STUDIES ON BIOCHEMICAL ANALYSIS OF FISH WASTE AND ITS APPLICATIONS

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SCIENCE AND TECHNOLOGY

BY

Miss. PRANOTI NAGESH KIRDAT
M. Sc.

UNDER THE GUIDANCE OF

Dr. (Mrs.) PADMA B. DANDGE
M.Sc. Ph.D.

ASSOCIATE PROFESSOR
DEPARTMENT OF BIOCHEMISTRY,
SHIVAJI UNIVERSITY, KOLHAPUR,
MAHARASHTRA, INDIA

2022

Department of Biochemistry
Shivaji University, Kolhapur

CERTIFICATE

This is to certify that the thesis entitled **"Studies On Biochemical Analysis Of Fish Waste And Its Applications,"** is being submitted here with for the award of the Degree of Doctor of philosophy in Biochemistry under the faculty of Science and Technology, Shivaji University, Kolhapur. The work reported in this thesis is based upon the results of original experimental work carried out by **Miss. Pranoti Nagesh Kirdat** under my supervision and guidance. To the best of my knowledge and belief the work embodied in this thesis has not formed earlier the basis for the award of any Degree or Diploma or similar title of this or any other university or examining body.

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Place: Kolhapur

Date:

Prof.K.D.Sonawane
Professor & Head
Department of Biochemistry,
Shivaji University, Kolhapur

Dr. (Mrs.) Padma B. Dandge
Research Guide
Department of Biochemistry
Shivaji University, Kolhapur

Department of Biochemistry
Shivaji University, Kolhapur

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ABBERATIONS

Symbol	Long form
%	Percentage
nm	Nanometre
ml	Millimetre
Mg	Microgram
Meq	Miliequivalent
kD	Kilo Dalton
Sec	Second
kPa	Kilopascal
eV	Electron volt
d.nm	Diameter in nanometre
r.nm	Radius in nanometre
mV	Millivolt
ASC	Acid soluble Collagen
ASE	Acid soluble extract
Conc.	Concentration
LA	Lactic acid
FA	Formic acid
OA	Oxalic acid
AA	Acetic acid
PA	Phosphoric acid
cP	Centipoise
FTU	Formazin turbidity unit
HyP	Hydroxyproline
IC	Inhibition concentration
w/w	Weight by weight ratio
w/v	Weight volume ratio
D/W	Distilled water
v/v	Volume by volume ratio
m/z	Mass to charge ratio

gm	Gram
Kg	Kilogram
m	Molar
mg	Milligram
µm	Microgram
mm	Millimetre
hr	Hour
sec	Seconds
mM	Millimolar
A	Absorbance
µl	Microliter
Min.	Minutes
No.	Number
N	Normal
RT	Room temperature
SD	Standard deviation
bp	Base pair
TNF	Tumor necrosis factor
IL	Interleukin
OFAT	One factor at a time method
pH	Hydrogen ion concentration
PUFA	Poly unsaturated fatty acid
SDS	Sodium dodecyl sulphate
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
NCCS	National centre for cell science

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1.Introduction

Fish is multipurpose and diverse commodity covering a wide domain of world. Fish is an intensive source of protein, micronutrients and numerous essential fatty acids, so it play distinct role by providing advantageous and nutritious contribution to variegated and healthy diet. The management policy and implementation actively impact on the global extent of fish production including both aquaculture and capture fisheries. Dissimilarity in fish utilization, processing and consumption within and between continents, regions and countries also influence on fish productivity. From last several years, problem due to fishery waste has increased and becoming a worldwide concern. It causes negative impact on some biological, technical and operational components as well as socio-economic factors. About 50% of fish tissues including tail, fins, head and viscera are discarded as 'waste'. The amount of waste produced through processing depends upon nature of species used, fishing areas and the product formed (Caruso 2015). The fish industry engenders remarkable quantity of waste at several phases of manufacturing. Majority of fish processing industries discard such waste in rigid mode, by incineration, disposal in sea or by landfilling manner. According to recent scenario, these conventional practices are affecting aquatic and terrestrial ecosystem with drastic environmental issues.

The fisheries and aquaculture sector contributes significantly to food security and nutrition, particularly in some of the world's most food-limited regions, while concurrently supporting lifestyle of millions of people throughout the world. Global capture fisheries production in 2018 reached up to 96.4 million tonnes, an increase of 5.4% from the average of earlier three years. Marine captured fish production was increased from 81.2 million tonnes in 2017 to 84.4 million tonnes in 2018, but less than in the year 1996 (86.4 million tonnes). Further, in 2018 global fish production was reached about 179 million tonnes of which 82 million tonnes derived from aquaculture production (table no.1.1 and figure no.1.1). Aquaculture accounted for 46% of the total production and 52% of fish for human consumption (FAO 2020).

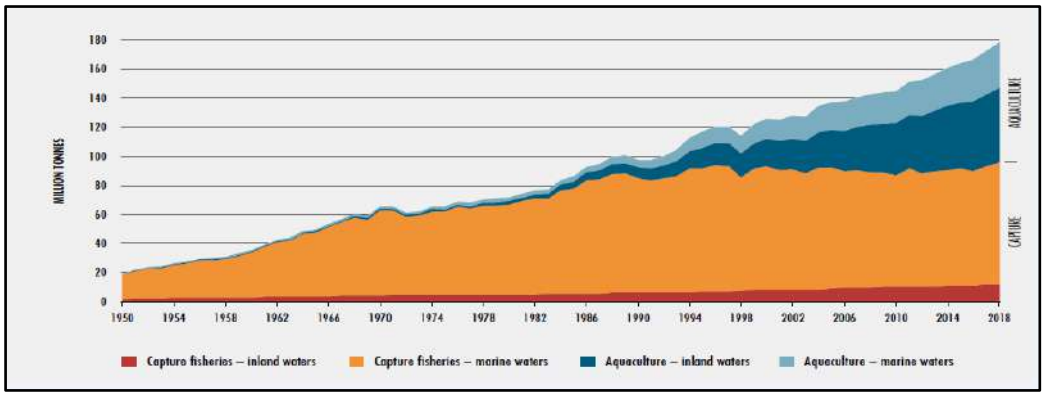

Figure 1.1.: World capture fisheries and aquaculture production (Source: FAO 2020)

Capture	1986–1995	1996–2005	2006–2015	2016	2017	2018	2019
Production(Million tonnes)							
Inland	6.4	8.3	10.6	11.4	11.9	12.0	12.08
Marine	80.5	83.0	79.3	78.3	81.2	84.4	80.40
Total capture	86.9	91.4	89.8	89.6	93.1	96.4	92.48
Aquaculture							
Inland	8.6	19.8	36.8	48.0	49.6	51.3	53.3
Marine	6.3	14.4	22.8	28.5	30.0	30.8	34.6
Total aquaculture	14.9	34.2	59.7	76.5	79.5	82.1	87.9
Total world fisheries and aquaculture	101.8	125.6	149.5	166.1	172.7	178.5	180.38
(Source: FAO 2020)							

Table 1.1.: Summary of worldwide aquaculture and fisheries production

Fish and fishery products remain most important food commodities in the world. In 2018, 67 million tonnes, or 38% of total fisheries and aquaculture production, were traded internationally (figure no.1.2). Of the whole total, 156 million tonnes were used

for human consumption, equivalent to an estimated annual supply of 20.5 kg per capita. The remaining 22 million tonnes were intended for non-food uses, generally to produce fishmeal and fish oil. Global food fish consumption increased at an average annual rate of 3.1% from 1961 to 2017. Per capita food fish consumption grew from 9.0 kg (live weight equivalent) in 1961 to 20.5 kg in 2018, by about 1.5% per year (table no.1.2). The top 20 producing countries accounted for about 74% of the total capture fisheries production. China has continued a major fish producer, accounting for 35% of overall fish production in 2018. Excluding China, a substantial share of production in 2018 came from Asia (34%), followed by the Americas (14%), Europe (10 %), Africa (7 %) and Oceania (1%). A growing share of fishmeal and fish oil, estimated at 25–35%, is produced from by-products of fish processing, which previously were often discarded or used as direct feed, in silage or in fertilizers (FAO 2020).

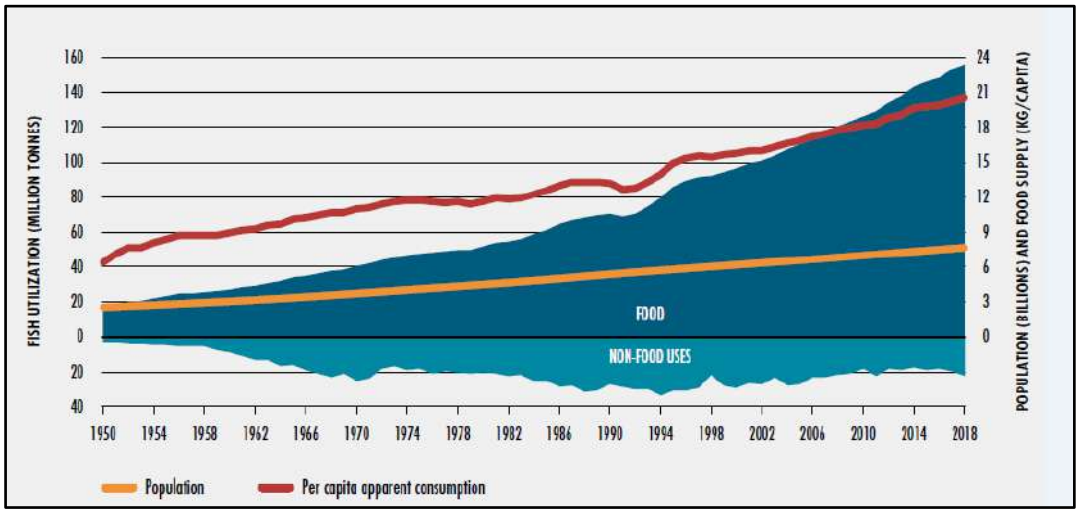

Figure 1.2.: World fish utilization and apparent consumption

(Source: FAO 2020)

	1986–1995	1996–2005	2006–2015	2016	2017	2018
Production (Million tonnes)						
Human consumption	71.8	98.5	129.2	148.2	152.9	156.4
Non-food uses	29.9	27.1	20.3	17.9	19.7	22.2
Population (billions)	5.4	6.2	7.0	7.5	7.5	7.6

Per-capita apparent consumption (kg)	13.4	15.9	18.4	19.9	20.3	20.5

Table 1.2.: Summary of worldwide fish utilization and consumption (Source: FAO 2020)

Excluding aquatic plants and other aquatic animals, the worldwide production of fish, crustaceans and molluscs was 177.8 million tonnes in 2019 and it was decreased by 1% than 2018. So, sum of capture production accounted for 92.5 million tonnes in 2019, a decrease of 4.3% compared with the earlier year. Aquaculture production was 85.3 million tonnes in 2019, an increase in 2018 up to 3.7 %. The involvement of aquaculture in total production of aquatic animals from capture and aquaculture collectively has grown gradually 39.9 to 48.0 % from year 2010 to 2019. The decrease in catches causes reduction in capture fisheries up to 80.4 million tonnes in 2019 which is less than 5% as compared to 2018. Global capture fisheries production in inland waters in 2019 reached the highest levels ever recorded at 12.1 million tonnes and it is continued. Its share in total global capture production remains around 13 %. Also, rise in catches can partially be attributed to enhanced reporting and assessment at the country level rather than entirely due to increased production (OECD/FAO 2019).

The majority of fishes are utilized to produce food products for human consumption and this share will lead to increase at 91% (178 million tonnes) by 2028. This represents in general increase of 16% compared to the average for 2016-18. The entire quantity of fish produced at the world level is estimated to be 196.3 million tonnes by 2028, an increase of 14% relative to the base period (average of 2016-18) and an additional 24.1 million tonnes of fish with seafood in absolute terms. Overall fish production increased at about 171 million tonnes in 2016, with aquaculture representing 47 % of the total and 53 % for non-food uses (including reduction to fishmeal and fish oil). The quantity of fish produced at the global level is projected to continue growing (1.1% p.a.), but at a slower rate than observed over previous decade (2.4% p.a.). Altogether, Asia is predicted to consume 71% (or 126 Mt), of the total food fish, while lowest quantities will be consumed in Oceania and Latin America (figure no.1.3). The proportion of fish meal being produced from waste is projected to increase from 25% in 2018 to 31% by 2028, while for fish oil it is projected to increase from 35% to 40%. Climate change, weather

variability and changes in the frequency and extent of extreme weather events are anticipated to have a major impact on availability, trade of fish and fish products mainly through habitat destruction, changes in fish migration patterns and natural productivity of fish stocks (OECD/FAO 2019).

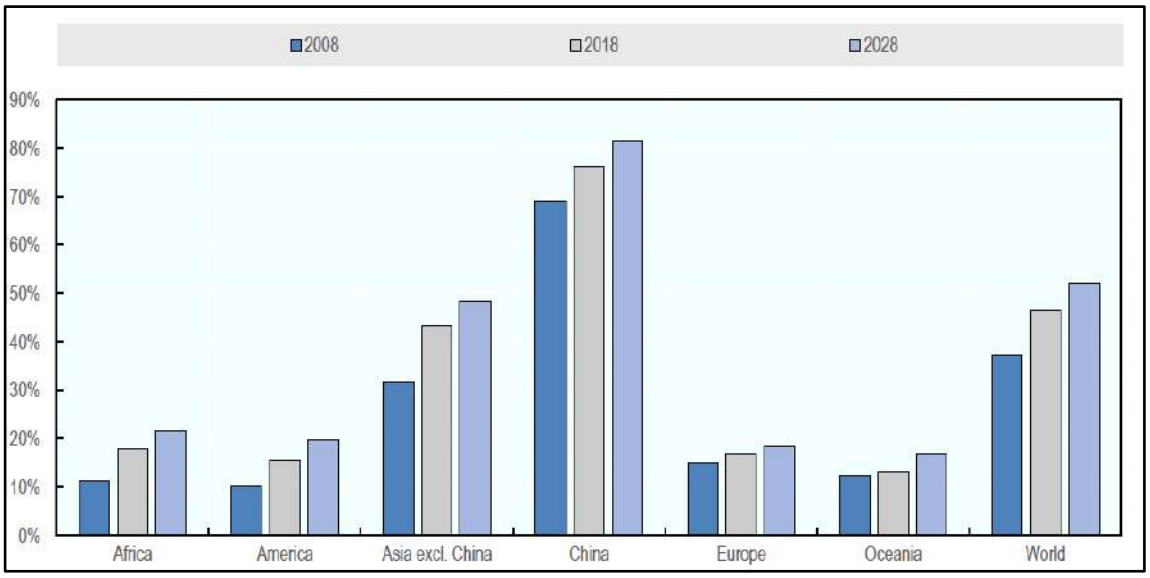

Figure 1.3.: Contribution of aquaculture to regional fish and seafood production (OECD/FAO 2019)

Fish is a vastly perishable food matter because of its moisture and nutrient contents. Seafood generated from wild or cultivated species causes formation of more amount of by-products upon processing or during capturing of targeted fisheries that may not be fully utilized. These by-products may serve as rich source of various biomolecules having significant health benefits. The conventional fishery by-products include fish meal, fish liver oils, fish maw, isinglass etc. Some other by-products derived from fish and its waste material include fish protein concentrate, glue, gelatin, pearl essence, amino acids, protamine, fish skin leather etc. (Pagarkar et al.2014).

Fish waste is abundant in potentially valuable oils, minerals, enzymes, pigments and flavours etc. that have multiple applications in food, pharmaceutical, agricultural and aquaculture industries. Organic fertilizers and composts produced from fish waste have significant advantages over chemical based products. Fish by-products can offer number of biomolecules involving proteins, lipids, oil, enzymes, micronutrients (riboflavin, niacin and vitamin A, D) as well as minerals (iron, iodine, selenium and zinc). In addition to fishmeal and oil production, there is potential in silage production, fertiliser, composting, fish protein hydrolysate and concentrate. Non-nutritional applications

include chitin and chitosan, carotenoid pigments, enzyme extraction, leather, glue, pharmaceuticals, cosmetics, fine chemicals, collagen and gelatin. There are countless other uses for this material and new uses are emerging all the time.

Collagen is present in almost all organs of vertebrates and is the main structural component of connective tissues like skin, bone, tendon, cartilage, blood vessels and teeth. Collagen is accommodating approximately 30% of animal body and also the most plentiful and ubiquitous animal protein polymer (Khan et al.2009). The unique collagen types are characterized by a huge complexity and diversity in their structure or shape, their splice variants, the presence of extra, non-helical domains, their assembly and function. Collagen has applications in pharmaceutical, nutraceutical field, cosmetics, agriculture because of its excellent biodegradability, biocompatibility and weak antigenicity. Commercially bovine and porcine derived collagen were used but with limited applications and also has ethical and legal issues. Therefore, marine species are the best suitable alternative for collagen. Fish wastes like skin, scales, fins, bone of various fishes are rich source of collagen (Berhie et al.2019). Extraction of collagen from this waste is economically feasible as well as cost effective and mainly helps to reduce environmental pollution burden on eco-system by this waste and there are less ethical issues regarding this.

Gelatin is a fibrous protein having molecular weight 20-200 KDa produced through thermal denaturation of collagen or partial degradation of animal bone and skin. The total or partial dissociation of collagen polypeptide during denaturation is occurred due to weakening and separation of hydrogen bonds which results in loss of original triple helical confirmation. The resulting polymer forms different coiled structure other than original protein designated as gelatin (Milovanovic and Hayes 2018). It is generally used in food, pharmaceutical, medical, cosmetic and photographic industries and has unique physical and chemical properties. Also, it incorporated as stabilizer, gelling, fastener, emulsifier and adhesive agent. It could also be used for diabetics and can reduce body weight. In nutraceutical industry, gelatin is one of the water-soluble polymers that can be used as materials to raise the food elasticity, consistency and stability (Ratnasari et al.2014). Because of gel like properties, it is an effective food packaging material and useful food additive in different food products like gelatinous desserts, gummy candies and many yogurts. It has various properties like thickening, emulsifying, binding and adhesive properties and also able to attract impurities in fruit juices, wine and vinegar. It

can be used in skin creams and lotions face masks, shampoos, hair conditioners, hair sprays, nail polishes, lipsticks also used for craft and decorative purposes.

Fish oil is the good source of polyunsaturated fatty acids (PUFA), especially omega-3 long chain ecosapentanoic acid (EPA) and docosahexanoic acid (DHA). Omega-3 fatty acids are polyunsaturated fatty acids characterized by presence of a double bond three atoms away from the terminal methyl group in their chemical structure. Omega-3 long chain fatty acid plays vital role in growth and development throughout the life and also beneficial in prevention and treatment of diseases related to coronary arteries (Suriani and Taulu 2015). Apart from this, it can be used to increase the omega-3 levels in meat and eggs when fed to land animals and is therefore, often added to animal feed. Fish oils are used in the medical and animal feed areas for supply of vitamin A and D; in production of soaps and detergents, paints and varnishes, floor coverings and oil cloths, oiled fabrics and in the processing of insecticides, alkalized resins, cosmetics, metal and processed leather. A growing market for fish oil supplements for human consumption is also imposing competing demands on the availability of sufficient fish oil supplies from wild caught fisheries due to health consciousness (Howieson 2017). Different fishes like silver carp, tuna, mackerel and sardine are good sources of fish oil. The health benefits of omega-3 fatty acids includes reduction in inflammation, fight against anxiety and depression, improves eye health, promote brain functioning, has application in feed and agriculture field and also, has role in skin and hair treatment.

Current research work deals with optimum utilization of fish processed waste (skin, scale, fins, tail and head) to extract commercially important biological macromolecules. Initial study deals with extraction, purification and characterization of collagen, gelatin and omega-3 fatty acids from waste material of Gethar (*Sarda orientalis*). After the biochemical analysis of these components, they are explored for various applications like biomedical, food and agriculture sector. Soil sample from fish waste dumping site was screened for isolation of fish waste degrading bacteria. After the extraction of all components from fish waste, remaining waste was subjected to microbial degradation. The hydrolysate generated after degradation was studied for its plant growth promotion ability. Thus, complete utilization of fish processed waste was carried out. Fruitful results can be obtained if this collagenous waste is treated in an eco-friendly manner using a biotechnological way which may help to minimize environmental pollution caused due to this waste.

1.2. Literature review

1.2.1. Fish preservation and processing

Fish is one of the protein rich foods that need careful handling. Fish spoilage is important aspect in fish processing. Due to high tropical temperature than actual body temperature of fishes, it initiates harmful activities of bacteria, enzymes and chemical oxidation of fat in fish. Therefore, all the processes during preparation, catching, landing, handling, storage and transport requires close attention to deliver a high quality product. The large amount of fish cannot be processed and utilized at same time so; preservation of fish is important aspect. It includes chilling, salting, drying, smoking, roasting and canning. The fish processing industries utilizes following steps for processing: Handling the catch, Removal of the scales and head, Cutting and Filleting of fish. The handling of fish includes catching, sorting, grading, chilling and storing of chilled fishes. Scales and head are inedible parts; it can be removed by mechanical scrapping. The cutting process involves gutting and washing. Gutting means removal of gut of fish and washing with clean water. Gutting and washing of the fish help to prevent bacterial attack before and during processing, preservation and storage. Fillets are nothing but flesh like material of fish was prepared by traditional method or filleting machine. Fillets are dripped in brine to enhance their appearance and to reduce the amount of drip and it also gives a salty flavour. Then put in a freezer at -35°C to- 40°C and stored at -23°C. After all the processing, the processed fish is subjected to formation of different food products like Fish mince, Surimi, Fish sauce and Fish meal (Ugochukwu 2017).

1.2.2. Fish processing waste

Fishery industries generate a large amount of solid waste such as gross fish waste, fish head, viscera, tails, skin, scales, bones, blood, liver, gonads, guts and some muscle tissues while liquid waste consists of wastewater used during fish processing. The average composition was fish was represented in figure no.1.4. Around 75% of the worldwide fish production is used for human utilization and the remaining 25% is treated as fish waste. The disposal of these bulk fish waste is quite complicated and expensive because they are rich source of organic contents such as 58% protein, bioactive peptides, collagen, gelatin, oil, enzymes as well as calcium, 19% fat and trace amounts of minerals mainly copper, phosphorus, magnesium, sodium, potassium, calcium, iron, zinc and manganese. Inappropriate discarding by traditional methods and sea dumping can lead to pollution and other environmental issues. (Ramkumar et al.2016).

