA graph of the ASC extracted from *Coryphaenahippurus*

Differential scanning calorimetry (DSC) Thermogram

Differential scanning calorimetry (DSC) can be used as an effective tool for identifying the denaturation temperature (T_d) of the collagen sample. The T_d was found out to 54.2°C for Acid soluble Collagen sample extracted from *Coryphaenahippurus* stating that the currently reported collagen generally poses good thermal stability in comparison with the previous report literatures. The enthalpy value (ΔH) for ASC was 0.61 J/g.

SDS PAGE:

According to the figure 7, there pertains a clear visibility of bands which infers that the sample contains Polypeptides from cytoplasmic proteins or membrane-associated but extrinsic to a membrane. Such proteins, tend to form well resolved narrow bands with sharp edges which defines those of collagen. And also, clear visibility of the migration of bands shows that there was no impurities present in the sample. With reference to a standard SDS gel corresponding to that of a fish collagen sample, we infer that our sample contains type I α1, α2 and β chains.

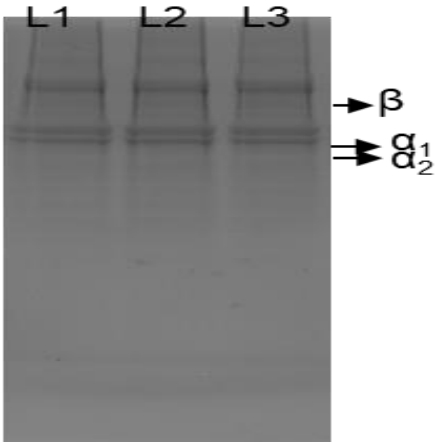

Fig 7 SDS PAGE image of the ASC (triplicates) extracted from *Coryphaenahippurus*

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Estimation of Hydroxyproline content

Hydroxyproline content present in the acid soluble collagen extracted from *Coryphaenahippurus* was estimated using modified Woessner method. Figure 8 shows the absorbance of theextracted sample collagen with that of standard Hydroxyproline. From the graph it was clear, that the sample contain proficient amount of hydroxyproline as its major content a characteristic feature of collagen. Collagen sample from *Coryphaenahippurus*contains 0.62 mg/ 1mg of sample.

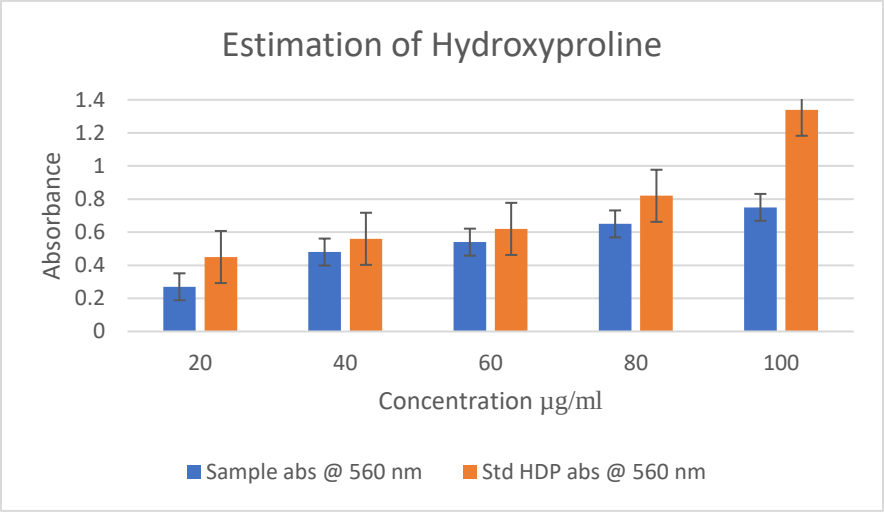

Fig 8 Estimation of Hydroxyproline content in the ASC extracted from *Coryphaenahippurus*

Turbidity Assay

Turbidity is considered as the direct measure of self-assembly nature of collagen. Gelation of collagen was closely monitored using spectrophotometer by measuring the absorbance at constant time intervals (fig 9). A sharp peak (Increase in absorbance) was seen at time interval of 50 seconds, stating that the time required for the self-assembly was 50 sec. A straight line preceding the sharp peak proves that the self-assembly gelation is constant and does not undergoes any denaturation even after a time period of 10 minutes.