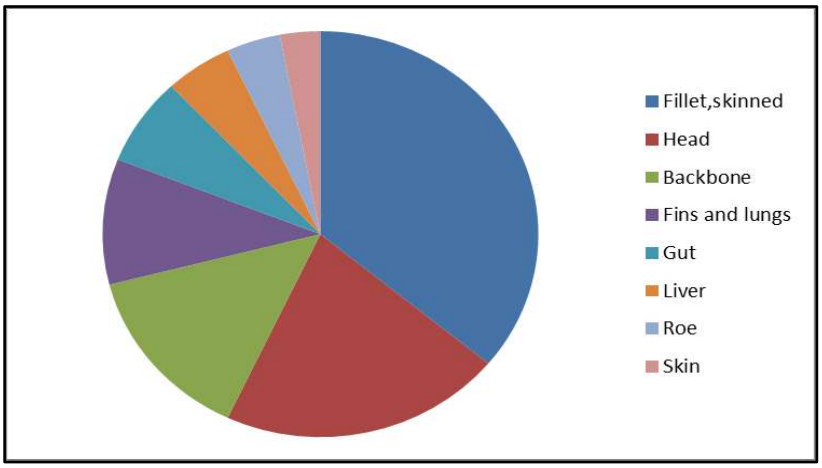

Figure 1.4.: Average composition of fish

1.2.3. Benefits of fish waste

Due to increasing demand of sea food, fish industry wastes are an important environmental contamination source. Fish industry waste and its liquid effluents affect not only surrounding area but also wider coastal zone at distinct ecosystem level. Thus, causes reduction in biomass, density and diversity of the phytoplankton's, zooplanktons and alter normal behaviour of natural food chains and food webs. A remarkable waste reduction approach for the industry is recovery of by-products from fish wastes so as to reduce the problems originated due to fish waste.

1.2.3.1. Animal feed

Recently, use of fish wastes is a substituent for animal feed to reduce the risk of environmental pollution and improve animal health benefits so as to increase currency production from animals. Treatment of fish waste such as head, bones, skin and sometimes whole fish was heated at 60, 80, 105 and 150°C for 12 hours reduces the moisture content to 10-12%. This quality of waste with reduced moisture content is suitable for animal feed as recommended by NRC (National Research Council), 1998. The large amount of minerals, protein (58% dry matter) and fatty acids (19%) like monounsaturated acids, palmitic and oleic acids were present in fish waste also 22% ash content indicates the mineral presence in fish waste (Arvanitoyanni and Kassaveti 2006).

1.2.3.2. Biodiesel/biogas

About 95-96% fuel was produced after filtration, primary and secondary treatments of fish waste. This oil was tested for its density, flash point, heating value, distillation test and sulphur content. The obtained oil was found to have equivalent properties for use in diesel engines. There is no information available about anaerobic

digestion of solid waste from fish industries. The produced biogas may be used to produce thermal and electrical energy (Arvanitoyanni and Kassaveti 2006).

1.2.3.3. Natural pigments

Carotenoids are the important colour pigments which imparts colour to the most of fish and shellfish. The orange-red integument and flesh like structure of more costly seafood like shrimp, lobster, crab, crayfish, salmon, redfish, red snapper and tuna possesses carotenoid pigments. The pigments extracted from fish waste can be effectively used in aquaculture feed formulation and the residue remaining after extraction may be used in preparation of chitin/chitosan (Arvanitoyanni and Kassaveti 2006).

1.2.3.4. Food industry/cosmetics

Some researchers found that, a various useful components can be extracted from fish waste including enzymes, proteins and bioactive compounds have potential antimicrobial as well as antitumor activities. Among different enzymes present in fish waste, protease is the most important industrial enzyme shows useful applications in food industry. Surimi is the new type of fish meat in which fishes are mechanically debones and persevered with the help of cryoprotectants. Fish stomach mucosa is used for the extraction of milk clotting enzyme which is an inexpensive substituent for rennet in cheese manufacturing. This approach may promote the development of new food industry. Some fish hydrolysates were utilized for food processing and preservation. Fish skin, bone and fin were acts as potential source for collagen. It can be used as best alternative to regular collagen in different food, cosmetics and biomedical industries (Arvanitoyanni and Kassaveti 2006).

1.2.4. Fish waste management

Waste management deals with the analysis of solid and liquid pollutants produced by fish processing industries. Environmental issues caused by industrial activity have become one of the major important aspects. The industry which is eco-friendly and socially responsible to nature and will actively help to increase economic strength of nation and also save environment. Waste minimization is one of the interesting approach to meet sustainability of waste management in fish processing and production process (Kurniasih et al.2018). Fishery waste contains useful as well as harmful components which affects adversely on eco-system. The large amount of solid fish waste is discarded near to coastal region; this will lead to change in normal behaviour of food chain. The huge amount of water is required for washing and processing of fish. This will create

liquid waste which is incorporated into water bodies without any treatment. It leads to decrease in oxygen level, change in pH, lowers concentration of ammonia and nitrogen as well as also effects on ecosystem of water bodies.

1.2.5. Composition of fish waste

1.2.5.1. Amino acids

Fish and its derivatives are rich source of proteins with high biological and nutritional values, hence it contains balanced amount of amino acids. Different amino acids are present in fish and its by-products. It includes glutamic acid, arginine, histidine, threonine, tyrosine, valine, methionine, isoleucine, leucine, phenylalanine and lysine. Thus, fish and its waste derivatives are used for fish meal, fish sauce, animal feed and for agricultural purpose (de Souza et al.2017).

1.2.5.2. Enzymes

Fish internal organs such as viscera and gut are great source of enzymes with high catalytic activity at low concentrations. Because of this, fish processing derivatives are used for enzyme extraction. Marine fish viscera have wide variety of proteolytic enzymes like pepsin, trypsin, chymotrypsin and collagenases which are extracted in large scale. Most of enzymes in fish and its derivatives possess high catalytic activity at different environmental conditions like low or high temperature, elevated nutrient concentrations, high pressure and high salt concentrations. Due to these properties, fish proteinases have many applications in food processing industries. The fish derived enzymes also used in isolation of bioactive compounds (Kim and Mendis 2006).

1.2.5.3. Bioactive peptides

There are different bioactive peptides extracted by using enzymatic extraction methods from fish waste or fish waste remaining after processing. Proteolytic hydrolysis of parent proteins using available enzymes and micro-organism releases inactive short amino acid sequences called as bioactive peptides. They have biological and nutritional properties which effects positively on normal functioning of organism’s body and also enhances the food quality (Phadke et al.2017). Bioactive peptides have large demand in pharmaceutical fields due to their beneficial effects on human physiological system.

1.2.5.4. Collagen

Collagen is a naturally occurring protein. About 30% of the total protein present in animal body is largely composed of this structural fibrous protein. It is one of the important component of extracellular matrix of various connective tissues such as the skin, cartilage, bone, tendons, ligament of vertebrates, while in invertebrates body walls,

cuticles showed presence of collagen. It helps in tissue remodelling, adhesion and also plays vital role in maintaining structure of different tissues. It is left handed triple helical structure of protein stabilized by hydrogen bond. It has a diameter of about 1.6 nm with length of about 300 nm. The molecule has specific characteristic repetitive sequence of triplet Gly-X-Y. The structural levels of collagen were given in figure no.1.5.

Different types of collagen are exhibited based on X and Y, that is amino acids linked to glycine. Generally, X and Y are the imino acids proline and hydroxyproline respectively. There are 29 different types of collagen are available. About 90% of the collagen present in our body is the fibril forming collagen. It includes collagen type I, II, III, V and XXVII. Type I is most abundant in all connective tissues except hyaline cartilage (Silvipriya et al.2016). Thus, this fibrous protein plays vital role in various connective tissues and helps to maintain structure of bones, relieve joint pain, improve skin health and also has some other health benefits.

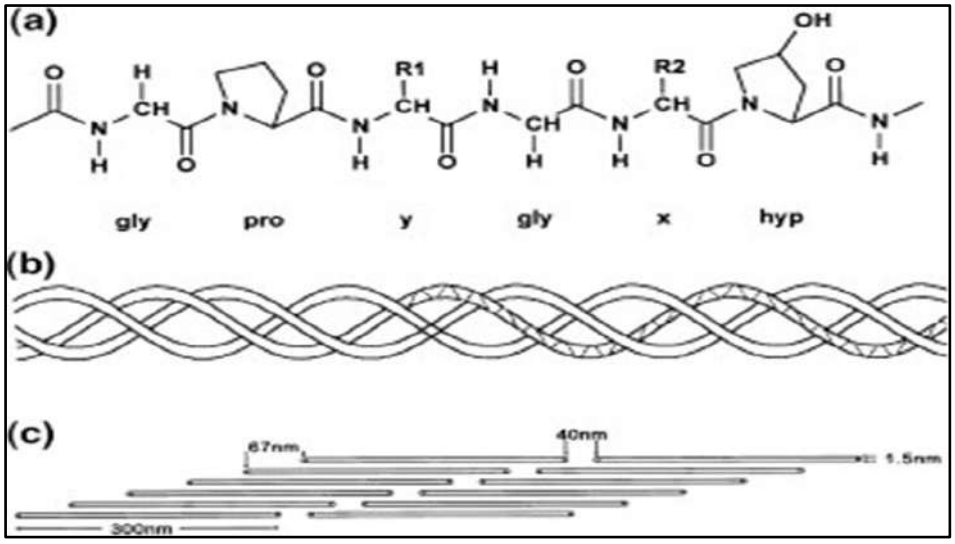

Figure 1.5.: Structural levels of collagen type I (a) amino acid sequence indicating primary structure (b) secondary and tertiary structure with left and right handed triple-helical and (c) collagen quaternary structure (Friess 1997)

1.2.5.5. Gelatin

Partial hydrolysis and heat dissolution at alkaline or acidic pH of skin collagen, bone and tendons of animals forms the proteinaceous hydrocolloid polymer gelatin. It is heterogeneous mixture of 300 to 4000 amino acids, single or multi stranded polypeptide with left handed proline helix confirmation (Kommareddy et al.2007). Chemical configuration of gelatin was showed in figure no. 1.6. It has high protein value having water soluble property and has ability to form transparent gel under

specific conditions. Molecular weight of gelatin ranges from 80 to 250 kD which contains 88% protein, 10% moisture and 1-2% salts. During the gel formation, it retains 50% moisture. Thus, Gelatin is an excellent biopolymer widely utilized in food and pharmaceutical industries (Wasswa et al.2007).

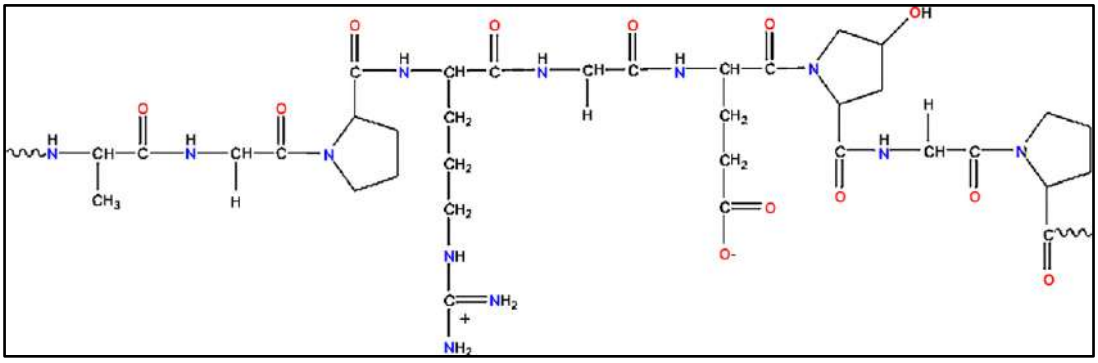

Figure 1.6.: Chemical configuration of gelatin (Chaplin 2012)

1.2.5.6. Oil

Fish oil is a rich source of polyunsaturated fatty acids (PUFA), especially omega-3 long-chain EPA and DHA. There are three types: eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and alpha-linolenic acid (ALA). These fatty acids also called as omega-3 oils or n-3 fatty acids. They are characterized by the presence of a double bond, three atoms away from the terminal methyl group in their chemical structure. Structure of fish oil containing EPA and DHA was depicted in figure no.1.7. Omega-3 long chain fatty acid plays crucial role in growth and development throughout the life. It helps in prevention and treatment of different coronary arteries diseases like arteriosclerosis, hypertension, arthritis, and impaired immune response. Also helps in development of central nervous system and prevention of heart disease (Suriani and Taulu 2015).

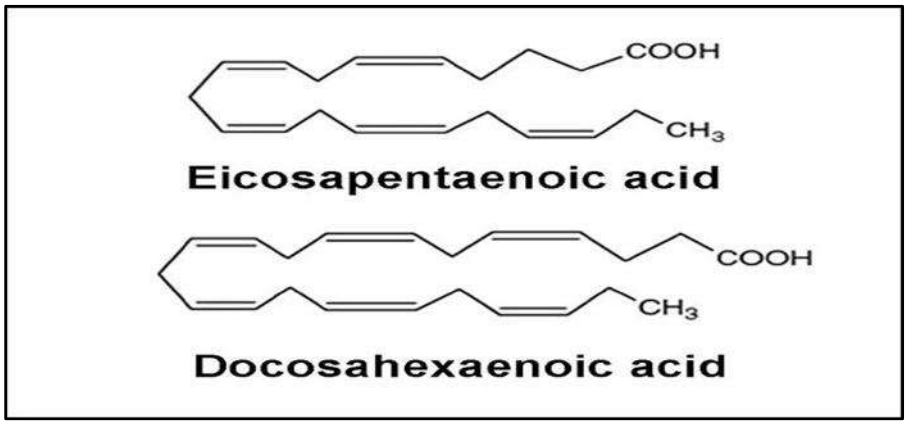

Figure 1.7.: Structure of Omega 3 fatty acids

1.2.6. Sources of collagen

The collagen obtained from bovine and porcine source was mostly used for industrial purpose. Skin and bones of cow and pigs were used for effective extraction. These two sources have different ethical and legal issues in different religions. Marine source is used as efficient alternative source for the extraction of collagen. It has some advantages, like high content of collagen, less immunogenic response, eco-friendly and showed negligible amount of contaminants. The waste materials derived from marine invertebrates and vertebrates were extensively used for this purpose. This will also help to reduce environmental threats caused due to fish waste. Besides all the traditional sources, some other sources like chicken, kangaroo tail, rat tail tendon, duck feet, sheep skin and frog skin also used. But, these sources may show some allergic and immunological response. Therefore, recombinant human collagen was under investigation which has lower immunogenicity as compared to other sources (Silvipriya et al.2015).

1.2.7. Types of collagen

About 25 to 30% of total protein of animal body is composed of most abundant fibrous collagen protein. It plays major role in maintaining biological and structural characteristics of extracellular matrix (ECM) and helps in physiological functions. Almost type I to type XXVIII different collagen forms has been studied. Out of this, type I, II, III and V are important in bone, cartilage, tendon, skin and muscle (Das et al.2017).

Family	Type	Distribution	Application
Fibril-forming	I	Skin, bone, tendon (non-cartilage), dermis, cornea, ligament	Membranes for guided tissue regeneration
	II	Cartilage, vitreous humor, nucleus pulposus, lung, cornea, skin, bone	Cartilage repair, arthritis treatment
	III	Extensible connective tissue (skin, lung, vascular system viz. artery), skin, vessel wall, reticular fibers of most tissues (lungs, liver, spleen)	Hemostats and tissue sealants
	V	Co-distributed with Type-I,	Corneal treatment

		especially in cornea	
	XI	Along with type II, vitreous body and cartilage	mAbs development for osteoarthritis
Basement membrane	IV	Basement membrane	Attachment enhancer of cell culture (mouse neuroblastoma) and diabetic nephropathy indicator
Microfibrillar	VI	Muscle, dermis, placenta, lungs, intervertebral disk, cartilage	Hemostat
Anchoring	VII	Dermal epidermal junction, skin, cervix, oral mucous	Treatment of dystrophic epidermolysis bullosa (DEB)
FACIT	IX	Along with type II in cornea, cartilage and vitreous body	
	XII	Tendon, perichondrium and ligaments	Regulator in early stages fibrillogenesis
	XIV	Along with type I in vessel walls, placenta, liver, dermis and lungs	
	XIX	Many tissues, human rhabdomyosarcoma	Antiangiogenic and antitumoral properties
	XX	Corneal epithelium, sternal cartilage, embryonic skin, tendon	
	XXI	Many tissues, blood vessel wall	Contribute to matrix assembly of vascular network during blood vessel formation
Transmembrane	XIII	NM junction, skin, hair follicle, intestine, lungs	Involved in inflammation and vasculogenesis, regulate bone mass
	XVII	Epithelia, skin hemidesmosomes	Teeth formation
Multiplexins	XV	Associated with collagens close to basement membranes, kidney, smooth muscle cells,	

		pancreas	
	XVI	Many tissues including keratinocytes and fibroblasts	Drug target and biomarker
	XVIII	Close structural homologue of XV, liver, lungs	Retinal structure, closure of neural tube
Miscellaneous	VIII	Endothelium	
	X	Hypertrophic cartilage	
	XXII	Tissue junctions	
	XXIII	Limited in tissues, mainly trans-membrane and shed forms	
	XXIV	Developing cornea, bone	
	XXV	Brain	
	XXVI	Testis, Ovary	
	XVII	Embryonic cartilage	
	XXVIII	BM around Schwann cells	

Table 1.3: Different types of collagen and their distribution in human body (Raman and Gopakumar 2018)

1.2.8. Collagen extraction methods

For extraction of collagen from fish waste includes two steps. First step deals with pre-treatment and second was effective collagen extraction.

1.2.8.1. Pre-treatment process

Pre-treatment of raw material (fish waste) is the crucial step during extraction. Different pre-treatment processes were applied on waste material to remove other biological constituents and to improve the efficiency of collagen extraction. Biological constitutes includes proteins, pigments, lipids and enzymes while fish bone and scale consist of calcium and some inorganic components which may interfere in collagen extraction. Alkaline pre-treatment by 0.1 M NaOH was used to remove non-collagenous proteins and pigments (Hamdan and Sarbon 2019). It also excludes the effect of endogenous protease. Fish bone and scales contains little calcium and some phosphate like components. It can be decalcified by using ethylenediamine tetraacetic acid (EDTA)

(Pati et al.2010). It releases phosphate into solution resulting in demineralization of waste. Lastly, the pre-treated sample is subjected to removal of fat and it was done by 10% butyl alcohol (Aminudin et al. 2015).

1.2.8.2. Extraction methods

There are various techniques employed for collagen extraction and based on this, collagen divided into discrete types. The types include alkali soluble, acid soluble (ASC), salt soluble (SSC), pepsin soluble (PSC) and ultrasound assisted collagen (UAC). Among these methods, acid and enzyme extraction methods are most common.

1.2.8.2.1. Alkali mediated collagen extraction

Many acidic and basic moieties are present in collagen. Extraction of collagen by alkali is not effective and produces very less yield. The alkali like CaO, $Ca(OH)_2$, Na_2CO_3 etc. were used for extraction. Acid-base neutralization under alkaline condition causes the cleavage of collagen into peptides. Yield depends upon concentration of alkali and time required for extraction. Alkaline method may be used to extract bone collagen but it requires more time, denaturation of protein structure and produce lesser yield. The collagen disintegrates and deteriorates in alkali solution, alters its structure, hydrolyses the peptide chains and reduction in isoelectric point. Thus, due to these disadvantages alkaline extraction of collagen is not suggested (Lian et al.2017).

1.2.8.2.2. Acid mediated collagen extraction

Acidic extraction is broadly applied method for productive extraction of collagen from marine waste products and it is designated as acid soluble collagen (ASC). Various concentrations of acetic acid are beneficially used as a solvent for extraction of collagen, since it generate increased yield and thermally stable collagen. It was reported that, collagen was effectively extracted by using 0.5 M acetic acid and gives more yield under controlled biological conditions (Chinh et al.2019). Principal monomeric subunits in collagen could be effectively solubilized in acetic acid. It has more extracting capacity and produces more yield than any other organic and inorganic acids (Veeruraj et al.2013). The concentration behind 0.7 M generates minimum yield and less active collagen. Inorganic acids also utilized for extraction but it gives rise to lower efficiency and low yield of collagen than organic acids (Hadfi and Sarbon 2019).

In addition to acetic acid some organic and inorganic acids such as citric acid, lactic acid, tartaric acid, formic acid, hydrochloric acid and sulphuric acid were employed for the extraction of collagen from fish waste (Bhuimbar et al.2019; Kothai and Premlatha 2018). Fish wastes including skin, scale, bone and fins are important by-

products from fishery industry which has more collagen content. Studies suggested that, acetic acid is applied to extract collagen form various fish processing waste including skin, scale, fins and swim bladder. The high collagen content about 50-70% was reported from skin waste (Nagai et al.2000; Pang et al.2013; Kumar et al.2016). Some studies suggested that, yield of collagen was dependent on fish species, raw materials, habit and habitat of fishes, biological conditions, type and time of extraction, concentration and type of acid used for pre-treatment processes. More concentration of acid may hydrolyse the collagen leading to lesser yield (Das et al.2017; Pamungkas et al.2019). Thus, acid extraction method was widely employed for collagen extraction.

1.2.8.2.3. Enzyme mediated collagen extraction

Commercially, collagen is retrieved from marine animals by using an acetic acid without inclusion of enzyme. Collagen extracted from sea food processing waste was less soluble in acidic condition. Acidic condition may give minimum production of extracted collagen. Therefore, enzyme mediated collagen extraction has been discovered to increase the yield which called as enzyme soluble collagen. Enzymes like pepsin, papain, collagenase isolated from microbial origin were implemented to increase the production of collagen (Junianto et al.2018; Rochima et al.2017; Min Li et al.2015). The acidic and enzymatic method of collagen extraction was depicted in figure no.1.8.

1.2.8.2.4. Salt mediated collagen extraction

Extraction of collagen from fish waste by using NaCl is carried out. But, it is rarely used due to its minimum yield (3.02%). The salt extracted collagen is referred as salt soluble collagen (SSC). Some researcher used 0.45 M sodium chloride for extraction of collagen from fish waste. Sodium chloride extracts free molecules that were not bounded by covalent interaction, thus it has least ability to solubilize collagen and produce smaller yield (Wang et al.2014).

1.2.8.2.5. Ultrasound mediated collagen extraction

Ultrasound is extensively employed method to enhance the shifting of mass by wet processes which are important for extraction and drying. Ultrasound generates excessive frequency sound waves (more than 16 kHz). Ultrasound mediated collagen extraction gives more yield of product in minimum processing period. Ultrasonic frequency of 20 kHz in presence of 0.5 M acetic acid exhibited larger yield in less extraction time. This wave does not alter the structural specificity of extracted collagen. Ultrasonic waves in combination with pepsin gives better yield than conventional

technique, reduces extraction time, greater efficiency and higher thermal stability without disturbing structural and functional quality of extracted collagen (Schmidt et al.2016).

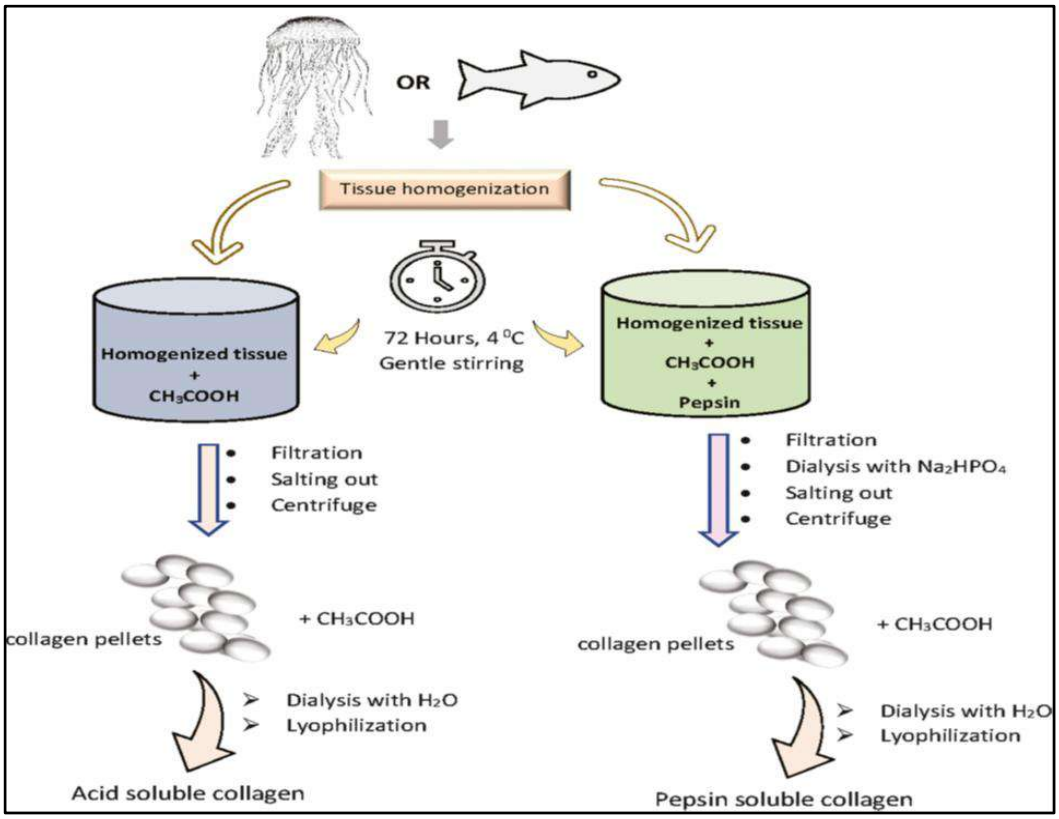

Figure 1.8.: Collagen extraction by acidic and enzymatic method (Felician et al.2018)

1.2.9. Applications of collagen

Collagen extracted from different marine sources has potential applications in biomedical and pharmaceutical industries, food additives and supplements, cosmetology and in healing materials.

1.2.9.1. Biomedical applications

The biodegradable, easily available, biocompatible and highly versatile biopolymer collagen has various applications in biomedical field such as tissue engineering, wound healing, ophthalmic surgery and delivery vehicle. This biomaterial used extensively to increase repair of bone, tendon, cartilage, skin, ligament and connective tissues. It has structural similarity with native collagen in human body so it also functions in cell support system. Collagen sponges were used for wound healing which contains keratinocyte and fibroblast. They are capable of proliferating cytokines and growth factors. Thus, provides skin regeneration and wound healing action in burn patients. Gel structure of collagen has special properties like flowability, injectability and

biocompatibility. Therefore, it effectively acts as delivery vehicle for drug, gene and proteins. Collagen fused with synthetic polymer and collagen based diffusion membranes were applied for controlled drug delivery and prolonged drug release therapy. Collagen-glycosaminoglycan fusion with insulin like growth factor-1 provides an efficient non-viral tool for gene delivery (eg. cartilage regeneration). Calcium phosphate coated (apatite) collagen acts as delivery vehicle for therapeutic proteins such as bone morphogenetic protein-2 (BMP-2) and carried out its safe release. Collagen-microsphere based delivery system was the safe way for delivery of protein based products (Muthukumar et al.2018).

1.2.9.2. Cosmetic potential

Collagen is major constituent of various cosmetic formulations because; it has potential benefits as a humectant and moisturizer. The type I collagen was effectively possess cosmetic potential, good activity to retain water therefore can be used as moisturizer. Collagen in its peptide form utilized as an anti-aging and anti-wrinkling product in cosmetic formulations for personal care. The analysis of molecular markers of collagen indicates no irritation and inflammation of human skin. Due to high molecular weight protein, it cannot binds to stratum corneum of the skin and carried out hydration of skin. Hence, it protects the wounded tissue from microbial infections by moisturization effect (Alves et al.2017).

1.2.9.3. Collagen supplements

Collagen is linked with different health benefits which lead to the development of collagen supplements industry. It has moisture absorption activity and its fractions have ample valuable nutritive fibres so it is used as protein source in human diets. Collagen synthesis will decrease as human gets older and tissues become thinner and weaker. Supplements of it are important to maintain collagen levels in skin, hair, nails and body tissues. Nutricosmetics are usually offered collagen in the form of liquids, pills or functional foods. Due to water absorption ability of collagen, it hydrates skin dermis and epidermis which helps to increase the smoothness and reduces wrinkling. Collagen and hydrolysed collagen contains natural creatine and arginine. These two components boost up muscle gain, decrease recovery time, reconstruct damaged joint structure and improve cardiovascular performances. Type II collagen is effectively used in rheumatoid arthritis. Collagen is effectively supplemented in sports nutrition (Hashim et al.2015).

1.2.9.4. Collagen as carrier

The lipids present in meat were oxidized due to various processes and leads to formation of other compounds which have unfavourable effect on quality and nutritive value of meat products and also decreases the shelf life of processed meat. Collagen is less allergic to humans and other animals, so it is potential carrier for biologically active substances like vitamins, drugs, minerals in medicine, pharmacology and nutraceutical. Collagen and its fractions are extracted from readily available, cheap, raw materials. Due to minimum production cost and different beneficial characteristics, it is a valuable agent in food industry including meat processing plants. Thus, collagen preparations are introduced into processed meat which improves its quality. Collagen fibres have better carrier capacity than collagen hydrolysates. Therefore, collagen fibres acts as carriers of rosemary extract in production of processed meat and its products which improve the antioxidant activity of meat (Waszkowiak and Dolata 2007).

1.2.9.5. Food applications

In current years, collagen has become key additive toward the evolution of healthy energy rich nutrients. Nutritive fibres were important for normal digestion and adsorption in the gastrointestinal tract. Improper diet as well as age is responsible for depletion in collagen production in body. Peoples refused to use collagen capsules, powder and injections to increase the amount of collagen. So, there is need to obtain collagen from nutritious food. Therefore, collagen integrates with numerous food and beverages to link up the required concentration of collagen in body. It is essential nutritive fibre which efficiently performs diverse biological functions. Some studies reported that, about 2-2.5% collagen decreases the time required for mincing and mixing components. Specially treated collagen may be employed for sausage casings. Due to cheap degree of alteration, ecological clarity and easy production method collagen fractions were mostly used for manufacturing of emulsion-type sausages in worldwide (Neklyudov 2002). Collagen or its fractions improves technological and rheological properties of many crude materials. So, it was approved that collagen fibre (heat treated) is native substitute to synthetic emulsifier and can be applied in acidic food products (Santana et al.2011). Collagen behaves as barrier that manages the relocation of oxygen, supplies permeability to water vapour as well as increase the shelf life of food. Thus, the collagen films or edible coatings can be utilized for wrapping, dipping, brushing or spraying the food (Rojo et al.2018).

1.2.9.6. Feed applications

For continuing different metabolic processes, animals need a stable nutrition that offers appropriate energy. The improvement in growth rate was detected in some birds supplemented with collagen in their regular diet. It has major role in broiler chicken feed which helps to increase the muscle growth and nutritional value. Poultry diet mainly consists of methionine and lysine as a source of energy and minerals. Collagen composed of methionine (6%) and lysine (19%) acts as a good protein supplement. Glycine in collagen has anti-inflammatory potential and provides protection against diverse diseases. Thus, due to presence of important amino acids, collagen provides valuable protein supplement for broiler chicken feed with minimum time period by increasing muscle content and health of poultry birds. The poultry feed along with collagen or collagen containing food enhances the growth rate, body weight and strength of muscle. Anti-inflammation and immunity improving capacity of glycine decreases the mortality. Antioxidant capability of collagen hamper the oxidative mechanisms of free radicals, thus remarkably decline the mortality rate. Collagen extracted from fish provides improved nutrient quality than commercial feed. Therefore, fish collagen can be supplied as substituent and productive feed in broiler chicken industry (Nurubhasha et al.2019).

1.2.9.7. Agricultural applications of collagen

The bio-composites derived from collagen and other proteins used in agricultural applications. It is used to enhance germination, nutrition, managed liberation of nitrogen to maintain the nitrogen balance and plant protection for different stages of plant development (Niculescu et al.2019). Collagen and its hydrolysates used in fertilization of horticultural crops and soil remediation. It is used to enhance the seed germination, plant growth stimulant, maintain nitrogen balance, minimize the use of insecticides and fungicides, increases fertilization rate and lowers the effect of iron deficiency caused due to calcareous soil. Collagen films have better elasticity, so that it can degrade easily and releases the tiny peptides. The water vapour permeability of film cause easy respiration of plants and seedlings, thus maintain the moisture content in soil (Niculescu et al.2017).

1.2.9.8. Hydrolysed collagen

Hydrolysed collagen (HC) or collagen peptides are tiny, 0.3-8 kD molecular weight structure synthesized from original collagen present in bones, skin and connective tissues of animals. Because of low molecular weight, HC has several benefits over native collagen like they are digested easily, absorbed easily and dispersed in the various tissues of human body. Different methods of HC extraction, effect on quality and molecular size.

The figure no.1.9 showed the structure of hydrolysed collagen. Hydrolysed collagen is rich in distinct amino acids like glycine, proline and hydroxyproline. During the assimilation of hydrolysed collagen, some proteases (e.g. pancreatic proteases; small intestinal brush-border proteases; peptidase) breakdown it into di and tri peptides or free amino acids (Sibilla et al.2015). The thermal analysis of collagen more than 40°C results in hydrolysis of collagen by proteolytic enzymes (alcalase, papain, pepsin, and others) and forms hydrolysed collagen. Solubility and functional properties (antioxidant, antimicrobial) of HC depends on the type and degree of hydrolysis and enzymes used for extraction. Acidic and alkaline methods are extremely corrosive and generate elevated salt concentration in the finishing product after neutralization. Thermal processing (100°C to 374°C) and pressure (22 MPa) treatment also used for extraction (Lopez et al.2019).

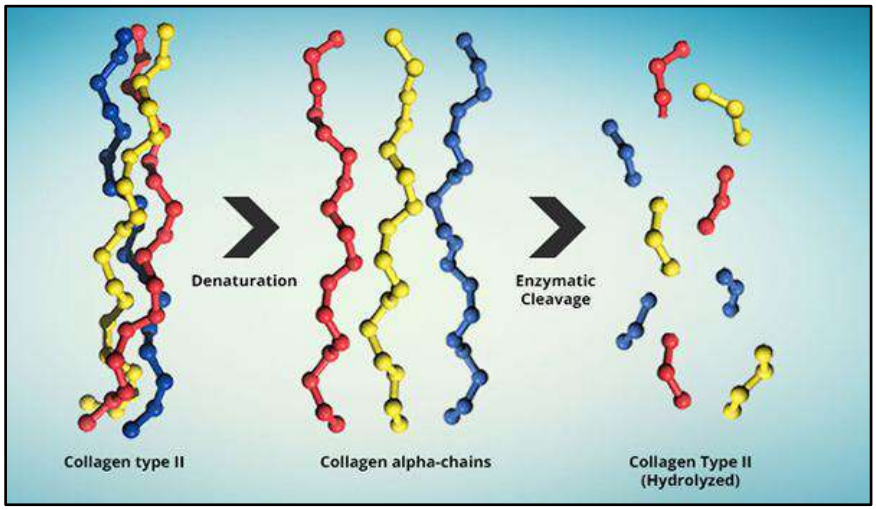

Figure 1.9.: Structure of hydrolyzed collagen

1.2.9.8.1. Applications of hydrolysed collagen

Hydrolysed collagen or collagen peptides maintain metabolic function of body by supplementing low molecular weight collagen which absorbed easily in the gastrointestinal track (GIT). Free amino acids and peptide fragments of HC speedily absorbed through the walls of intestine into blood stream. Collagen peptides were assist nutritional development of skin, hair and nail. Lysine and arginine also present in HC, lysine helps to maintain muscle growth and metabolism while arginine acts as precursor for the energy storage molecule creatine. Thus, hydrolysed collagen is beneficial for athletes and body builders for maintaining muscle growth, expansion of metabolism and supply the structural material for curing of injuries (cartilage, ligaments, tendons, bones

and discs). Some amino acids involved in the formation and repair of collagen of body which supplied by hydrolysed collagen. Due to presence of more amount of glycine, HC provides protection against the toxic substances like chemicals, pollutants, alcohol or tobacco.

HC also helpful in curation of mood related problems by increasing alertness, enriched energy, enhanced concentration, steady mood and improved sense of well-being. Collagen peptides include 18% nitrogen which is helpful to regulate positive nitrogen balance (Haltiwanger 2014). It was observed that, collagen peptides extracted from fish skin exhibit anticancer potential and provides inhibitory action against Angiotensin I converting enzyme. ACE inhibitory activity is initiated by aromatic acids present at C-terminal end. It gives rise to apoptosis of tumor cells by inducing caspases activity. Thus, hampers the cancer cell proliferation (Baehaki et al.2016).

1.2.10. Types of gelatin

The hydrolyzed form of collagen is gelatin synthesized from common sources of collagen such as cattle bones, pig skin and fish. The collagen derived from connective tissues of vertebrates is a major component of mammalian gelatin. It includes the type A gelatin from porcine and type B from bovine sources with molecular weight between 10 to 400 kDa. Fish waste and the waste derived from fish processing industries contains ample amount of collagen which is a major source for gelatin. In some countries, edible insect gelatin can be used as an alternative source of gelatin (Mariod and Adam 2013).

1.2.11. Gelatin extraction methods

1.2.11.1 High pressure processing (HPP)

It is a non-thermal preservation technique employed for inactivation of micro-organisms in food without changing its freshness and nutritional value. This is also designated as Ultra High Pressure (UHP) or High Hydrostatic Pressure (HHP) and considered as one of the best method for gelatin extraction. At certain pressure up to 500 Mpa, it does not cause significant change in collagen structure and extend the protein structure. Because of high pressure it brings protein denaturation and alters the balance of non-covalent bond association which stabilize the native arrangement of numerous proteins. Thus, results in easy extraction of gelatin from fish waste (Jaswir et al.2017).

1.2.11.2 Alkaline extraction method

Gelatin can be extracted from skin and fin of sliver carp fish using alkali such as calcium hydroxide and is common method for extraction from fatty fishes. Raw sample was soaked in solution for 4 weeks. Longer pre-treatment or high concentration of alkali

may reduce the quality of gelatin. The pre-treated gelatin solution was filtered and mixed with 5% (w/w) chloridric acid. The extracted gelatin solution concentrated in rotary vacuum evaporator and dehydrated at 50-60°C for the formation of dry and thin layers of gelatin (Tavakolipour 2011).

1.2.11.3 Acidic extraction method

Acidic method has more yield and good quality of gelatin than alkaline method. Skin of some marine fishes was soaked in 0.1 M sodium hydroxide to remove non-collagenous proteins (Hue et al.2017). Various organic acids like acetic, citric, lactic, malic or tartaric of 0.05 M were added in 1:3 (w/v) ratio. All the extraction processes were carried out under constant stirring for 4 hours (Khiari et al.2011). The extracted gelatin was dehydrated and dried.

1.2.11.4 Enzymatic extraction

Food and pharmaceutical industries has wide applications of microbial protease. Among these alkaline protease produced by *Bacillus licheniformis* was mostly studied. Chemical method has some negative effects on quality and purity of gelatin. Thus, this microbial protease is an effective agent for gelatin extraction from marine waste (Kouhdasht et al.2018).

1.2.12. Applications of gelatin

1.2.12.1. Biodegradable food packaging film

Biodegradable food packaging films has increasing demand than traditional, commonly used packaging polymers due to its large source, sustainability, eco-friendly nature, effective applications and more compatible to various foodstuffs. Gelatin is one of the interesting special materials produced by partial hydrolysis of collagen and increasing demand in various food industries for packaging and coating purpose. Among the distinct biopolymers having film forming ability, it is the best suitable material with advisable film forming properties. Gelatin films were effectively resisting the solvents with significant extent of flexibility and antioxidant activity. The gelatin biopolymer is hygroscopic in nature, thus it has huge moisture contents which expand or dissolve when exposed to the food surface. The different polymers in combination with gelatin were used to synthesize composite films for food packaging. Gelatin films were used to increase shelf life of food and agricultural products. Thus, gelatin play a crucial role in packaging and considered as excellent packaging agent having efficient oxygen barrier capacity which required for packaging resolution than other biopolymers (Hanani et al.2014).

1.2.12.2. Health benefits of gelatin and gelatin-derived peptides

Gelatin and its derived peptides have substantial biological advantages, like antioxidant, antihypertensive, anticancer, antiphotoaging and cholesterol lowering capacity. Due to presence of specific linkages of amino acids, several gelatin peptides derived from fish skin exhibit the ability to scavenge free radicals and reactive oxygen species and also chelate the metal ions. The peptides like His-Gly-Pro-Leu-Gly-Pro-Leu, Pro-Ala-Gly-Tyr and peptides containing hydrophobic amino acids (Gly, Pro, Ala, Val, Leu) at N-terminus and amino acids like Tyr, Met, Ile, Glu, Trp at C-terminus correlated with the high radical scavenging activity.

The gelatin hydrolysates acquired from Atlantic salmon skin using flavourzyme contains peptide Gly-Pro-Ala-Glu and Gly-Pro-Gly-Ala. It inhibits the activity of dipeptidyl-peptidase IV and therefore, gelatin derived peptide can be effectively employed in functional foods and pharmaceutical products against type 2 diabetes. The enzymes including protamex, trypsin, neutrase, savinase and alcalase were implemented for the extraction of gelatin hydrolysates from squid revealed greater cytotoxic effect against cancer cell lines MCF-7 (human breast carcinoma) and U87 (glioma); especially alcalase extracted gelatin were employed for this reason. The free radical scavenging and metal chelating ability of gelatin hydrolysates derived from blacktip shark skin using papaya latex crude enzymes minimizes the oxidation of human low density lipoprotein (LDL) cholesterol by 8.3–39.2%. The peptide Leu-Ser-Gly-Tyr-Gly-Pro from tilapia skin gelatin has potential to protect antioxidative system of mice, thus causes less damage to lipids and collagen (Liu et al.2015).

1.2.12.3. Feed application

Fish derived gelatin acts as protein source and natural stabilizer in diets for the several fish species instead of commercial binders like starch, agar, molasses and wheat meal. Gelatin extracted from dried fish skin has greater amount of crude protein. Regular fish feed in addition of 2% gelatin improves the quality, chemical and physical properties of feed also influence positively on growth parameters of fish. It helps to increase protein amount, flexibility, durability, stability and functional properties of fish feed also decrease the water solubility of normal diet. Thus, fish derived gelatin can be applied in normal fish feed to improve its characteristics (Dubakel et al.2015).

1.2.12.4. Gelatin in plant growth promotion

Greenhouse studies reported that, animal derived gelatin can be acts as bio-stimulant during seed treatment, thus it helps to improve plant growth and performance.

Gelatin capsule treatment significantly increases the crop growth, improves nitrogen uptake, expansion of total leaf area and dry weight. It also helps to induce expression of amino acid and nitrogen transporter genes that may be responsible for root nitrogen uptake improvement (Wilson et al.2018).

1.2.13. Types of omega-3 fatty acids

Omega-3 fatty acids are generally considered as long chain polyunsaturated fatty acids (PUFA). They have more than one carbon-carbon double bond. The first double bond is between third and fourth carbon from tail end so considered as omega-3-polyunsaturated fatty acids. The main types are alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). ALA is found is vegetables source like flaxseeds, canola (rapeseed) oil, soybeans, pumpkin seeds, walnuts and their derivative oils while EPA and DHA is mainly found in marine animals like fatty fishes includes sardine, salmon, mackerel, tuna (Gammone et al.2018). Also, some amount of EPA and DHA are present in waste material of marine animals such as skin, scales, head and tail. EPA is 20 carbons long and DHA is 22 carbons long. The daily requirement of both fatty acids ranges between 250-500 mg per day. These two fatty acids perform various important roles in normal functioning of human body.

1.2.14. Omega-3 fatty acid extraction methods

1.2.14.1. Supercritical fluid extraction method

In last few years, supercritical fluid extraction (SFE) has been used extensively to extract fish oil from fish by-products. It uses medium temperature and oxygen free media which reduces the oxidation of omega-3 fatty acid during extraction. SFE avoids the co-extraction of other polar lipids with inorganic impurities and only allows extraction of lower polar lipids. Because of carbon dioxide, it can be used as alternative method for conventional physical and chemical extraction method. Maximum extraction by this method requires moisture content less than 20% and raw material must be finally cut. The major limitation of this method is high production cost, use of high pressure equipment and the freeze dried machine which maintain moisture content below 20% (Rodriguez et al.2012).

1.2.14.2. Microwave assisted technique

This technique requires minimum time for solvent extraction (soxhlet) and therefore considered as efficient method. Microwave power of 100 W and extraction time of 10 minute using hexane as solvent effectively extracts omega-3 fatty acids. This

technique extracts good quality of oil but may effect on colour and odour of it. Due to high heat generation, it may leads to destruction of omega-3 fatty acids (Li et al. 2018).

1.2.14.3. Enzymatic hydrolysis technique

Among the various methods, enzymatic hydrolysis is suitable method of extraction which includes enzyme and water. Generally, protease, lipase and alcalase are used for extraction. Different temperature range, enzyme concentration and incubation time are important parameters in this technique. Enzymes catalyse hydrolysis reaction which produces more amount of omega-3 fatty acid than any other method (Iberahim et al.2018).

1.2.14.4. Wet rendering process

Wet rendering has been widely used extraction method having some advantages over other. By excluding harmful chemicals, less extraction cost, does not disturb the environmental balance and maintain the natural state of fish waste components. This process involves lower temperature for shorter time with centrifugation and higher temperature for longer time without centrifugation. It uses water as a solvent instead of harsh chemicals which results in cost effective, non-toxic and easily available. Thus, this technique is the green method for extraction of omega-3 fatty acids from fish waste material (Rahman et al.2018).

1.2.15. Applications of omega-3 fatty acids

1.2.15.1. Anti-inflammatory role

Omega-3 polyunsaturated fatty acids (PUFA) mainly EPA and DHA inhibit the synthesis of pro-inflammatory eicosanoids. By using different substrates, it uses same cyclooxygenase (COX) and increase the production of anti-inflammatory eicosanoids without blocking the COX. Due to slow action of omega-3-PUFA, takes more time for effect. It is the best alternative anti-inflammatory agent for long term chronic inflammatory stage. EPA and DHA minimize the pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6. It also initiates the cascade of adhesion molecules followed by injury. This causes increasing movement of immune cells, such as neutrophils, monocytes, B cells and T cells. In both chronic and acute inflammation omega-3 PUFA carried out inflammatory response by inhibiting the inflammatory hallmarks. It also inhibit section of lipopolysaccharides (LPS) in cardiac disease by limiting the nuclear factor kβ (NF-kβ) and toll-like receptor 4 (TLR-4) inflammatory pathways. Thus, omega-3 PUFA can be effective agent in cardiac inflammatory response (Ye and Ghosh 2018).

1.2.15.2. Omega-3 fatty acids and fetal development

Proper supplementation of EPA and DHA during pregnancy provides various benefits for infant. DHA with other nutrients were transferred through placenta from mother to foetus. The long chain omega-3 fatty acids, EPA and DHA play major role in fetal brain and retina development, cell membrane functions, eye and hand co-ordination. It also prevents the premature birth of baby by increasing gestation period. It is caused due to decreasing production of prostaglandin E2 and prostaglandin F2a thus; reduce the inflammation of uterus. It was also suggested that, EPA and DHA supplementation may protect children against allergies (Swanson et al.2012).