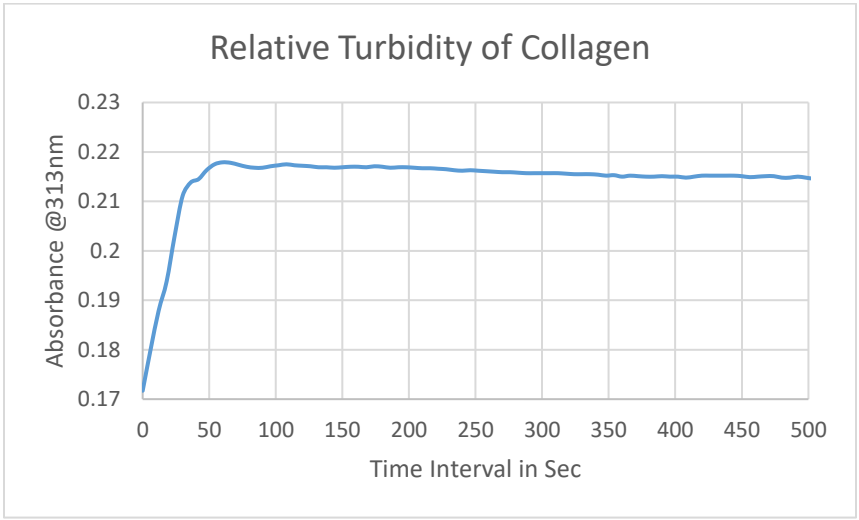

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Fig 9 Estimation of relative turbidity of the ASC extracted from *Coryphaenahippurus*

Wound Scratch Assay

Wound Scratch Assay is used to estimate the wound healing ability of a compound of interest. Most of the collagen isolated from nature sources were extensively studied for their use in tissue engineering. Acid soluble Collagen extracted from fore skin of the skin *Coryphaenahippurus* was also subjected to wound healing assay and the results were shown in the figure 10 (Before Incubation period) and figure 11 (After Incubation period). From the figure it was clearly evident that the collagen extracted from *Coryphaenahippurus* poses very good wound healing ability and the same can be used for various tissue engineering applications including skin graft, ointment for fire burn and as a Bio-band aid. After the incubation period of 24 hrs, the collagen sample with a concentration of 100 µg/ml showed highest percentage of reformation i.e 88.36% (fig 12) proving the wound healing activity of collagen extracted from *Coryphaenahippurus.*