1.2.15.3. Anticancer effect

The two main omega-3 polyunsaturated fatty acids that are eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA) were studied extensively for its anticancer potential. Combination of these two fatty acids showed their anticancer activity by effecting multiple targets in distinct stages of cancer development consist of cell proliferation, cell survival, angiogenesis, inflammation, metastasis and epigenetic abnormalities that are important for development and progression of cancer. Daily uptake of 250-500 mg of omega-3 fatty acids decreases the risk of common cancers including skin, colorectal, prostate and breast. EPA and DHA have varying effects on cancer cells indicating their importance in daily routine. DHA initiates the cell death by suppressing cell proliferation while EPA reduces the loss of adipose tissue and protect skeletal muscle. The EPA and DHA efficiently prevent the cancer initiation and development, inhibit cell cycle, increases cell death, reduce inflammation, angiogenesis and metastasis by both epigenetic and genetic changes. Thus, provides supportive agent in anticancer treatment (Jing et al.2013).

1.2.15.4. Neuroprotective agent

There are some evidences that omega-3 PUFA have therapeutic potential in neurology and psychiatry. Omega-3 PUFA provides effective protection in spinal cord injury (SCI) and traumatic brain injury (TBI). Docosahexaenoic acid (DHA) is efficiently acts as a neuroprotective agent against injury. It provides protection to central nervous system (CNS) from damage by kainic acid. It reduces neuronal cell death, prevents cell loss, decrease oxidative stress by mild TBI, reduces trauma induces in cellular homeostasis and inflammatory process. Thus, improve the neurological function. In neurotrauma, it was observed that combination of proper amount of omega-3 PUFA immediately after injury provides a significant therapeutic potential because it helps in various mechanisms of body and provides effective benefits (Titus 2017).

1.2.15.5. Feed applications

Omega-3 polyunsaturated fatty acids is the vital nutritional component may utilized in poultry feed lead to better production, enhanced quality of chicken and poultry products which is also beneficial for human consumption. Poultry feed supplemented with omega-3 fatty acids has remarkable role in lowering the cholesterol and lipid content in blood and egg yolk. The increased content of fatty acids in poultry feed showed positive effect on chicken development. It helps in nervous system development, increase immunity, good quality of meat and eggs also maintain the normal functioning of chicken body. Thus, fatty acids will ultimately enter into human body which provides protection against cardiovascular diseases, having anti-tumor and anti-inflammatory effects. Direct incorporation of omega-3 PUFA in poultry feed increases the amount of important fatty acids in broiler chicken meat. Utilization of fatty acids leads to neutralization of oxidants and causes increase in antioxidant level which minimizes effect of oxidative stress. Thus, use of omega-3 fatty acids in poultry feed supplements increases the nutraceutical value of poultry products and also provides health benefits (Alagawany et al.2019).

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CHAPTER II

MATERIALS AND METHOD

2.1 Chemicals

All chemicals used in this study were highly purified, analytical grade and purchased from local suppliers. Bovine achilles tendon collagen, type I calf skin collagen, p- dimethylaminobenzaldehyde, Bovine serum albumin, standard docosahexanoic acid (DHA), coomassie brilliant blue R-250 gallic acid, quercetin and DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) were purchased from Sigma-Aldrich, USA. Acrylamide, bisacrylamide, agarose, ammonium persulphate, Bio-Rad precision plus protein standard (10-250 kD), leucine, hydroxyproline, gelatin, NaCl (sodium chloride), NaOH (sodium hydroxide), $CuSO_4$ (copper sulphate), Na_2CO_3 (sodium carbonate), $FeCl_3$ (ferric chloride), folin ciocalteu reagent, EDTA (ethelenediamine tetra-acetic acid), TEMED (N,N,N,N'- tetra-methyl ethylenediamide), IMVIC test reagents, ascorbic acid, chloroform, methanol, butanol, acetic acid, lactic acid, formic acid, phosphoric acid, oxalic acid, sodium dodecyl sulphate and all other chemicals required for current study were brought from Hi-media and Sisco Research Laboratories (SRL) chemicals, India. The ingredients used for value added food products preparation were of food grade.

2.2. Optimization of extraction method

The yield of interested end product is influenced by the different extraction conditions. Acidic extraction is commonly employed method for collagen and gelatin recovery from fish waste. Type of solvent, its concentration, time of extraction and temperature as well as recovery conditions has effects on final yield of product. Hence, optimization of these conditions is necessary for maximum recovery. The one factor at a time method is employed for extraction of acid soluble collagen, gelatin and omega-3 fatty acid from fish waste.

2.2.1. One factor at a time method

Many scientists and engineers make use of one factor at a time (OFAT) method for designing experiments, in which only single factor is varied at a time while other factors remains unchanged and response is recorded. This technique requires more experiments to obtain results hence its very lengthy and time consuming. The changes of one constituent at a time in increasing way only describe about their influence on the production (Czitrom 1999).

2.3. Extraction methods

2.3.1. Acid soluble collagen extraction

The ideal procedure for extraction of collagen from marine sources is acid solubilisation. In this protocol weak acids are effectively utilized for collagen recovery. Acetic acid is referred as most significant solvent for extraction with 0.5 M concentration and it was called as acid soluble collagen (ASC). It was proposed that acidic environment resembles positive charge primarily on collagen molecules. Solubilisation of collagen in the extracting acidic solution mainly depends upon the concentration of acid utilized and its swelling properties (Kiew and Don 2013). Thus, the higher repulsive forces between precursor tropocollagen lead to better solubilisation of collagen in extracting solvent.

2.3.2. Acid mediated gelatin extraction

Gelatin is by-product of collagen formed after its partial hydrolysis by heat treatment. Gelatin derived from acid treated precursor referred as type A while type B gelatin was derived from alkali treated precursor. Acid pre-treatment enable to remove impurities from waste so 0.1 M sodium hydroxide (alkali) effectively utilized to remove non-collagenous protein impurities. After this, sample was subjected to acid extraction followed by final extraction with water. The various organic acids were employed for acid mediated gelatin extraction from waste. Concentration of acid and time of heating influences on gelatin yield. Heat treatment during extraction causes disruption of hydrogen bonds in collagen molecule and results in irreversible solubilisation of three dimensional structure of collagen to form gelatin (Kim et al.2020).

2.3.3. Solvent extraction of omega-3 fatty acid

Among all commercial techniques, solvent extraction followed by enzymatic concentration is the only method carried out on laboratory scale. Interaction among the solvents and hydrophobic or hydrophilic regions of molecule causes pure lipids soluble in multiple solvents. The main principle of solvent extraction includes addition of solvent to the reaction mixture which helps to remove one or more components from it. Chloroform: methanol is preferred as binary solvent system, generally employed for efficient extraction of fish oil from waste. Chloroform-methanol may be the best lipid extractant and gives more yield than other solvents, but it is absolutely not safest from health and environmental aspects, thus the research is carried out to find out best replacement for it (Mohanarangan 2012).

2.4. Purification techniques

After the production of sufficient amount of product, next important step is its purification. Steps involved in purification of collagen, gelatin and omega-3 fatty acid can be summarized as below.

2.4.1. Precipitation

The basic step used for protein purification and concentration is precipitation by using salts. It is carried out by changing solvent conditions in order to alter the solubility of interested protein than other proteins in reaction sample. Generally, ammonium sulphate is mostly preferred salt for precipitation and it's more advantageous than any other precipitants because of its good ability to stabilize protein structure, highly soluble, comparatively inexpensive, easy availability of pure material and lesser density of a saturated suspension than any other salting out components (Burgess 2009).

Increase in salt concentration results in saturation of solution which causes decrease in solubility of protein and it get precipitated in the reaction solution called as "salting out". Increase in salt concentration at low ionic strength results in increasing solubility of proteins referred as "salting in". Some examples of precipitants other than ammonium sulphate include NaCl, TCA, ethanol and acetone etc. After precipitation it was subjected to dialysis to remove unwanted substances that may be interfere with the further experimental procedures.

2.4.2. Centrifugation

Centrifugation is a mechanical process that utilizes an applied centrifugal force to separate the components of a mixture according to density and/or particle size. Random Brownian motion results in net movement of solute or suspended particles from regions of higher concentration to regions of lower concentration, a process called diffusion. Thus, diffusion works in opposition to centrifugal sedimentation, which tends to concentrate particles (Zheng et al.2013). The centrifugal force is proportional to the rotation rate of rotor. The centrifugal force (speed of centrifugation) and time has effects on separation. The more centrifugal force causes effective separation between small and large proteins but may cause structural changes in protein structure.

2.4.3. Dialysis

Process of dialysis separates molecules from solution based on their diffusion rate through a semi permeable membrane. Figure no.2.1 depicted the process of dialysis. Technique efficiently eliminates unwanted particles and prevents their possible interference in further purification process. Unwanted particles and small molecules

diffuse through semi-permeable membrane i.e. dialysis bag, into the adjoining medium (buffer or water) leaving behind the protein into dialysis bag. This technique is also useful for concentrating solution.

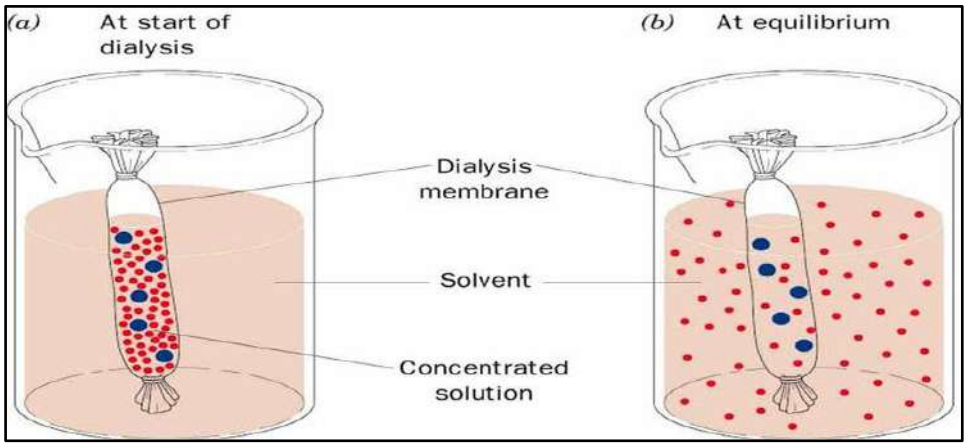

Figure 2.1.: Process of dialysis (Source: Wikipedia)

2.4.4. Filtration

In filtration process solid particles in a liquid or gaseous fluid are removed by use of a different filter medium (Whatmann's filter paper) which permits fluid to pass from it and retains solid particles and separation of mixture achieved. These filter papers useful for qualitative or quantitative analysis of biomolecules or fine separation mixtures. It has different paper grades o which it can be used for various applications. Whatmann filter paper composed of cellulose fibres of high quality cotton containing more alpha cellulose which gives high wet strength and not allows any impurities to enter into the filtrate or sample. Hence, filtration is a basic purification technique for any biological samples or mixtures.

2.5. Characterization techniques

Various characterization techniques were employed for efficient characterization of macromolecules extracted from fish waste. The biochemical and biophysical analysis determines structural and functional properties of respected molecules.

2.5.1. Ultra violet-visible spectroscopy (UV-Vis)

UV-vis spectrophotometer is a scientific tool useful in structural elucidation of bio-organic molecules. It is a powerful technique for superior characterization and quantification used in scientific research. This technique has advanced properties like accuracy, efficacy, quick analysis (speed), easy handling and cost effective. UV-vis

spectroscopy is an absorption spectroscopy involved ultraviolet and visible spectral region employed for quantitative and qualitative investigation (determination) of different metal ions, organic constituents and biological macromolecules. The principle behind it is, Beer-Lambert law based on relation between concentration of sample concentration and intensity of light. This law stated that absorptive capacity of a dissolved substance is directly proportional to its concentration in a solution. Whereas, Lambert law states that path length and concentration of sample is directly proportional to the absorbance of the light.

At fixed path length, concentration of sample in solvent can be determined by measuring absorbance of the respective solvent. Absorbance was measured when electrons in solution get excited at lower transition to upper transition state. Molar extinction coefficients or calibration curve can be resolved for quantification. Therefore, analyte concentration of solution is calculated at fixed path length by computing its absorption and also implemented for qualitative as well as quantitative determination of protein and nucleic acid at absorption wavelength 280 nm and 260 nm respectively. The mechanism of UV-visible spectroscopy was showed in figure no.2.2.

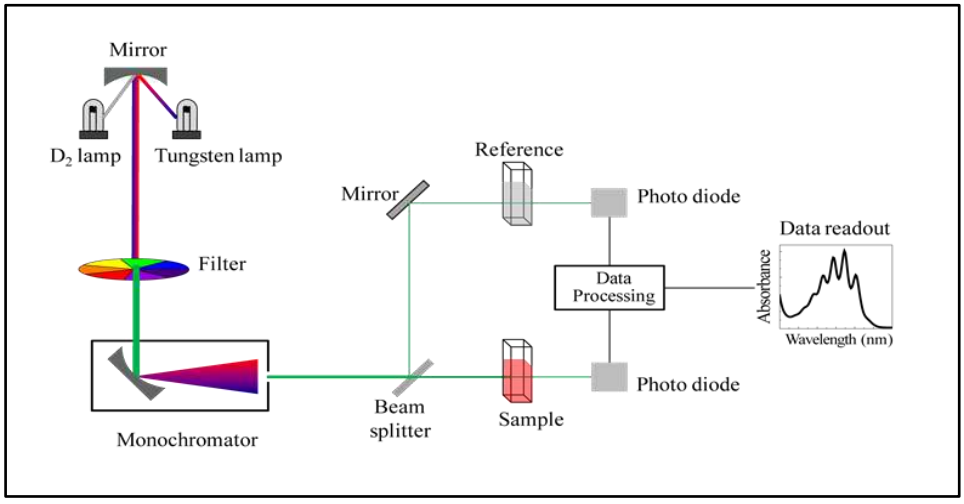

Figure 2.2.: Mechanism of UV-visible spectroscopy (Source: Wikipedia commons)

2.5.2. SDS-PAGE electrophoresis

Gel electrophoresis is most frequently executed for electrophoretic separation of biological molecules. Technique gives good results with better resolution and is comparatively easy to carry out than any other separation procedures. Thus, it is generally employed for qualitative as well as quantitative determination of the protein

molecules (Garfin 2003). An analytical approach of SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) is normally applied for molecular weight determination based on electric mobility. Mainly in this technique influence of properties of biomolecules are important like nature (amphipathic), charge of molecule and pH of medium. Figure no.2.3. illustrated the process of SDS-PAGE gel electrophoresis. By this qualities and properties it acts as an effective reducing agent employed to degrade native polypeptide into unfolded structure and gives negative charge to protein. Polymerization of acrylamide-bis-acrylamide forms gel like network for appropriate separation of proteins of precise size. Consequently, due to electric mobility charged protein fragments migrate from one site to another and separation was achieved. Separated proteins in gels are examined by staining with anionic dye CBB R-250 or by silver nitrate followed by destaining to visualize protein bands (Meyer et al.1965; Garfin 2003).

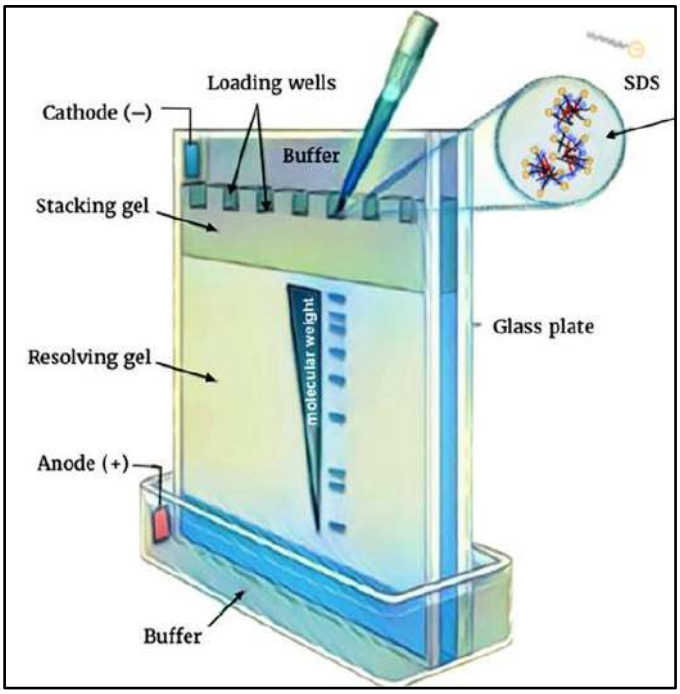

Figure 2.3.: Process of SDS-PAGE gel electrophoresis (Source: Researchgate)

2.5.3. Hydroxyproline content determination

Hydroxyproline evaluation is easy, colorimetric process employed to determine concentration of collagen as well as its partially hydrolysed product gelatin. An oxidizing agent p- dimethylaminobenzaldehyde brings out oxidation of hydroxyproline which results in formation of yellow colored end product. HyP is a prime imino acid helps in equilibration of triple helical structure of collagen. It also plays vital role in maintenance

of thermal stability of protein by establishing hydrogen bonds between polypeptides present in protein. Higher degree of denaturation temperature of collagen and gelatin is related to the more amount of hydroxyproline in it (Rosenbloom et al.1973). HyP is the main component of collagen and it is present in very less concentration or totally absent in any other proteins, thus HyP determination is used to calculated collagen from waste material.

2.5.4. Fourier transform infrared spectroscopy (FTIR)

Fourier transform infra-red spectroscopy is the analytical method applied for the qualitative and quantitative exploration of various composites using infrared absorption of molecules and also gives details about secondary arrangement of proteins (Pelton and McLean 2000). Mechanism of FTIR spectroscopy was given in figure no.2.4. Due to the slow speed of scanning, conventional IR instrument is not suitable for fast data processing thus, FTIR spectroscopy is invented. Basically, FTIR is mostly related to the vibrations and stretching of molecules which have distinctive vibrational frequencies in the IR range and therefore, it is suitable for identification of entire organic molecular assemblies and constituents. The FTIR frequency range is between 4000-400 cm^{-1}. Infrared radiations were absorbed by the bonds existing in the atoms in the molecule and its bending as well as stretching is occurred to generate FTIR spectrum.

Infrared radiations fall on sample and part of it was absorbed while remaining part of radiations is transmitted through the sample. As a result of this, molecular fingerprint of analyte was generated by combination of absorption and transmission peaks. Upon bombardment with the IR radiations, molecules get excited and shifted to the higher energy level from lower energy level. During this transition energy transfer is occurred with the help of molecular bond vibrations, rotations, electron ring shifts and translations. Each specific component has its characteristic vibrational frequency in the infrared region. The FTIR is a quick method utilized for structural analysis of that give complete information about collagen at its molecular level like nature of functional groups, forms of connection and conformation between bonds.

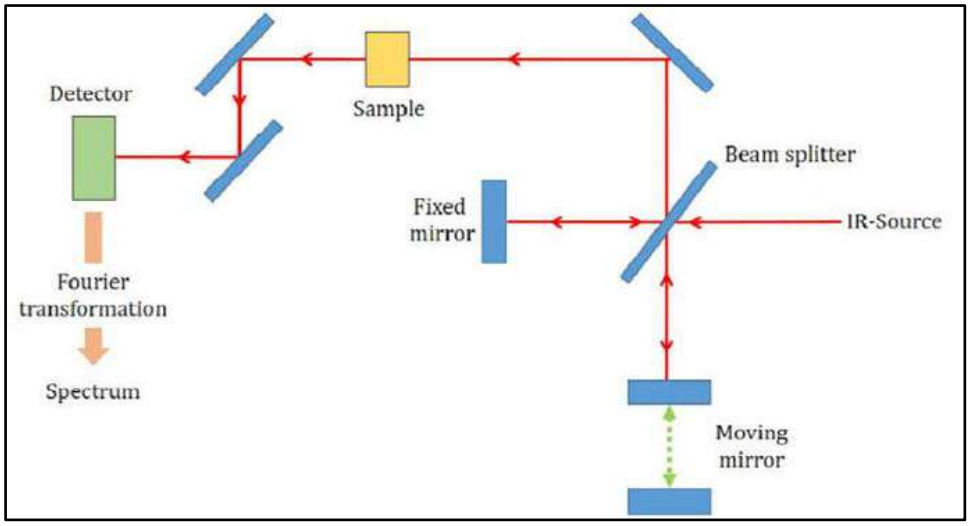

Figure 2.4.: Mechanism of fourier transform infrared spectroscopy (Source: Researchgate)

2.5.5. X-ray diffraction technique (XRD)

X-ray diffraction (XRD) is more convenient analytical technique to acquire information about arrangement of crystalline substances like crystal size and lattice spacing. The diffraction pattern of compounds is used for its identification and characterization. In XRD, monochromatic radiation (X-ray) was fall on atom and photons in it was scattered in every lattice phase. The constructive interference is created by scattered rays that construct peaks for the particular sample. The mechanism of XRD was given in figure no.2.5. XRD is used to analyse diffraction patterns of different biological components, thin films or powder, solid etc. Collagen contains more amount of imino acid and repeating (Gly-X-Y) motif in sequence and therefore it displays triple-helical conformation with semi crystalline organization as well as also interpret distribution and alignment of collagen fibril (Bigi et al.2001).

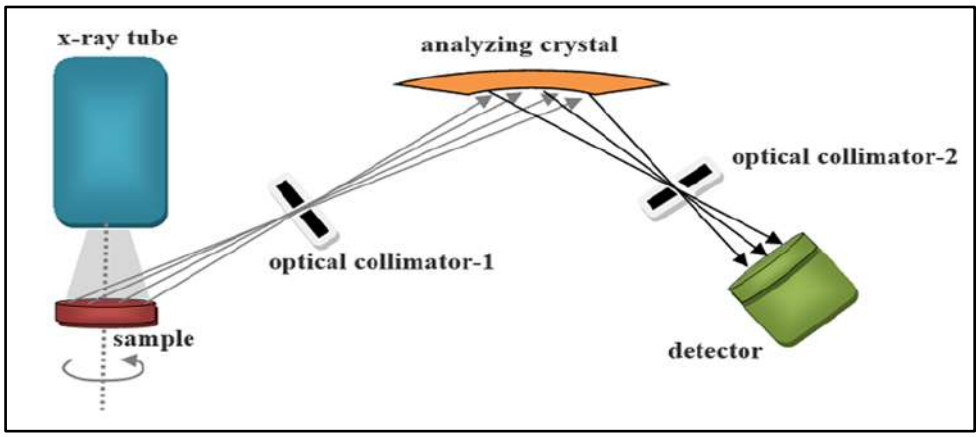

Figure 2.5.: Mechanism of X-ray diffraction (Source: Nuclear ecology)

2.5.6. Scanning electron microscopy (SEM)

Scanning and transmission electron microscopy (SEM, TEM) is the modern analytical techniques of microscopy. SEM studies surface of various materials, chemicals as well as biological samples to compute and assesse fine particulars of it through image analysis. The narrow electron beam produces images of better resolution and magnification with more depth of field thus, helps to evaluate surface topography of the analyte. In SEM, electron beam is focused on the sample surface and upon interaction it will generate different signals associated with surface topography and composition of sample. For SEM characterization of biological material, it should be totally dry and secured with chemical fixative for firmness and structural integrity. After that, the material was covered with gold on stab and placed on sample analyser stage of electron microscope to generate good resolution image of it. Figure no.2.6 represented the diagram of SEM. Thus, this microscopy analyse orientation of collagen fibrils and provides information about its structural properties (Raspanti et al.1996; Starborg et al. 2013).

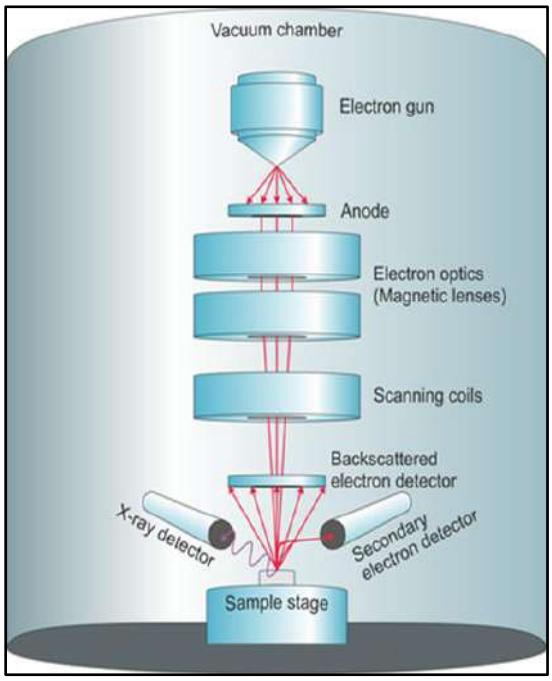

Figure 2.6.: Diagram of scanning electron microscopy

(Source: Applied micro spectroscopy)

2.5.7. Differential scanning calorimetry (DSC)

DSC is an important thermo analytical method employed for characterization of the temperature dependent conformational changes in structure of proteins as well as other biomolecules. The capacity of a material to store energy is referred as heat capacity

(Cp) which is determined by DSC as function of the temperature. Block diagram of DSC was shown in figure no.2.7. Basically, it evaluates the heat capacity difference between two cells under the continuous and concurrent heating of two cells (Ibarra-Molero and Sanchez-Ruiz 2006). Several industries like printing, polymers, pharmaceuticals, paper, semiconductors, food, manufacturing and electronics broadly utilizes DSC. This technique helps to measure the different parameters comprising induction period of oxidation, reaction energy and temperature, heat of melting and fusion, glass and crystalline region conversion temperature, specific heat and energy and also determines melting as well as denaturation temperatures.

The stable and continuous increase in the temperature, results in start of unfolding of protein while the alteration in heat flow raises significantly and attains an extreme point (Tm) and after complete unfolding it will start to decline, generating a peak in the heat flow *vs.* temperature graph. The purified protein exhibited only single peak which describes the heat absorption related to the denaturation of the protein (Ibarra-Molero and Sanchez-Ruiz 2006). Hydroxyproline content present in collagen helps in the thermostability of collagen which can be assessed by differential scanning calorimetry (Komsa-Penkova et al.1996). This method records the change of calorimetric energy due to heating procedure that effects on collagen denaturation. The continuous heat results in unfolding of protein by absorbing heat and DSC was recorded (Bruylants et al.2005).

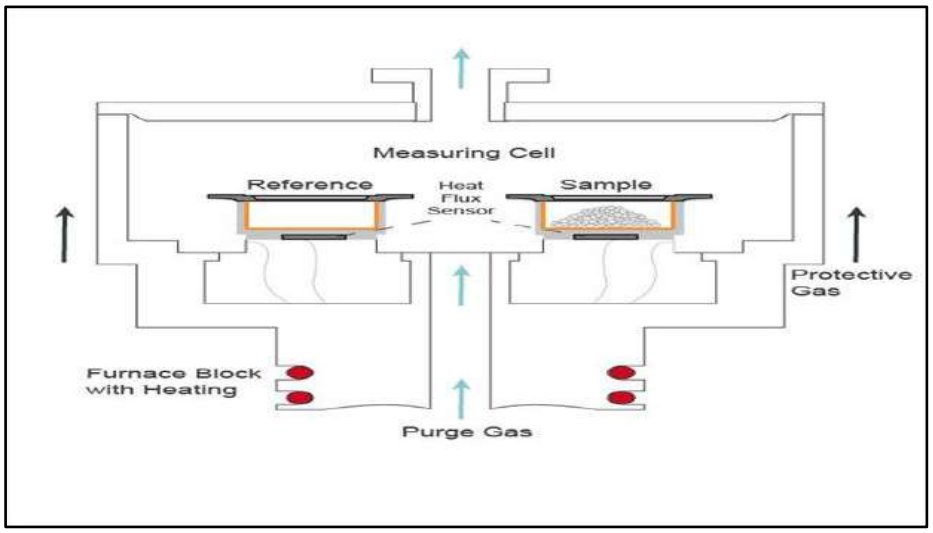

Figure 2.7.: Block diagram of DSC (Source: Particle technology labs)

2.5.8. Particle size analysis

Particle size analyser with zeta potential is used to measure particle and molecular size from less than a nanometre to several microns using dynamic light scattering and

zeta potential by using electrophoretic light scattering. Particle diameter has effect on zeta potential. Because particles of small diameter are easily affected by the random movement of fluid flow and other particles, then the absolute value of effective zeta potential of small particles is greater than that of large particles. Particle size analysis is employed to analyse the size distribution of particles in a given sample. It can be applied to study particle size of solid materials, suspensions and emulsions. The most common techniques to determine particle size distribution are dynamic image analysis (DIA), static laser light scattering (SLS, also called laser diffraction), sieve analysis and dynamic light scattering (DLS). The particle size analyser instrument is based on different technologies such as high definition image processing, analysis of Brownian motion, gravitational settling and light scattering (Rayleigh and Mie scattering) of the particles. In biological field, it is used to measure size of protein aggregation.

2.5.9. Zeta potential

Electrostatic repulsion helps in the stabilization of maximum aqueous colloidal structures. Interaction between the charged colloidal particles causes interactive repulsion at prolonged distances. Some colloidal particles exhibited particular ionisable assemblies that are covalently attached to their surfaces. The pH of the particles varies according to the pH of the surrounding medium, thus it may have positive, negative or net zero charge. The pH of the medium has intense effect on the net charge of the suspended particles and also provides stabilization during its aggregation. Zeta potential is to evaluate total charge of the colloidal particles. Zeta potential is the potential difference existing between the surface of a solid particle immersed in a conducting liquid (e.g.: water) and the bulk of the liquid. Figure no.2.8 depicted the mechanism of zeta potential.

Micro electrophoresis method is utilized to define the zeta potential of colloidal suspensions. The voltage is applied through a couple of electrodes at both end of the cell comprising the particle diffusion; velocity of the charged particles is recorded and determined as unit field strength as their mobility. Particles having high positive or negative zeta potential repelled from each other and causes very less flocculation of particles in suspension. The particles with less zeta potential unable to inhibit the accumulation of particles. Particles having zeta potential value between -10 mV and +10 mV may acts as neutral while particles having value much negative than -30 mV or more positive than +30 mV are generally behaves as steady particles (Clogston and Patri 2011).

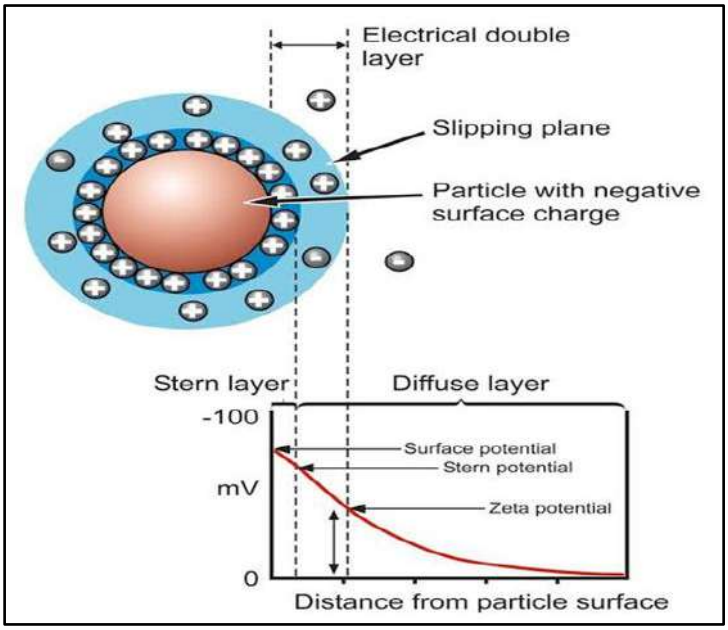

Figure 2.8.: Mechanism of zeta potential

(Source: Encyclopaedia of membranes)

2.5.10. Gas chromatography mass spectroscopy analysis (GCMS)

Chromatography is commonly utilized practise for the evaluation of saturated, mono and polyunsaturated fatty acids in foods as well as biological samples. It is beneficial technique because of rapid separation of mixtures comprising several types of acids, along with more sensitivity given by various types of detectors, such as mass spectrometers (Brotas et al.2020). GC-MS is an instrument that associates the features of gas-chromatography and mass spectrometry to detect different organic compounds presents in the organic material, which contains alkanes, fatty acids, alkenones, sterols etc. The Gas Chromatography/Mass Spectrometry (GC/MS) distinguishes chemical mixtures (the GC component) and recognizes the constituents at molecular level (the MS component). GC can separate various volatile and semi-volatile compounds but not always specifically distinguish them whereas MS can selectively distinguish many compounds but not always separate them. It has very low detection limit that is about sub-ng (nanogram) is measured.

Currently, in GC-MS ion source is directly inserted into capillary column. At 300ºC the effluent comes out from the GC (temperature may goes up to 400ºC). When effluent (single component) release from the GC, it gets enter into electron ionization detector which is commonly used MS detector. Ions can be generated by ion trap or time-of flight. Upon production, these ions are bombarded by a stream of electrons results into its fragments. The mass to charge (M/Z) ratio is calculated as mass of the fragment

divided by the charge. The charge is always +1 while M/Z ratio signifies the molecular weight of the fragment. The quadrupoles of the computer scan (continuous cycle) these fragments one at a time until the range of M/Z is retrieved. This will create the mass spectrum which is a graph of signal intensity (comparative abundance) versus M/Z ratios (particularly molecular weight). Each component has a specific fingerprint and software is easily available to offer a library of spectra for unknown components (Sneddon et al.2007). Figure no.2.9 showed the block diagram of GCMS.

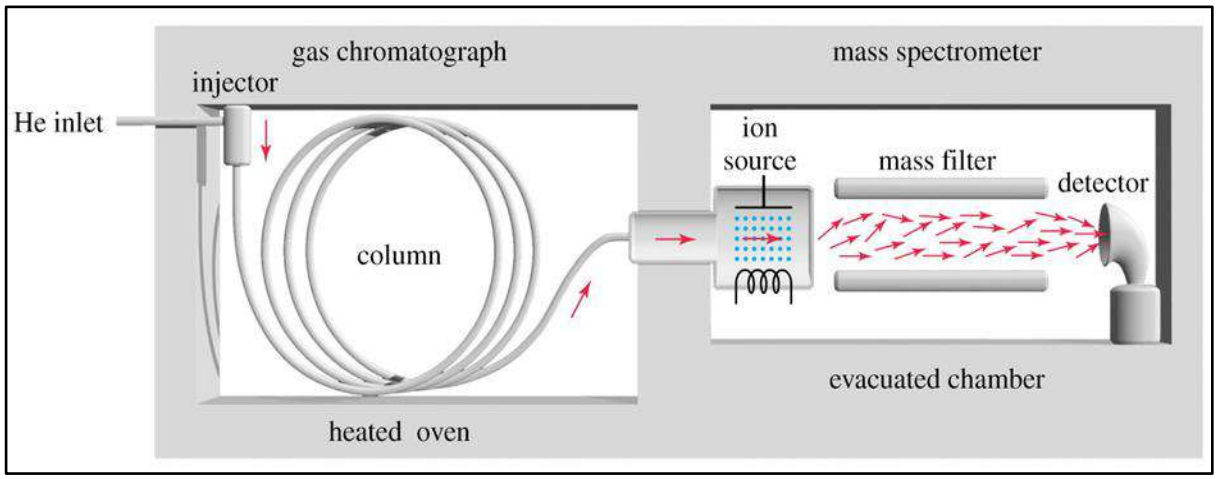

Figure 2.9.: Block diagram of GCMS (Source: Organic spectroscopy)

2.6. Molecular identification of microorganism

16S rDNA sequencing procedure is commonly employed for molecular identification of microorganisms particularly bacteria. This method offers sequence of isolated bacteria and after that it was compared with previous bacterial sequences present in database for identification. BLAST (Basic Local Alignment Search Tool) can be utilized for query sequence comparison with known sequences in several databases (Altschul et al.1997).

2.6.1. Phylogenetic analysis

Phylogenetic analysis is the study of the evolutionary development of a species or a group of organisms or a particular characteristic of an organism. The query sequence was compared with already known 16S rDNA sequences present in GenBank database. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura 1980). The 16S rDNA gene sequence was employed to carry out BLAST with the database of NCBI GenBank database. Based on maximum identity score first ten sequences were selected and aligned using multiple

alignment software program Clustal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 7.

2.7. References

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Objectives of thesis:-

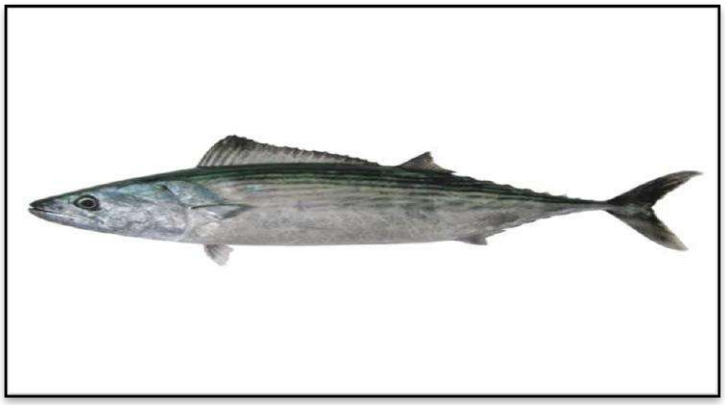
- Collection of fish waste from fish processing industries.

- Extraction, purification and characterization of collagen, gelatin and omega-3 fatty acids extracted from fish waste.

- Application of residual fish waste.

Classification of fish

Kingdom	:- Animalia
Phylum	:- Chordata
Class	:- Actinopterygii
Order	:-Scombriformes
Family	:- Scombridae
Genus	:- *Sarda*
Species	:- *orientalis*
Common name	:- Gethar



CHAPTER III

EXTRACTION, CHARACTERIZATION AND PURIFICATION OF COLLAGEN FROM WASTE MATERIAL OF GETHAR (*Sarda orientalis*)

3.1. Introduction

Fishing industry is one of the conventional food sector which offered adequate quantity of food to consciously increasing population. Fish and its foodstuffs are one of the most-traded food commodities worldwide. The fishery industry in India rises speedily day by day. India stands 2nd and 7th place worldwide in case of fish captures from inland and marine sources respectively. Between these two, fish production through inland sources increases tremendously indicating requirement of fish as a food source in non-coastal areas of India (FAO 2014). The fish processing is the agro based occupation that make use of fishery products as a raw material to generate new products. During fish processing, waste material also produced which increases environmental difficulties. For example, fishing industry utilizes more amount of water (80%) for cleaning purpose, thus generates 20 m^3/tonnes of waste water (Kurniasih et al.2018).

An ample quantity of waste (50% to 70%) generated from original fish raw materials during processing of fish products. This waste contains scales, skin, bone, viscera and head which composed of high nutritive components which can be supplemented as feedstuff or fertilizer along with conventional products. The improper disposal of waste into water bodies or dumping site may disturb ecological balance. Sometimes, poisonous gases may release from it which creates offensive odour. Therefore, optimal exploitation of these wastes for manufacturing of value added by-products is a promising approach to upsurge the income of producers and to minimize the disposal management cost of these wastes (Nagai and Suzuki 2000 and Wang et al.2007).

Collagen is a most important structural protein in extracellular matrix of numerous connective tissues in the body (i.e. skin, bones, ligaments, tendons, and cartilage). It is endogenously synthesized protein by organisms made up of three helical chains of amino acids. The polypeptide chains composed of repeated sequence of (Gly-X-Y)n, in which X and Y positions are usually occupied by proline and 4-hydroxyproline.The Gly-Pro-Hyp is the most common tripetide (10.5%) found in collagen (Shoulders and Raines 2009; Davison et al.2019; Silva et al.2014).

In current scenario, the commercial collagen is easily obtained from skin and bone of bovine and porcine. Due to the outbreak of bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and foot-and-mouth disease (FMD), there are some limitations on consumption of collagen and supplemented products produced from these animals. Also there are some ethical issues behind the use of collagen extracted from bovine and porcine sources. Thus, there is need to find out

alternative source of collagen especially from aquatic/marine origin to overcome these problems (Gaurav and Suresh 2015).

Therefore, the more research is carried out for the utilization of marine by-products as it is a potential source of collagen. These marine derivatives offer some advantages such as simple extraction, more collagen content, easy absorption by human body due to its small molecular weight, biocompatibility, minimum risk of animal and pathogenic diseases, eco-friendly, low amount of biological contaminants and toxins, fewer religious and ethical limitations as well as less regulatory and quality control complications (Jafari et al.2020).

Collagen extracted from marine origin has good biocompatibility, biodegradability, better cell attachment properties and weak antigenicity. Thus it has many applications in food, cosmetic, pharmaceutical, tissue engineering and biomedical sectors (Gomez-Guillen et al.2011). Collagen and collagen containing hydrolysate possesses superior gelling ability, texturizing, solidifying, and water holding capacities. Also it has swelling and solubility capabilities, emulsifying and foaming abilities, adhesion-cohesion properties, protective colloid function and film developing capacities. Due to this wide range of different properties, it generally utilized in food, cosmetic and pharmaceutical fields (Gaurav and Suresh 2015). The surrounding environment and body temperature of fish species has direct effect on thermal stability and applicability of collagen from several sources. In pharmaceutical sector, the collagen can be employed for generation of wound bandages, transparent implants and as transporters for drug delivery. It acts as a healing agent to treat burn patients, for reconstruction of bone as well as for surgical purposes related to dental, orthopaedic and cosmetic region. Also utilized for formation of edible coverings in meat processing industries (Mahboob et al.2014).

Due to good antioxidant potential, collagen is added into the skin moisturizing cosmetic products and can be analysed by numerous techniques, comprising DPPH (1,1-Diphenyl-2-Picrylhydrazyl), FRAP (Ferric Reducing Antioxidant Power), and CUPRAC (Cupric Reducing Antioxidant Capacity). Antioxidants are composites that attach to free radicals and very reactive molecules to prevent its oxidation which results in prevention of cell destruction. Collagen and some other peptides act as good antioxidant agent. The use of collagen containing products in cosmetics is the best treatment to decrease oxidative stress on the skin (Ardhani et al.2019). This supplementation can protect normal skin cell function by inhibiting free radical accumulation. Biomolecules and their

hydrolysate having strong antioxidant potential is widely applicable in anti-aging research as it reduces the oxidative stress which is the key reason of aging (Nurilmala et al.2019).

Gethar (*Sarda orientalis*) also called as striped bonito. It is tuna like fish of length 102 cm. It belongs to species of marine perciform fish and family *Scombridae*. It is distributed through the Indo-Pacific and East Pacific region and occurs at depths from 1 to 167 meters from sea surface. The present work was designed for; (i) collection of fish waste; (ii) optimization of extraction method; (iii) extraction of acid soluble collagen and (iv) purification and characterization of collagen for further potential applications.

3.2. Materials and methods

3.2.1. Collection of waste

The skin, fin, tail waste of *Sarda orientalis* (Gethar) were collected from fish market Ratnagiri (MS, India) and Gadre fish processing industry, Ratnagiri (MS, India) by maintaining suitable conditions. The same type of fish waste was collected from local fish market Kolhapur for further studies.

3.2.2. Proximate analysis of fish waste

The standard methods approved by AOAC (1980) were carried out to determine proximate composition of fish waste. The content of moisture, fat, ash and protein was evaluated on wet (WWB) and dry weight basis (DWB).

3.2.3. Pre-treatment of waste

The collected waste was separated into skin, fin, tail and other waste parts. These are washed twice with cold tap water and scrapped with knife to remove flesh and other contaminants. After that, waste was washed with cold distilled water and cut into small pieces with the help of scissor for collagen extraction. All process of pre-treatment and extraction were carried out at 4^{o}C.

The cleaned waste material was subjected to pre-treatment by NaOH and butyl alcohol according to method of Kumar and Nazeer (2013) with slight modifications. The non-collagenous proteins were removed by 0.1 M NaOH. The waste pieces (10 gm) were soaked into 0.1 M NaOH with 1:30 (w/v) ratio for 24 hr. The solvent was changed after every 4 hr. After incubation period, the soaked material was washed with cold demineralized water till neutral pH to remove traces of alkaline solution. Then, deproteinized waste material was subjected to defatting by 10% butyl alcohol with 1:30 (w/v) for 48 hr. The defatted residue was washed with cold demineralized water till pH 7.0 and used for further extraction.

3.2.4. Effect of NaOH

Pre-treatment with NaOH was useful for the removal of non-collagenous proteins on fish waste. To study the effect of NaOH, the method of Widowati (2017) was slightly altered. The waste pieces of fish (5 gm) were soaked in 0.1 M NaOH solution (1:30 w/v) for 24 hr. The NaOH solution was changed after 4 hr interval to know the effective time for removal of non-collagenous impurities. The protein concentration was measured by Lowery method using BSA as a standard.

3.2.5. Extraction of ASC from waste and effect of extraction conditions

ASC (acid soluble collagen) was extracted according to method of Bhuimbar et al. (2019) with minor modifications. Entire process of extraction was carried out at 4ºC. The optimization of method was carried out with respect to acid type, concentration of acid and extraction time. The pre-treated fish waste was suspended in 0.5 M concentration of acetic, lactic, formic, phosphoric and oxalic acid and kept for incubation at 72 hr to carry out extraction. Also inorganic acids such as hydrochloric acid (HCl) and sulphuric acid (H_2SO_4) were tested for extraction. After swelling, the skin was squashed and viscous solution was filtered through muslin cloth. The clear solution was obtained by centrifugation at 5000× g for 20 min. The 2 M NaCl was used for the precipitation of protein. The precipitate was collected by centrifugation at 5000 × g for 20 min. The sticky gel like nature of collagen was dissolved in minimum volume of respective acid (1:5 w/v). The final solution was dialyzed against cold demineralized distilled water for 48 hr using dialysis bag MW cut-off 12 kD, lyophilized and used for characterization and applicatory studies. The acid which gives maximum collagen yield was utilized for further optimization studies. The molar concentration of acid was optimized from 0.1 M to 1 M while extraction time ranges from 4 to 72 hr. Also waste: acid ratio (w/v) was optimized. The optimization of process was carried out to determine suitable condition of extraction which gives maximum yield. The acid which gives maximum yield in less extraction time was further used for mass production of collagen from waste.