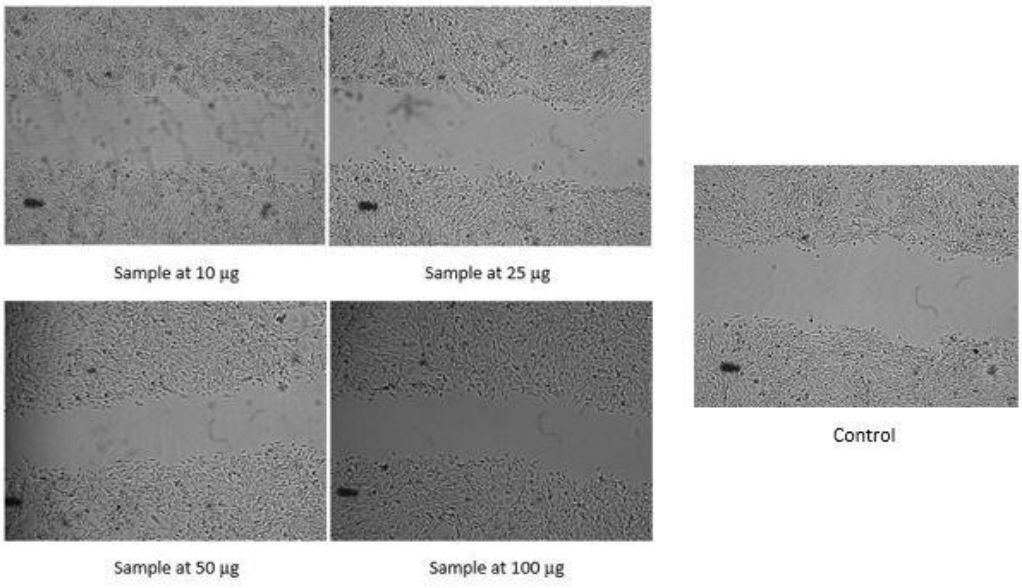

Fig 10 Scratch Assay of the ASC extracted from *Coryphaenahippurus*before the incubation period of 24 hrs

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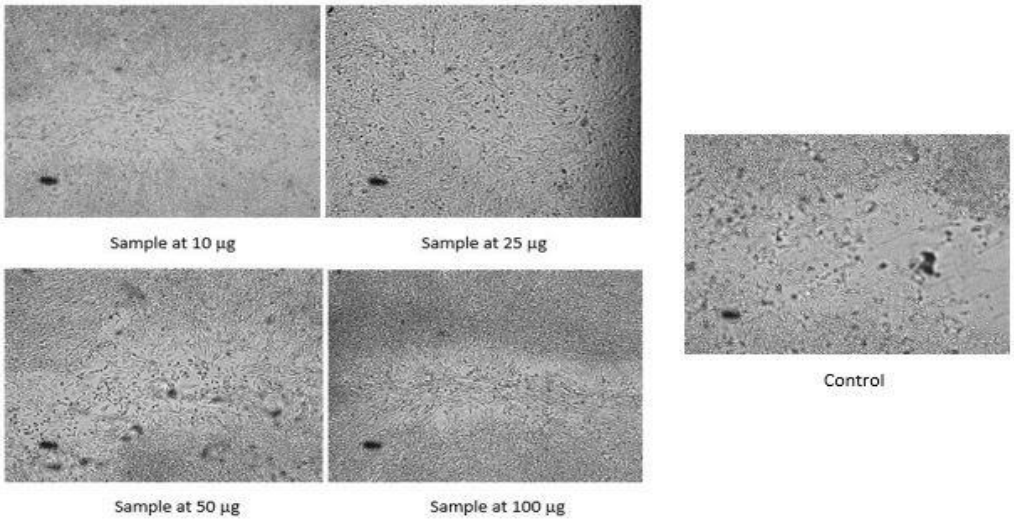

Fig 11 Scratch Assay of the ASC extracted from *Coryphaenahippurus*after the incubation period of 24 hrs

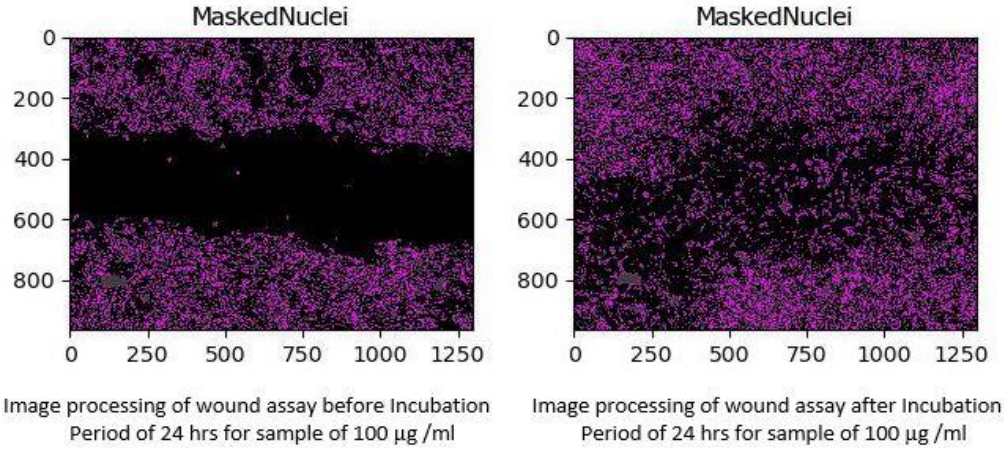

Fig 12 Image processing result of Wound Scratch Assay of the ASC extracted from *Coryphaenahippurus*