3.2.6. ASC yield and hydroxyproline content

Yield of ASC on wet weight basis was computed using formula:

$$\text{Yield (\%)} = \frac{\text{weight of freeze dried ASC (g)}}{\text{weight of initial skin (g)}} \times 100$$

Hydroxyproline analysis of collagen samples was accomplished by the method of Neuman and Logan (1949) using L-hydroxyproline as standard. Percentage of hydroxyproline was calculated as:

$$\text{Hyp (\%)} = \frac{\text{conc.of Hyp (µg ml}-1)}{\text{conc.of protein (µg ml}-1)} \times 100$$

3.3. Characterization of extracted ASC

3.3.1. UV Visible spectrum

The UV-Visible spectrum of acid extracted collagen was determined according to method of Zhang et al. (2011) using a Shimadzu spectrophotometer UV-1800, Japan in the range of 200-400 nm. 1 mg of ASC was dissolved in respective acid to determine UV-visible spectra.

3.3.2. Electrophoretic pattern of ASC

The separation of collagen fragment based on their molecular weight was carried out by using SDS-PAGE according to the method of Laemmli (1970). Polyacrylaminde gel was prepared using 8% resolving gel and 5% stacking gel. About 100 µl protein samples with gel loading dye was loaded into the gel wells and electrophoresis was carried out. After electrophoresis, staining was performed by coomassie brilliant blue R-250 followed by distaining. The bands were observed on gel and then it was compared with standard collagen to determine molecular weight.

3.3.3. Fourier transform infrared spectroscopy (FTIR)

The different functional groups attached to collagen during extraction were analysed by Fourier transform infrared spectroscopy. FTIR analysis of gethar extracted collagen was carried out using Nicolet iS10 Mid FT-IR spectrometer (Thermo electron scientific, Madison, USA) in the range of 500-4000 cm^{-1}.

3.3.4. Scanning electron microscopy (SEM)

The scanning electron microscopy was used to study structural morphology of acid extracted collagen (TESCAN, Czech Republic).

3.3.5. Differential scanning colorimetry (DSC)

The differential scanning colorimetry (DSC) analysis of Gethar extracted ASC was studied according to method of Liu et.al.2012. The DSC was performed on DSC Q20 V24.11 calorimeter (Netzsch-Geratebau GmbH, Germany). The instrument was standardized for enthalpy and temperature by using indium as the standard and the measurements were carried out when the samples were continuously eradicated with ultrahigh-purity nitrogen at 50 cm^3/min. The lyophilized protein sample was precisely weighed into aluminium pan, wrapped and scanned from 25ºC to 400ºC at a heating rate of 5ºC /min. For reference a vacant wrapped aluminium pan was used. The highest

temperature was noted by the software referred as maximum denaturation temperature (Tmax).

3.3.6. Particle size analysis of collagen with zeta potential

The zeta potential of ASC along with particle size analysis was evaluated according to method of Chen et al. (2016) with slight modifications. The particle size was helps to analyse size of collagen based on intensity.3 ml of collagen dispersion was prepared in distilled water and homogenized for its hydrolysis. The hydrolysed sample was subjected to particle size analysis. For zeta potential, collagen sample was dispersed in acetate solution (0.05gm/100 ml) and kept for stirring for 6 hr. The zeta potential and particle size analysis of sample was studied by using Malvern Zetasizer Ver. 7.11.

3.3.7. Amino acid analysis

The amino acid composition of extracted collagen was checked on a Waters-PICOTAG amino acid auto analyser high performance liquid chromatography (Model: Waters 501) connected to the automatic amino acid estimating software. The Waters-Pico Tag column (size:- 3.9×150 mm) was employed for this purpose.

3.4. Functional properties of ASC

The ASC with different functional properties is suitable for various applications in food, pharmaceutical and agricultural field. So there is need to analyse Functional properties of ASC which includes emulsifying and foaming properties, water holding capacity, turbidity and oil absorption capacity.

3.4.1. Turbidity of collagen

Turbidity of collagen was determined as per method of Shon et al. (2011) with little modifications. About 10% (w/v) collagen was dissolved in double distilled water (DDW) and kept undisturbed for 1 hr. After incubation absorbance was measured at 600 nm (Shimadzu spectrophotometer UV-1800, Japan) and it was expressed as turbidity of collagen sample.

3.4.2. Solubility of collagen

The method of Shon et al. (2007) was slightly modified to determine solubility of gethar extracted collagen. At neutral pH, 0.7 gm of collagen powder was dissolved in a centrifuge tube containing 7 ml of 10 mM imidazole buffer. Pre-wrapped (black paper) tubes were vortexes for 15 sec and kept in undisturbed condition for 5 min. After incubation tubes were centrifuged at 7000 × g for 25 min. The supernatant was removed and tubes were dried and weight was taken. The solubility was expressed as;

$$\text{Insolubility (\%)} = 100\ \frac{\text{insoluble sample weight}}{\text{sample weight}}$$

$$\text{Solubility (\%)} = 100 - \text{insolubility (\%)}$$

3.4.3. Viscosity of collagen powder

The methodology of Gomez-Guillen (2000) was little bit modified to determine viscosity of collagen sample. 5% (w/v) collagen was dispersed in distilled water (D/W) and heated at 70ºC with constant increase in temperature. The viscosity was recorded by Brookfield digital viscometer (Model DV-II) in centipoises (cP) units.

3.4.4. Determination of water-holding capacity

The water holding capacity of acid extracted collagen was determined according to method of Pan et al. (2018). 10 mg of collagen was dispersed in 1 ml D/W and allowed to incubate at 20ºC for 1 hr. The tube was vortexes after every 15 min. The resultant sample was centrifuged at 5000 × g for 15 min. The supernatant was removed carefully without disturbing pellet remaining at the base and this tube was weighed. Water-holding capacity is the ratio of the weight of the absorbed water per milligram of collagen sample.

3.4.5. Oil absorption capacity

Oil absorption capacity (OAC) of collagen was studied according to method of Wani et al. (2015) with slight changes. About 0.5 gm of collagen was mixed with 10 ml soyabean oil and kept for incubation at room temperature (RT) for 30 min. After incubation, centrifugation was carried out at 4000 × g for 25 min at RT The volume of supernatant (free oil) was measured and OAC was calculated as gm of oil/gm of collagen.

$$\text{OAC (gm)} = \frac{V0 - V1}{W}$$

3.4.6. Emulsifying properties of ASC

The method of Pearce and Kinsella (1978) was executed for determination of Emulsion activity index (EAI) and Emulsion stability index (ESI) of collagen with minor modifications. In this experiment, 6 ml of collagen (1%, 3% and 5% w/v) and soyabean oil (2 ml) was mixed and homogenized for 10 min. After emulsion formation 100 µl sample was pipetted at 0 and 10 min then mixed with 5 ml of 0.1% SDS solution. The absorbance of solution recorded instantly (A_0) and after 10 min (A_{10}) at 500 nm using a spectrophotometer (Shimadzu UV-1800 Japan). The following formulae were used to calculate EAI and ESI;

$$\text{EAI } (m^2g^{-1}) = \frac{2 \times 2.303 \times A0}{0.25 \times \text{protein weight in g}}$$

$$\text{ESI (min)} = \frac{A0}{\Delta AXt} \quad \text{where } (\Delta A = A0 - A10,\ t = 10 \text{ min})$$

3.4.7. Foaming properties of ASC

The process utilized by Celik et al. (2019) was used to determine foaming capacity (FC) and stability (FS) of collagen. 1%, 3% and 5% w/v collagen solution was homogenised for 5 min at RT. After foam formation, the whipped solution was transferred to 25 ml measuring cylinder. Total volume of beaten solution was recorded immediately at 0 min and after 30 min by incubating it at 20ºC. The FC and FS of collagen was determined by using following formulae;

$$\text{Foaming capacity (\%)} = \frac{VT - V0}{V0} \times 100$$

$$\text{Foaming stability (\%)} = \frac{Vt - V0}{V0} \times 100$$

3.4.8. Effect of pH on collagen solubility

The effect of pH on collagen solubility was evaluated by method of Rodriguez et al. (2014) with minor modifications. 1 ml of collagen dispersion was taken into an Eppendorf centrifuge tube and pH was adjusted from 2.0 to 14 with either 6 N HCl or 6 N NaOH. The final volume of each pH tube were adjusted to 1.5 ml with distilled water and adjusted to same pH. The reaction mixtures were centrifuged at 5000 × g for 25 min at 25ºC and protein content was determined by Lowery method. Relative solubility was calculated with respect to pH having highest solubility.

3.4.9. Effect of NaCl on collagen solubility

Effect of NaCl on collagen solubility was studied to determine effective concentration of NaCl at which precipitation of collagen in acidic solvent was occurred. For this purpose, the methodology of Jongjareonrak et al. (2005) was used with some changes. About 10 ml of acidic solvent was precipitated by using NaCl ranging from 0.5 M to 3 M concentration. After precipitation all reaction mixtures were centrifuged at 5000 × g for 25 min at 25ºC and protein content was determined by Lowery protocol. Relative solubility of each sample was calculated with respect to NaCl concentration having highest solubility.

3.4.10. Sensory assessment

The sensory analysis of fish waste extracted collagen was carried out as per protocol of Aichayawanich and Parametthanuwat (2018) along with calf skin collagen as control. The sensory characteristics like appearance, color, fishy odour and overall

acceptability were tested by non-trained 7 member panel from Food Science and Technology department, Shivaji University, Kolhapur using 5 point hedonic scale (from 1:- dislike very much; 2:- dislike slightly; 3:- neither like nor dislike; 4:- like slightly and 5:- like very much).

3.5. Result and discussion

3.5.1. Proximate composition of fish waste

The Ward and Courts (1977) stated that, pre-treatment process during extraction eliminate several cross linked constituents present on waste as well as used to remove impurities and unwanted ingredients. More values of protein and ash were observed when raw material taken on dry weight basis for analysis. However, proximate composition may be influenced by seasonal variation and habitat of fish. Proximate composition like moisture, protein, lipid and ash content of fish waste on wet and dry weight basis were tabulated in table no.3.1. The result showed that, gethar fish waste contains 65.11±0.27% moisture, 34.69±0.38% protein, 6.48±0.24% lipid and 1.20±0.05% ash on wet weight basis while 15.79±0.27 moisture, 72.07±0.56 protein, 8.39±0.18 lipid and 2.52±0.28 ash was calculated on dry weight basis. The obtained value is slightly similar to the proximate composition (%) of small spotted catshark (Blanco et al.2019), shark, rohu and tuna (Hema et al.2013) as well as deep and shallow sea orbicular batfish (Pan et al.2018).

Samples	Moisture (%)	Protein (%)	Lipid (%)	Ash (%)
WWB	65.11±0.27	34.69±0.38	6.48±0.24	1.20±0.05
DWB	15.79±0.27	72.07±0.56	8.39±0.18	2.52±0.28

Values were means ± SD from triplicate determinations

Table 3.1.: Proximate analysis of fish waste

3.5.2. Effect of NaOH on non-collagenous protein removal

The non-collagenous proteins were removed by pre-treatment process. High temperature may degrade or denature collagen so, all experiments was carried out at cold condition (4ºC). For pre-treatment, alkaline solutions were preferred instead of acidic. The alkaline solutions were more effective to remove non-collagenous proteins and have lower level of protein loss than using acidic (Suptijah et al.2018). During pre-treatment, reaction between fish waste and NaOH take place which results in chemical changes in solution. The de-proteinase action was occurred on fish waste hence, excess NaOH turn to turbid during soaking condition. By the action of NaOH, the disruption of telopeptide

region on collagen molecule was happened which causes enlargement of fish tissues and successive removal of protein. High NaOH concentration with prolonged soaking time will increases the amount of dissolved proteins. Thus, it may dissolve collagen protein along with non-collagenous proteins (Zaelani et al.2019). The figure no.3.1 depicted the decrease in non-collagenous protein concentration within 24 hours. The results were confirmed that, pre-treatment with 0.1 M NaOH for 24 hr effectively eliminate non-collagenous impurities without damaging collagen structure.

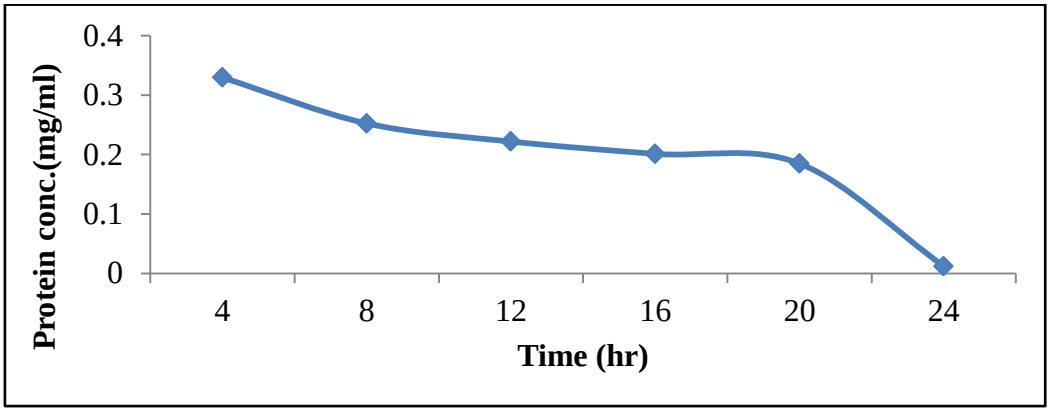


Figure 3.1.: Effect of NaOH on non-collagenous protein removal

3.5.3. Influence of extraction parameters on collagen yield

There are numerous parameters such as acid type, molar concentration of solvent, extraction time and waste to acid ratio affect the yield of collagen from fish sources. Effect of each of these parameters should be considered and optimized to determine suitable conditions to extract collagen.

3.5.3.1. Effect of acid

The different fish waste like skin, tail and fin were subjected to acidic extraction by using various organic and inorganic acids. The figure no.3.2 gives the general extraction procedure of collagen. The swelling of collagen was occurred in acidic solvent and loses its fibrillar appearance, thus generates viscous solution. Figure no.3.3 showed, the effect of acid on collagen yield. Among organic acids, lactic acid exhibited maximum collagen yield (34%) followed by acetic acid (26%), formic (6%) and phosphoric acid (2%). Cheng et al. (2009) reported that, utilization of inorganic acid (hydrochloric and sulphuric acid) for the extraction of collagen from animal tissues results in lesser efficacy and yield than the organic acids. It produces shorter amino acid chains of results in weak stability of collagen structure.

Liu et al. (2015) stated that, organic acid causes break down of inter strand crosslinks in collagen and solubilisation of chain. The exposure of protein to acid induces its hydration. The solubility of protein totally depends on type of acid, ionic strength and pH which effects on swelling characteristics. The H+ ion of acid causes entry of water in collagen fibres and enriches its recovery from the raw material (Gomez-Guillen 2001; Skierka and Sadowska 2007). Oxalic acid was also an organic acid but is not preferred for extraction because it disrupts the collagen structure during precipitation. The yield was less than collagen from skin of medusa fish extracted by lactic acid which was found to be 45% (Bhuimbar et al.2019) but more than 13.6% of collagen from black pomfret extracted by acetic acid (Maboud et al.2014).

The yield variation of collagen is due to difference in fish species, protein content of fish, its habitat conditions as well as pre-treatment and extraction method (Junianto et al.2018). Sometimes, combination of acid with enzyme (pepsin) may increase the extraction yield (Munasinghe et al.2014). Alkali was not suitable for extraction of collagen because it lost the ability of collagen to produce fibrils at neutral pH under physiological conditions (Hattori et al.1999).

Figure 3.2.: Collagen extraction procedure from fish waste

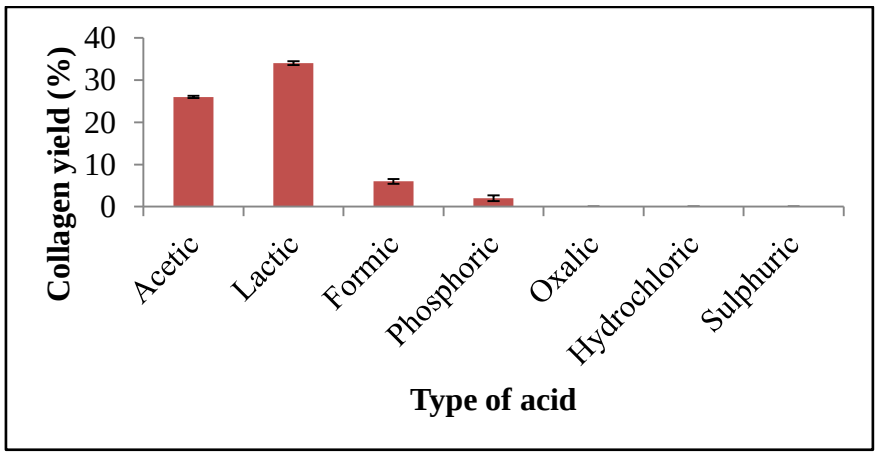


Figure 3.3.: Effect of acid on collagen yield

3.5.3.2. Effect of solvent concentration

Among various organic and inorganic acids employed for extraction, lactic acid gives more yield. Hence, optimized to find out its effective molar concentration ranging from 0.1 to 1 M while other component remains constant. Mostly collagen extraction was carried out using 0.5 M acetic acid because of its high productivity (Wang et al.2008; Veeruraj et al.2012; Tamilmozhi et al.2013), indicating 0.5 M is the effective concentration for extraction. In current study, 0.2 M lactic acid (11.46±0.23%) extracts more collagen than other acids (figure no.3.4) but slightly reduced at 0.3 M and 0.4 M that is 11.21±0.46% and 11.04±0.26% respectively. Comparatively better yield was obtained till 0.7 M (9.43±0.33%) beyond this it was start to decrease and very low yield was recorded at 1 M (1.28±0.41). It was found that, the increasing acid concentration beyond 0.7 M decline collagen yield due to degradation by excess acid (Jafari et al.2020). The similar study was carried out using 0.1 M to 1 M acetic acid on the collagen yield of sole fish skin (Arumugam et al.2018) and hybrid catfish skin (Kiew and Don 2013).

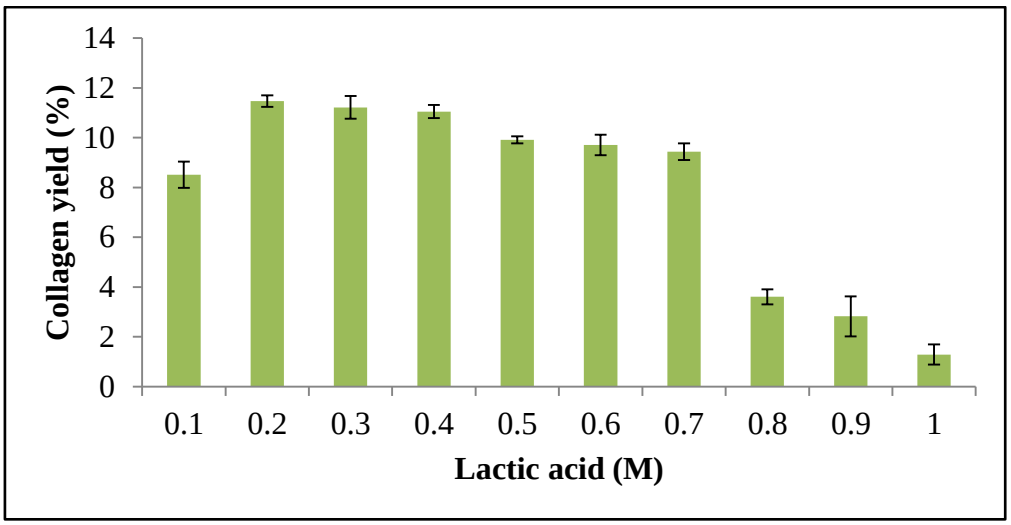


Figure 3.4.: Effect of solvent concentration on collagen yield

3.5.3.3. Effect of extraction time

The high yield of collagen was obtained for 0.2 M lactic acid, thus it was selected to optimize extraction time ranging from 4 to 72 hr. Extraction is strongly influenced by diffusion mechanism, which is time-dependent. The mass transfer rate explains the impact of extraction time on collagen yield. Therefore, increased extraction time is related to the more recovery of collagen. However, prolonged extraction period may cause disintegration of the peptides. In this condition, collagen polypeptide chains were

broken by the acid solution resulting decomposition of it and reduce the final extraction yield.

Additionally, lengthy extraction time would make the extraction procedure inappropriate for industrial scale-up (Jafari et al.2020). The effect of extraction time on collagen yield (%) was shown in figure no.3.5. It was found that, the yield was increased up to 11.44±0.53% within 40 hr. Later on, slight decrease was observed and further, it was constantly improved up to 72 hr (9.91%±0.44). Thus, optimum extraction time for gethar waste collagen was recorded as 40 hr with maximum yield. The obtained result was closely related to extraction time of sole fish skin collagen in which 36 hr gives more yield and after that it reduced (Arumugam et al.2018). But, reported extraction time in present study was more than 15 hr for collagen from wami tilapia (Alfaro et al.2014).

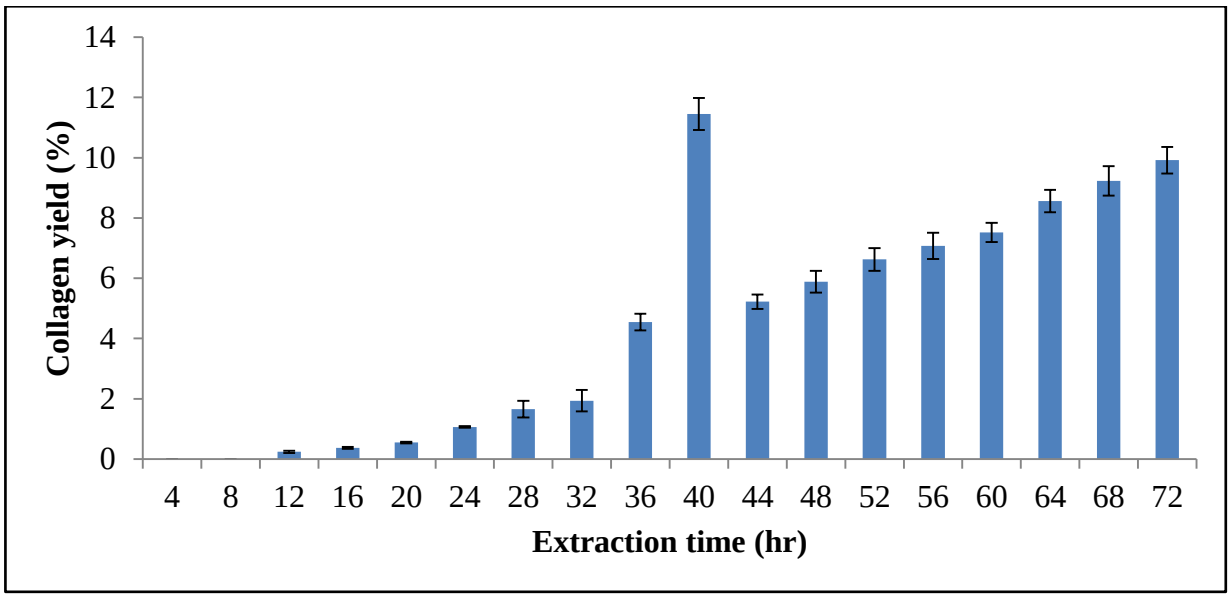


Figure 3.5.: Effect of extraction time on collagen yield

3.5.3.4. Effect of solid to liquid (S/L) ratio

The solid to liquid ratio is the quotient of quantity of solid collagen source divided by liquid solvent utilized for extraction. Commonly, increasing solvent amount results in improved interaction between free protons and amino acids of collagen chain thus stimulates separation of crosslinks present in the collagen helix. The reduced S/L ratio rises depolymerisation rate of peptides, since a more amount of acid causes disintegration of collagen chains, thus generates high quantity of lower molecular weight peptides (Arumugam et al.,2018). The figure no.3.6 depicted the effect of S/L on collagen yield. The 1:30 ratio gives maximum yield of collagen (12.59±0.42) and further it was decreased. The obtained ratio was less than 1/10 of collagen from skin of tilapia

and cod (Salamone et al.2016; Carvalho et al.2018) but higher than 1/60 of collagen from grass carp skin and giant croaker (Liu et al.2015; Coelho et al.2017). As the amount of solvent is improved, the fish tissue will be exposed to a more quantity of new solution, thus enhancing the degree of solubilisation of collagen peptides (Jafari et al.2020).

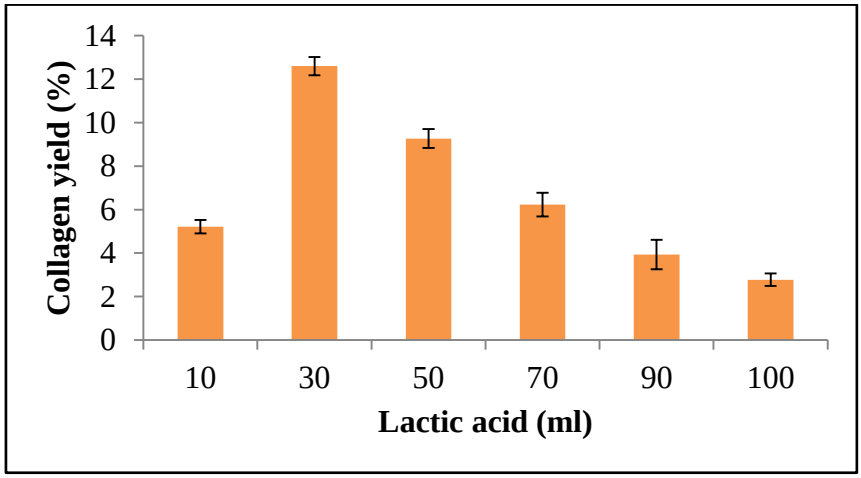


Figure 3.6.: Effect of solid to liquid ratio on collagen yield

Additionally, collagen extraction temperature was kept constant at 4ºC and it was depends on type of substrates used. The denaturation temperature of collagen was between 30-40ºC and therefore, it was more beneficial to carry out overall extraction procedure at 4ºC. The constant extraction temperature avoids degradation of protein and maintains its structural integrity (Coelho et al.2017; Jafari et al.2020).

3.5.4. Hydroxyproline content

The hydroxyproline (HyP) content is directly proportional to protein amount and is the major amino acid present in collagen (Lopez et al.2018). Hydroxyproline is a marker imino acid that stabilizes triple helical collagen structure. Additionally, hydroxyproline is exclusively present in collagen and showed very insignificant concentration in other proteins and can be used to estimate collagen quantitatively. Hydroxyproline content of collagen obtained after different acid treatments was tabulated in table no.3.2. Lactic, acetic, formic and phosphoric acid extraction exhibited 5.83±0.36 mg g^{-1}, 3.38±0.14 mg g^{-1}, 2.77±0.46 mg g^{-1} and 1.95±0.30 mg g^{-1} of hydroxyproline respectively. The lower amount was observed in phosphoric acid treated sample due to loss of collagen during extraction. HyP was detected in oxalic acid as well as HCl and H_2SO_4 extracted sample as they were ineffective for collagen extraction. The HyP amount in all acid samples was much less than swim bladder of catfish (Lopez et al.2018).

Sample	Hydroxyproline mg g^{-1} sample
Lactic acid	5.83 ± 0.36
Acetic acid	3.38 ± 0.14
Formic acid	2.77 ± 0.46
Phosphoric acid	1.95 ± 0.30
Oxalic acid	00
Hydrochloric acid	00
Sulphuric acid	00

Table 3.2.: Hydroxyproline content of ASC

3.5.5. Structural properties

3.5.5.1. UV-Visible absorption spectra

The basic and simple way to characterize collagen is to scan the sample by using UV-visible spectroscopy with range 200-800 nm. The UV visible spectra of lactic acid extracted ASC was given in figure no.3.7 with absorption maxima at 269 nm. The protein has maximum absorption at 280 nm but tryptophan did not present in collagen while very less amount of tyrosine was detected. The spectrum was nearly similar to collagen from skin of tuna, dog shark and rohu (Hema et al.2013; Kumar and Nazeer 2012).

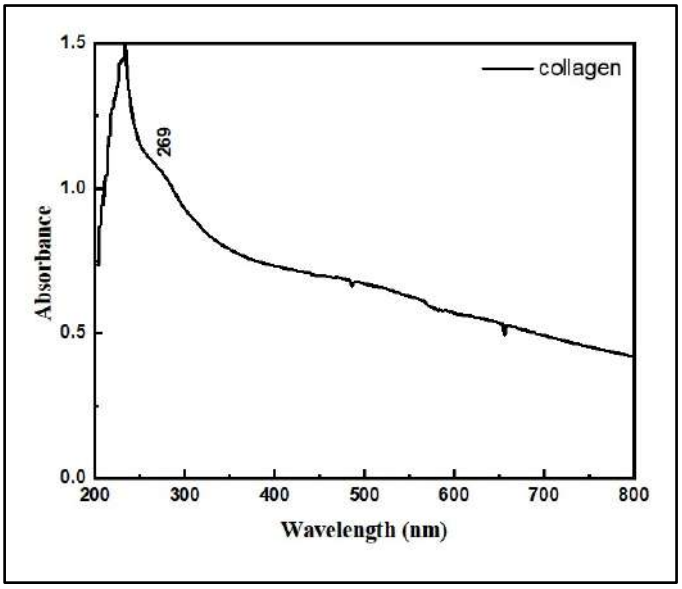


Figure 3.7.: UV–Vis absorption spectra of ASC

3.5.5.2. SDS-PAGE analysis

The figure no.3.8 depicted the SDS-PAGE analysis of acid extracted collagen along with standard calf skin collagen. Depending on electrophoretic mobility and subunit arrangement, it was concluded that collagen from gethar fish waste was type I collagens and were composed of monomer of β chain and dimer of α chain (α1 and α2). The bright β band was observed due to association of 2 α subunits while light band was because of α subunit (Seixas et al.2020). The presence of two different subunits confirmed that extracted collagen is the type-I (Ogawa et al.2003). Similarly study was observed in collagen from skin of black pomfret, nile tilapia and cuttlefish (Maboud et al.2014; Potaros et al.2009; Krishnamoorthi et al.2017) exhibited two α chains (α1 and α2) and one β component in the structure. The band pattern of collagens extracted using various acids are similar with that of standard.

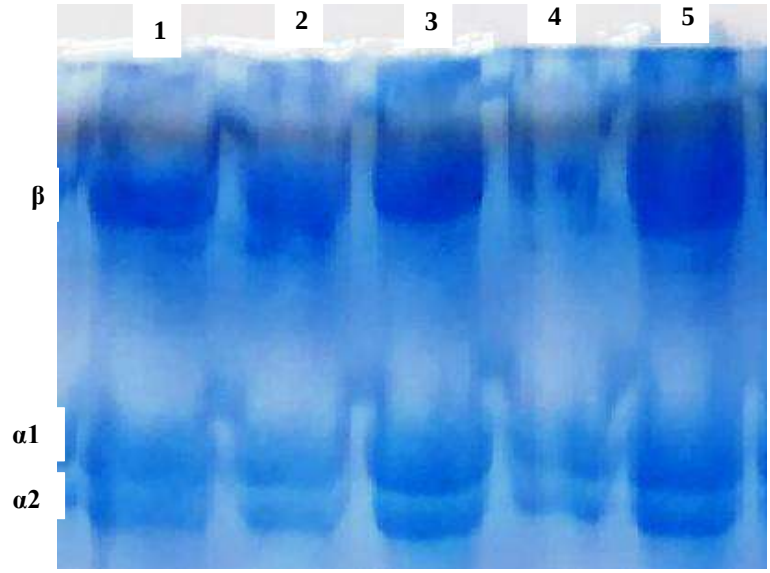


Figure 3.8.: SDS-PAGE of collagen: lane 1-standard calf skin collagen and lane 2-5 showed collagen extracted using lactic acid, acetic acid, phosphoric acid and formic acid respectively

3.5.5.3. FTIR spectroscopy analysis

The Fourier transform infrared spectroscopy is used to analyse various functional groups attached to protein. The figure no.3.9 depicted FTIR spectra of gethar waste extracted collagen. The weak stretching present at 1216 cm-1 corresponds to the vibrational modes of amide III functional groups (Payne and Veis 1988). The stretching at 1612 cm-1 represents the amide I bond. The broadening of –OH group was analysed between 3000-4000 cm-1 wave number range. The deep widening at 3370 cm-1 was occurred due to amide A (N-H stretching). The –OH stretching band was disintegrated into fragments whose frequencies were correlated to various O-H bond lengths, i.e. water molecules forms array in the different vibrational energies (Bridelli et al.2017). The conformation of the protein backbone may have positive influence on FTIR spectra of protein molecules. The theoretical studies of the vibrational force fields of the polypeptide chain revealed that α-helix, β-sheet and β-turn structures generates distinct positions for the amide I, II, and III bands (Payne and Veis 1988). Similar type of spectra was observed for collagen from elasmobranch by-products (Seixas et al.2020) and bone of torpedo scad and tigertooth croaker marine fishes (Kumar et al.2012).

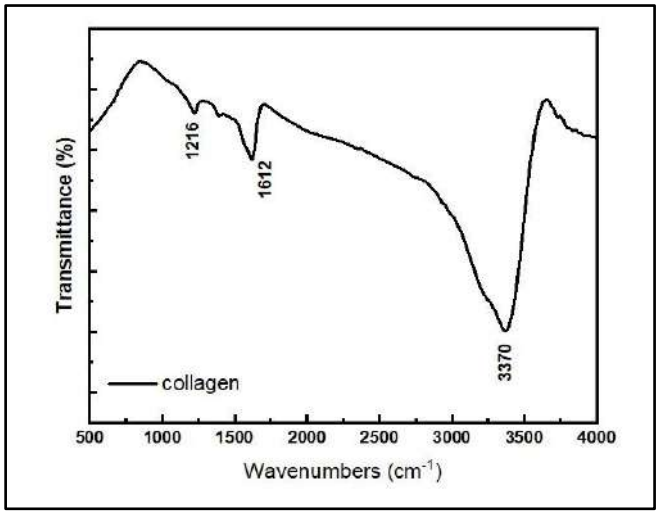


Figure 3.9.: FTIR spectra of ASC

3.5.5.4. SEM analysis

The SEM analysis of extracted collagen was shown in figure no.3.10. Under low magnification it exhibited highly porous nature with random windings of coil-like structures which specifies the fibrous nature of collagen with smooth and regular surface. At more magnification, coil-like fibrils were observed as a sheet interrelated to each other. The space between inter coiled sheets provides porosity to collagen, which will promote easy incorporation of any important drug into collagen structure (Shanmugam et al.2012; Arumugam et al.2018). The similar SEM structure was reported for collagen from scallop mantle, skin of cuttlefish and sole fish (Choi et al.2013; Shanmugam et al.2012; Arumugam et al.2018).

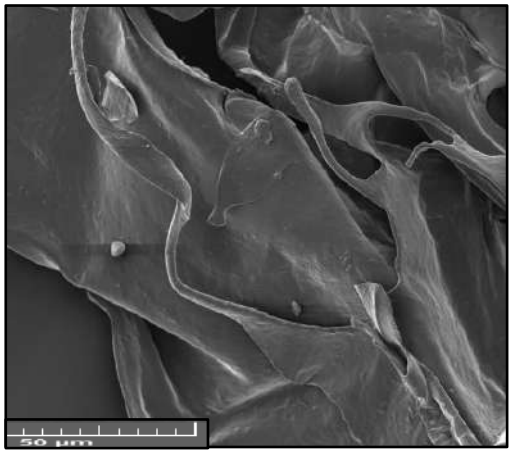


Figure 3.10.:SEM image of extracted ASC

3.5.5.5. DSC analysis

DSC remains as an incomparable method to evaluate the thermodynamic steadiness of proteins in a particular buffer condition. DSC determines heat capacity as a function of temperature. The DSC analysis of gethar extracted ASC was depicted in figure no.3.11 which exhibited single broad endothermic peak at 88.64ºC and three smaller peaks at 61.56 ºC, 129.82 ºC and 244.46 ºC. These peaks describe the thermal denaturation (Td) of collagen due to heating. The variation in thermal stability of collagen is occurred due to physicochemical changes during extraction. The 61.56ºC was observed because of alteration in covalent cross linkages which was more than 54.18°C and 59.17°C of chicken collagen (Akram and Zhang 2020) but less than bovine skin collagen (63-65ºC; Zhao and Chi 2009). The other peaks were associated to the constant conformational modifications of super helix and accordingly with the destruction of materials (Huang et al.2016).

The thermal stability of collagen was related to body and environmental temperature of fish habitat. The Td values determine the unfolding of helix assembly of protein to the random coil that alters the physicochemical properties of protein (Akram and Zhang 2020). It was studied that, thermal stability of collagen of various species depends on imino acid concentration (proline and hydroxyproline). The more number of imino acids imparts higher stability to collagen to maintain its helical structure (Abedin et al.2013). The Td values were accompanying with the amino acid profile and were justified with amount of hydroxyproline and proline (Okazaki and Osako 2014). The collagen with high thermal stability, good heat resistance and superior structural strength might be useful as probable substitutes for mammalian collagen (Huang et al.2016). Thus, extracted ASC possess good thermal stability so it can be utilized in nutraceutical, cosmetics and biomaterials product.

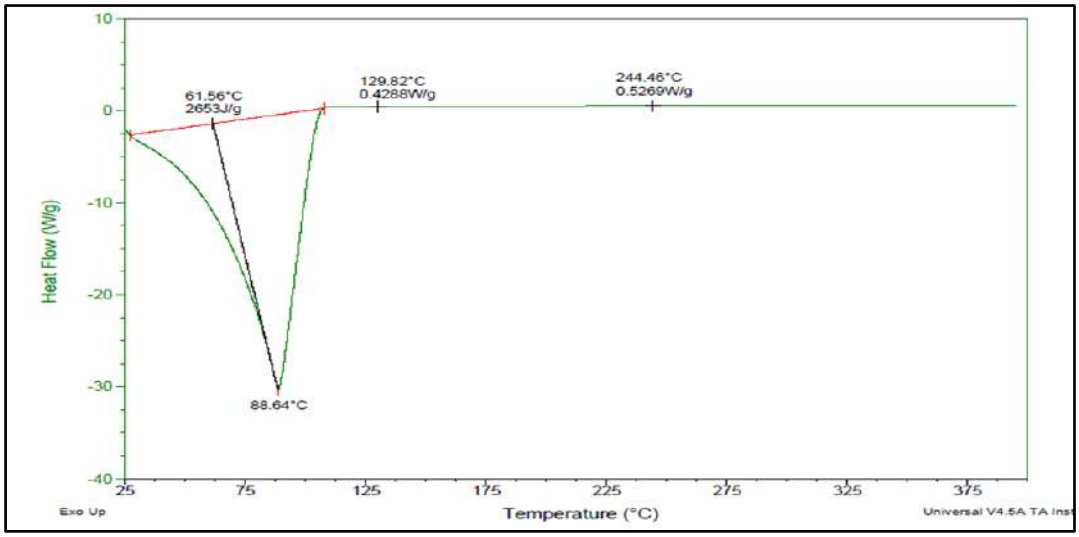


Figure 3.11.: DSC analysis of ASC

3.5.5.6. Zeta Potential

Generally, zeta potential technique was employed to study the surface net charge variation of the proteins along with collagen. At pH more than sample compound isoelectric point (pI); there is negative charge on surface of it. Usually, at the pH above and below of the protein pI, it possess net negative and positive charge respectively. This was occurred due to the de-protonation and protonation of the amino acids, respectively (Benjakul et al.2010). The protein has least solubility when the pH of mixture is same as to its pI. In aqueous sample, the protein has zero net charge while positive and negative charges counterbalances each other at their pI. At the pI, electrostatic repulsion will be reduced and precipitation as well as an aggregation of collagen is occurred. The zeta potential of gethar fish extracted ASC was illustrated in figure no.3.12 and it was recorded as -0.345 mV. The similar results were observed for collagen from skin of seabass (Sinthusamran et al.2013) and bamboo shark (Kittiphattanabawon et al.2005). Constant increase in pH results in decrease of charge on collagen. The pI of collagen was in the acidic range as it was related to the greater amount of acidic amino acids such as aspartic and glutamic than the basic including lysine, histidine and arginine. Also alteration in amino acids composition and distribution on the surface domains of collagen molecules may effect on pI (Muhammed et al.2018; Upasen et al.2019).

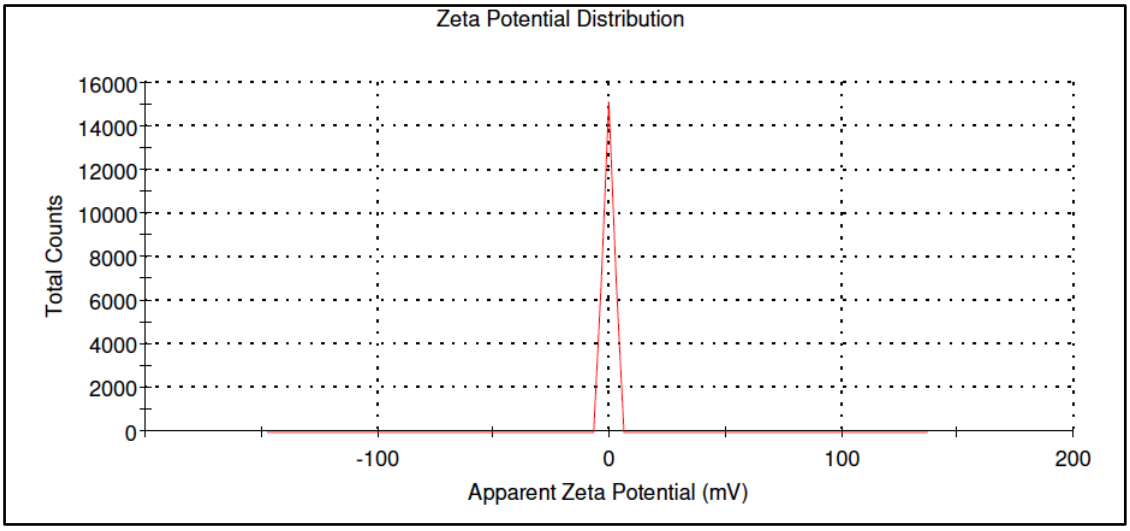


Figure 3.12.: Zeta potential of ASC

3.5.5.7. Particle size analysis

Particle size analysis was carried out to characterize size distribution of particles in a specific sample. The size distribution of gethar extracted ASC based on its intensity was represented in figure no.3.13. The mean particle size of ASC ranges from 3.12-14.51 d.nm (diameter values in nanometres).

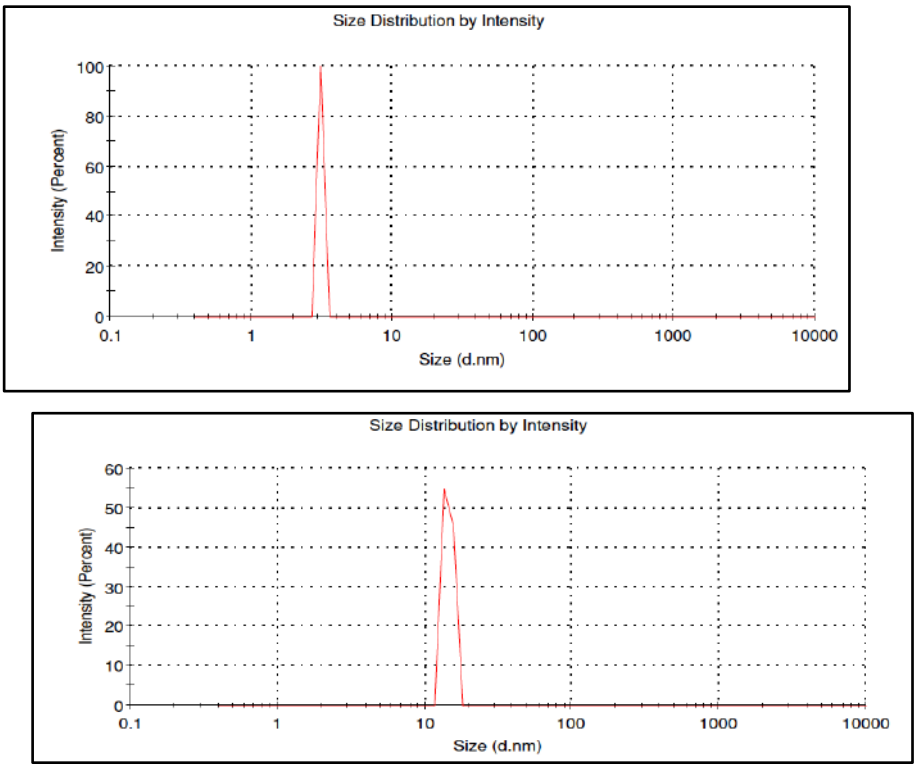


Figure 3.13.: Particle size distribution of ASC

3.5.5.8. Amino acid composition

The amino acid composition of gethar extracted ASC was tabulated in table no.3.3. The collagen contains more amount of proline, glycine (329 residues) and alanine (132 residues) while comparatively low amount of hydroxylysine (6 residues) which is general characteristic of collagen (Lee et al.2009). The stability of helices depends on content of imino acid. Therefore, the quantity of proline (111 residues) and hydroxyproline (82 residues) was found to be significantly higher and may have probable effect on thermal stability and structural integrity of collagen (Akram and Zhang 2020). The higher degree of proline hydroxylation effects on the triple helical structure of collagen (Xu et al.2017). About 6/1000 residues of hydroxylysine were reported in extracted ASC which was similar to collagen from shark skin (Kittiphattanabawon et al.2010). The analogous amino acid composition was observed in red snapper, black drum and sheepshead seabream skin collagen as well as *Axinella cannabina* and *Suberites carnosus* sponge collagen (Zaelani et al.2018; Ogawa et al.2003; Tziveleka et al.2017).