Cell viability

Finally the cell cytotoxicity of the collagen sample extracted from the skin of the fish *Coryphaenahippurus*was evaluated using MTT assay and the results were depicted in the figure 13. From the graph it was clear even at a higher concentration of 100 µg/ml, the sample does not show any kind of toxicity towards S3T6 cell line. About 93.20 % of cell remains intact after 24 hrs of time interval from the inoculation of sample.

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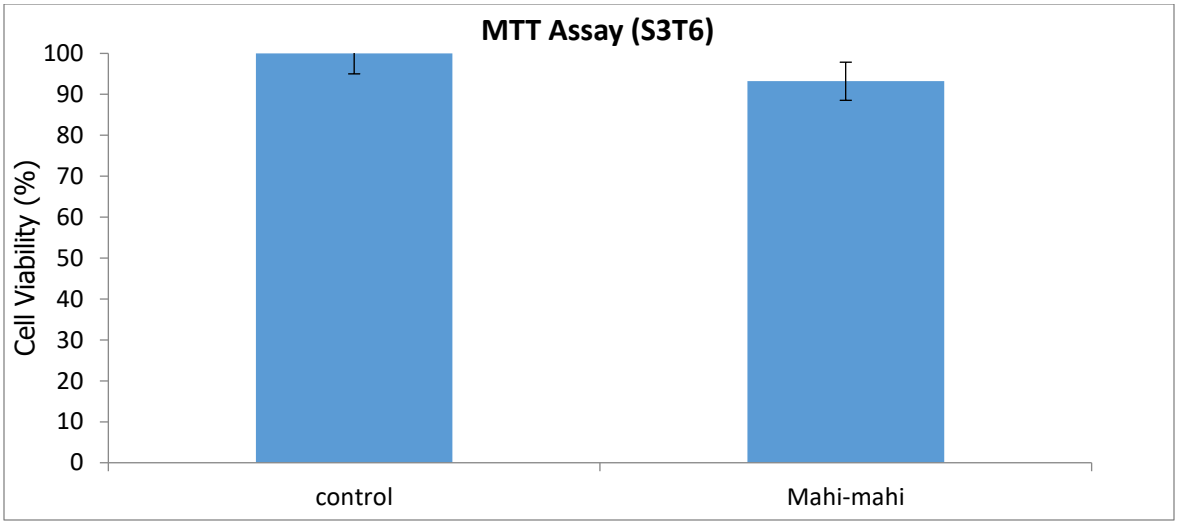

Fig 13 MTT Assay of the ASC extracted from *Coryphaenahippurus*

IV. Conclusion

Current study focused on extraction of thermo-stable, bio-active, Non-toxic variety of collagen from marine waste such as fish skin. Fore skin from the fish *Coryphaenahippurus*was used as a novel source for extraction of Collagen. Acid Soluble Collagen (ASC) was extracted using acetic acid and the same was characterized with UV, FTIR, SEM, MALDI-TOF, TGA and DSC. UV, FTIR and MALDI-TOF showed significant peaks which are the characteristic signals for the presence of collagen in the sample. SEM revealed the complex nature of the fibular protein and the same was confirmed by a fibril assay. Results of TGA and DCS depicted the thermo-stable nature of the current collagen sample. SDS PAGE results of the collagen sample posturized the alpha (α1, α2) and beta (β) chains of the globular protein structure. While with the turbidity assay, self-assembly nature of the protein was proved, Hydroxyproline content was estimated to be 0.62 µg/mg of the sample. Finally, wound healing assay and MTT assay were carried out and percentage of migration and Cell viability at a concentration of 100 µg of sample was found out to be 88.36% and 93.20% respectively. With all the result of extensive and a comprehensive study, it was concluded fish waste especially skin from species such as *Coryphaenahippurus*can be a great source for the extraction of Collagen with versatile Tissue engineering applications.

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