Amino acid	No.of amino acid per 1000 residues	% of amino acid
Alanine	132.33±1.24	13.23
Arginine	54.33±1.27	5.43
Aspartic acid	42±0.86	4.2
Cysteine	0	0
Glutamic acid	72.66±1.21	7.26
Glycine	329.33±1.24	32.93
Histidine	5.66±0.47	0.56
Isoleucine	14±0.89	1.4
Leucine	22.66±0.94	2.26

Lysine	26±0.86	2.6
Hydroxylysine	5.66±0.42	0.56
Methionine	15.66±0.89	1.56
Hydroxyproline	82±0.97	8.2
Proline	111.33±2.05	11.13
Serine	26.33±1.71	2.63
Phenyl alanine	5.33±0.14	0.53
Threonine	26.33±0.47	2.63
Tyrosine	5.33±0.54	0.53
Valine	23±0.81	2.3

Table 3.3.:Amino acid analysis of extracted ASC

3.5.6. Functional properties

3.5.6.1. Turbidity of ASC

Turbidity value reveals concentration of residual lipid and other colloidal material that were appear in collagen. As concentration of collagen increases, the turbidity value also increases and values are largely relying on performance of the clarification (filtration) process. The protein-protein interaction leads to the aggregation of particles having size larger than wavelength of light which results in more turbidity value. Protein aggregation in collagen dispersion occurred by heating at 60ºC for 30 min and increases the turbidity of solution (Kim et al.1994; Gomez-Guillen 2000). The turbidity of gethar waste extracted collagen was found to be 0.229±2. It was least than turbidity of skate skin collagen 0.28±0.04 (Shon et al.2011).

3.5.6.2. Solubility of collagen

The solubility of collagen was more at acidic pH (2.0-5.0) with relative solubility higher than 80% and decline suddenly at the neutral pH. Some collagens exhibited variation in solubility between pH 6.0 to 10.0. The difference in solubility of proteins with respect to pH was occurred due to variation in pI (Foegeding et al.1996). At acidic pH of 1.0, the some amount of collagen undergoes degradation results in solubility

reduction. At pH near to pI, there is reduction of molecular charges on collagen molecule occurred leads to declined solubility (Montero et al.1991). The solubility of extracted ASC was 57.23±0.37% while insoluble amount was 42.30±0.41% which was less than skate skin collagen (82.7±1.87%) (Shon et al.2011). The alterations in pH maxima for solubility among collagens from skin and bone might be happened due to various molecular characteristics and conformations between collagen while skin collagen exhibited a lesser degree of molecular cross-linkages and weaker connections than bone collagen (Kittiphattanabawon et al.2005).

3.5.6.3. Viscosity of collagen

The stronger electrostatic repulsion between molecular chains of collagen results in more viscosity which is important physicochemical characteristic of it. Type of acid used has impact on relative viscosity of collagen. The hydrogen bond balances triple helical alignment of collagen is irreversibly damaged and transformed into random coil arrangement. The destruction of hydrogen bonds was occurred due to heat treatment which minimizes viscosity of collagen. At high temperature molecules transfer quickly and enhance the molecular exchange, as thermal energy increases, molecules become extra moveable, thus reduces the viscosity (Lower 2013; Hadfi and Sarbon 2019). The large proportion of more molecular weight β and γ-chain in the solution at specific temperature causes increase in viscosity. But, the weak configuration may be formed due to reorganisation of α, β and γ-chain which could lead to the reduction in viscosity (Shahiri et al.2012; Pan et al.2018). The temperature more than 40°C disorganizes hydrogen bonds and random coil alignment of collagen molecule results in dissociation of it (Normah and Nur-Hani Suryati 2015). The viscosity of ASC was found to be 2.18±0.04 cP which was more than orbicular batfish (1.27±0.12 cP) but less than chilean mussels collagen (2.35±0.27 cP) (Pan et al.2018; Rodriguez et al.)

3.5.6.4. Water holding capacity

The solubility, particle size, micromorphology and physicochemical atmosphere of protein are responsible for variation in water holding capacity (Wu et al.2011). Proteins with more water absorption and retention capacities are estimated to have comparatively more polar residues with the property to produce hydrogen linkages with water (Hou et al.2012). Water holding capacity (WHC) is the function of proteins to inhibit water from being removed or excluded from their three-dimensional structure. Water binding and holding capacity is the very significant feature in development of confectionary food products. The sample having these two characteristics contains

amount of water in the form of hydrodynamic water, bound water and physically entrapped water. The polar and non-polar species attached to water showed effect on water binding properties. The increase in water holding capacity is observed due to the higher hydrophilic properties of lower molecular weight peptides (Barzideh et al.2014). The water holding capacity of gethar extracted ASC was recorded as 19.7µl/mg which was more than ASC from orbicular batfish (25.22 µl/mg) and tilapia scale (9.4-15%) (Pan et al.2018; Huang et al.2016). Most important functional characteristics of proteins are associated to their interconnection with water, thus the choice of proteins with a suitable WHC is prime in food development. The dominant protein-protein interactions and the zero net charge on protein results in lower value of WHC (Akram et al.2020).

3.5.6.5. Oil absorption capacity (OAC)

Oil absorption capacity is related to texture contributed by interaction between oil and other compounds. The OAC of a protein is correlated to its non-polar amino acid residues. Hydrophobic association among the non-polar amino acids of protein component and hydrocarbon residues on oil regulate OAC of protein. The OAC value determines the number of non-polar amino acids present on ASC. The result was in accordance with the contact angle investigation, where ASC possesses more hydrophobic groups than PSC (Chen et al.2019). OAC of gethar extracted ASC was found to be 12.2±0.21 gm/gm of collagen which is higher than OAC of scallop gonad protein (5.2 mL/g) (Han et al.2019) but much less than OAC of red stingray ASC (41.41 ±0.47 mL/g) (Chen et al.2019). As stated by Maruyama et al.(1998), proteins with greater value of OAC provide superior shape retention in food, such as meat or candy products ;thus the collagen extracted from gethar waste will be employed in nutraceutical industries.

3.5.6.6. Emulsifying and foaming properties

Emulsification denotes the mixing of two or more components to produce a uniform dispersion of performance in which particles in the form of a liquid, distributed in another liquid (Guo and Ruan 2006). It is an essential characteristic of protein which reflects the ability of protein to associate with water and oil to generate emulsion. It consists of emulsifying activity and stability. Emulsifying activity index (EAI) states that, the property of protein to produce emulsion in presence of water and oil while emulsifying stability index (ESI) is the ability of protein to conserve the oil-water emulsion without its separation and to resist the external stress situations (Zhang et al.2006). The collagen and other higher molecular weight polypeptides act as efficient stabilizer for protein film than lesser peptides in oil-in-water emulsion. Protein

hydrolysate behaves as surface-active materials that stimulate an oil-in-water emulsion due to its hydrophilic and hydrophobic moieties and their charges (Chi et al.2014). The reduction in repulsive intensity increases the probability of oil droplet aggregation. The oil droplet aggregation decrease the association between oil and water required for foaming, thus it decreases the EAI and ESI of collagen.

The foaming capacity (FC) of protein defines the function of protein to produce film at the air-water boundary. The protein with fast adsorption on newly formed air liquid interface throughout bubbling and causes unfolding as well as molecular reorganization at the interface, reveal improved foaming capacity than proteins that adsorb gently and obstruct unfolding at border (Damodaran 1997). The decline in FC and foaming stability (FS) may occur due to reduced solubility and weak electrostatic repulsion between the collagen molecules which was not sufficient to inhibit accumulation of molecules. This accumulation decreases the inter linkage among protein and water essential for foaming results in lesser FC and FS of collagen (Chen et al.2019). The relationship between protein and water has vital role in generation of foam and its stability. Ultrasound treatment can be applied to increase the FC and FS properties of collagen (Akram et al.2020).

As per table no.3.4, 1% ASC has EAI 15.30 m^2g^{-1} and ESI 27.29 min. Further increase in protein concentration from 1% to 5% lowers emulsion forming ability as well as stability. Oppositely, foaming capacity and stability of ASC was enhanced with increased protein concentration. The 5% ASC has 21 ± 0.87% foaming capacity and 15 ± 0.39% foaming stability (table no.3.4). Standard calf skin collagen has greater EAI, ESI and foam properties than extracted ASC; this might be because of source and purity of standard collagen. Remaining lipid in collagen has been recognized as a prime component to influence on emulsifying, foaming and flavour properties. Thus, elimination of residual lipids from collagen results in enhancement of these characteristics (Shon et al.2011). The similar results were obtained for ASC from black ruff skin collagen (Bhuimbar et al.2019). The emulsifying and foaming capacity of gethar waste extracted ASC was mush lesser than ASC from skin of brown bullhead and collagen hydrolysate from skin of spanish mackerel (Chen et al.2013; Chi et al.2014). Therefore, it can be concluded that studied ASC acts as an alternative source for commercial collagen. Due to its good emulsifying and foaming properties it can be employed in baking, beverages, and minor food ingredients (Chen et al.2019).

Sample	Emulsifying Activity Index	Emulsion Stability Index	Foaming capacity	Foam stability
	(m^2g^{-1})	(min)	(%)	(%)
1 % ASC	15.30 ± 0.04	27.29 ± 0.12	6.0 ± 0.02	2 ± 0.09
3 % ASC	8.13 ± 0.03	13.28 ± 0.04	16 ± 1.28	11 ± 2.4
5 % ASC	4.86 ± 0.03	11.32 ± 0.08	21 ± 0.87	15 ± 0.39
Calf skin collagen	56.23 ± 0.14	30.63 ± 0.03	30 ± 0.03	22.5 ± 0.95

Values were means ± standard deviation from triplicate determinations.

Table 3.4.: Emulsifying and foaming properties of ASC

3.5.6.7. Effect of pH on collagen solubility

Isoelectric point (pI) was used to determine influence of pH on protein solubility. When pH of protein aggregation is more or less than pI, then repulsive power between charged residues and chain of protein increases. Thus, it results in enhancement of protein solubility. When the pH is close to pI, then the overall charges on protein molecules are zero and there is rise in hydrophobic interaction. Therefore, it forms precipitation and aggregation of the protein molecule (Jongjareonrak et al.2005; Iswariya et al.2018). Singh et al. (2011) stated that most of collagen exhibited higher solubility in acidic environment and after that reduction was observed. The figure no.3.14 depicted the effect of pH (2-14) on the relative solubility of collagen. The ASC from gethar fish waste has maximum solubility between pH 1-3 (solubility at pH 2.0:- 99.36%) and after that slightly decreased up to pH 8.0 (75.59%). The gradual reduction of solubility was detected up to pH 14 (40.75%). Similar result was obtained for collagen from puffer fish and silver catfish skin collagen (Kirti et al.2015; Hukmi and Sarbon 2018).

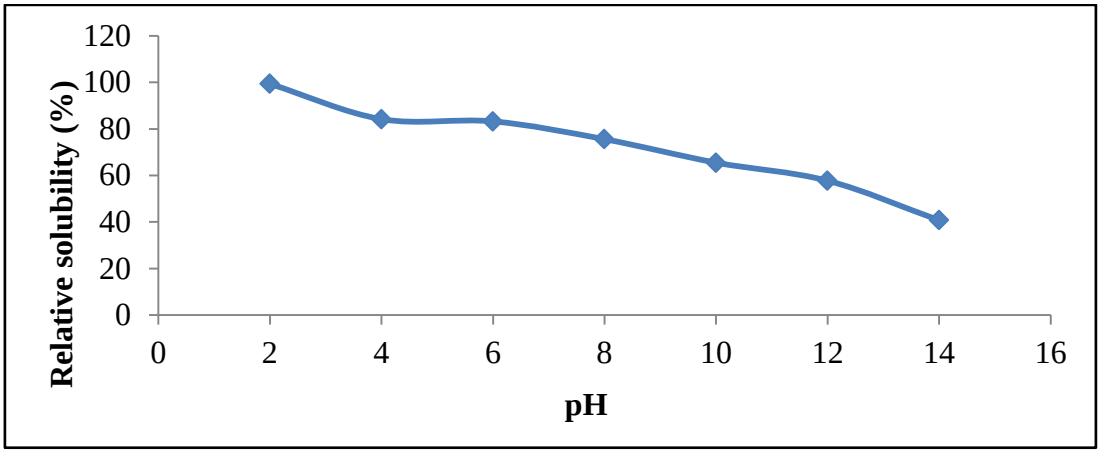


Figure 3.14.: pH dependent relative solubility of ASC

3.5.6.8. Effect of salt concentration on collagen solubility

Effect of NaCl was studied to determine optimum concentration of salt to precipitate collagen from acid solvent. At low NaCl concentration, collagen solution exhibits salting in effect while increase in concentration results in salting out. The figure no.3.15 represents the effect of NaCl concentration on collagen solubility. Solubility was maximum at 2M NaCl (99.96%) and further it was decreases up to 76.50% as NaCl concentration was increased up to 3 M. The improvement in hydrophobic interaction reduces the collagen solubility after 2 M (Abdelaal et al.2020). This result was similar to the solubility of the tilapia skin (Zeng et al., 2009), puffer fish skin (Kirti et al.2015) and brown stripe red snapper fish (Coppola et al.2020). As The more co-operation between ionic salts and water results in precipitation of protein (Kirti et al.2015). The ASC exhibited better solubility at low NaCl concentration (0.5 to 2 M) because the salt ions attaches weakly to charged clusters on the protein molecule (Damodaran 1996).

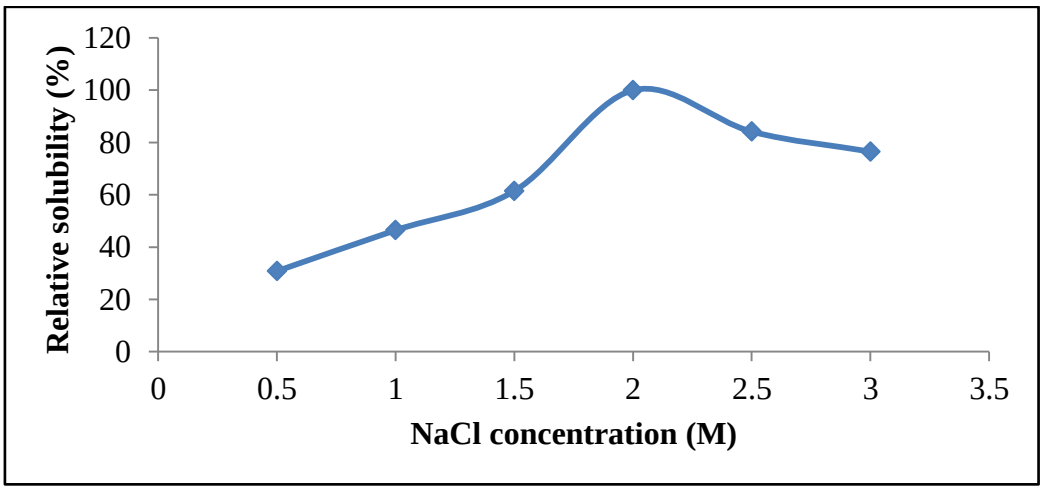


Figure 3.15.: Effect of NaCl on ASC precipitation

3.5.6.9. Sensory evaluation

The quality of food products in market were determined by studying its sensory characteristics. The 5 point hedonic scale was used for this purpose. The consumer dislikes food product, if score is less than 3. The sensory analysis of appearance, colour, fishy odour and overall acceptability was carried out. The result was shown in figure no.3.16. The similar analysis was obtained for red cheek barb scale collagen (Aichayawanich and Parametthanuwat 2018). The sensory analysis score of colour, appearance and overall acceptability of extracted ASC was more than 3 hedonic points. It has slight fishy odour due to raw materials and extracting solvents used. Thus, due to good sensory properties ASC can be exploited for different food applications.

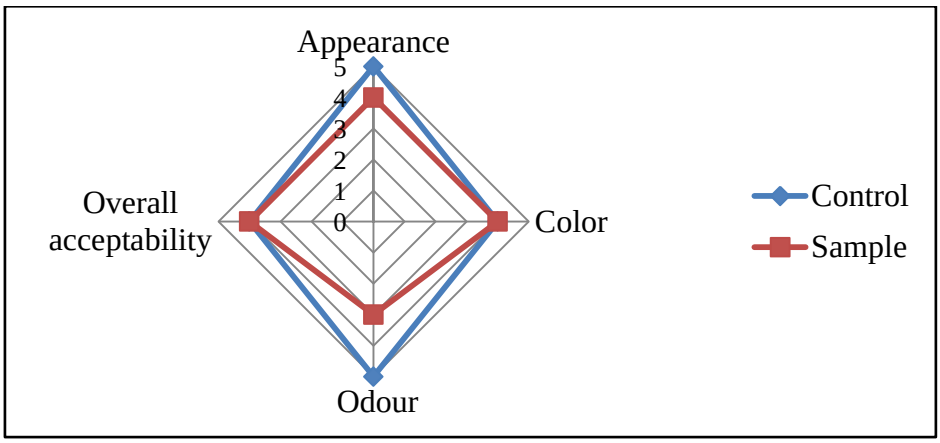


Figure 3.16.: Sensory analysis of extracted ASC

3.6. Conclusion

In this study, fish waste that ultimately responsible for reducing environmental pollution burden was effectively utilized for extraction of medically and industrially important collagen. Lactic acid with 0.2 M concentration was found most efficient among other acids used for collagen extraction from waste material of gethar (*Sarda orientalis*). The 40 hr extraction time and 1:30 (w/v) was sufficient to generate better yield of collagen and also it reduces prolonged extraction time so it can be applicable at small scale level. Electrophoretic pattern, UV visible spectra, FT-IR, SEM, zeta potential and particle size analysis confirmed type I collagen with two α chains (α1 and α2) and one β chain. Extracted collagen exhibited significant emulsifying, foaming properties, water holding and oil absorption capacity as well as possess good solubility, turbidity and viscosity. Thus, it can be useful in formulation of functional foods enriched with collagen. It also has maximum solubility at pH 2.0 and effectively precipitated by 2 M

NaCl. Thus, fish waste was successfully utilized for the extraction of collagen and development of protein rich functional foods as well as in pharmaceutical field due to its antimicrobial, antioxidant, anti-diabetic and anti-tumour properties.

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CHAPTER IV

EXTRACTION, CHARACTERIZATION AND PURIFICATION OF GELATIN AND OMEGA-3 FATTY ACID FROM WASTE MATERIAL OF GETHAR (*Sarda orientalis*)

4.1. Introduction

The seafood industry generates large amount of fish waste through various processes. Different parts of fish have been utilized by fish processing industries to making of fishmeal as well as some by-products for the pharmaceutical and nutraceutical fields as a supplementary component. Each year fishing industries disposes significant quantity of waste after fish processing which include trimmings, fins, tail, frames, head, skin, scales and viscera. Some of these products are used but the majority components are discarded as waste causing various types of environmental pollution. These wastes contains large amount of nutrients like protein with good biological importance, unsaturated fatty acids, vitamins and antioxidants, minerals as well as physiologically valuable amino acids and peptides which acts as substrate for fish meal production (Oladapo and Awojide 2015).

About 75% waste was produced after fish processing and it was major environmental pollutant. Skin and bone part of waste contains higher amount of collagen protein (Guillen et al.2002). Gelatin is one of the largely employed biopolymer (polypeptide) in nutraceutical, pharmaceutical and cosmetic sectors. Due to its distinct physical properties, it utilized both as food additive and functional food in the nutraceutical. However, it plays major role in various food components as a thickener, stabilizer, adhesive and emulsifier in biodegradable films. Also act as a gelling, foaming and microencapsulation agent in several food products like confectionary, jelly, yoghurt, ice cream, cheese, and canned foods (Koli et al.2012). In addition to this, it comprises bioactive properties which includes antimicrobial, antioxidant and also exhibited antihypertensive ability by inhibiting angiotensin converting enzyme (ACE) (Atma and Ramdhani 2017).

Due to diverse functional properties, gelatin has classified into two classes: first one is correlated with gelling process and another related with the surface behaviour of gelatin. The degree of collagen transformation into gelatin is associated with the strength of pre-treatment and extraction method as a function of pH, temperature and extraction period. Depending on the acid or alkaline pre-treatment process, two categories of gelatin were obtained, called as type-A (isoelectric point at pH ~8–9) and type-B (isoelectric point at pH ~4–5) respectively (Sousa et al.2017; Karim et al.2008).

During last few years, the neurological disorders like Bovine Spongiform Encephalopathy (BSE) have been identified in several countries and it may be exported from cow meat and meat products (Baziwane and He 2003). On the other hand,

consumption of pork meat has ethical issues in some religions. Therefore, there is need to find out new source of gelatin, like marine fish wastes which is generated in more amount during fish processing by industries and local markets.

The major distinguishing feature between fish and mammalian gelatin is the quantity of imino acids proline and hydroxyproline, which balances the ordered configuration during gel formation of gelatin. Due to fewer amount of these imino acids gelatin possess low gelling and melting temperature (Tavakolipour 2011). The generation of gelatin from fish waste mainly from fish skin has much importance due to its characteristic properties and qualities. Fish and its products were accepted by almost all religions, so there are negligible ethical issues (Kittiphattanabawon et al.2005). Thus, it imparts a solution for implementation of vast amounts of fish wastes generated.

The total world fisheries accounts about 141.6 million tons (FAO 2006) and it was increases day by day. Thus, there is need to convert this enormous quantity of fish waste into beneficial products such as fish gelatin (Herpandi et al.2011). To get gelatin from unsolvable native collagen, a proper heat was applied which will disrupt the non-covalent bonds. Therefore, it will cause sufficient swelling and break the intra and inter molecular linkages leading to successive solubilisation of collagen (Kim and Cho 1996). The extraction of gelatin from waste material involves acid or alkaline treatment to cleave the collagen crosslinks followed by treatment with hot water. Heat treatment is important to destabilize the triple helix confirmation of collagen to transform its helical configuration into a coiled structure, resulting in a gelatinous nature at cooling temperature (Dincer et al.2015).

There is a necessity to carry out more research to acquire essential fatty acids EPA and DHA for the use of its probable application in food and pharmaceutical trades because these fatty acids has important role in diagnosis and treatment of human disorders. The extraction of these fatty acids from discarded fish waste is economically feasible and also it is a cost effective alternate method to decrease problems of waste disposal and to prevent environmental pollution from it (Rai et al.2013; Nascimento et al.2015).

The increasing demand of highly purified fish oil specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has led researchers to invent nontoxic procedures for the separation of suitable components. Fish oil is present in the form of omega-3 polyunsaturated fatty acids (PUFA) are widely utilized as pharmaceutical supplements, food flavourings and health supplements (Espinosa et al.2002).

Various clinical and epidemiologic studies have revealed positive effect of omega-3 PUFA in new-born improvement, cancer, cardiovascular disorders as well as in mental illnesses such as depression, attention-deficit hyperactivity syndrome and dementia. The De Novo synthesis of PUFA was not carried out in humans and therefore these are essential fatty acids which are acquired from diet. Fish oil, marine animal oil and vegetable oil were naturally available dietary sources of omega-3/6 PUFA. As per the European Food Safety Agency (EFSA) recommended dietary intake of EPA+DHA was 250 mg per day. Thus, PUFA containing fish oil has more importance in nutrition sector and its production has been one of the increasing research area in last few years (Ferdosh et al.2016).

Fish and fish tissues exhibited comparatively high autolytic activities and more amount of polyunsaturated fatty acid and thus they are susceptible to both lipolysis and oxidation. Due to this, there are some difficulties during extraction of oil from fishes. Large quantity of PUFA causes hydrolytic spoilage of fish oil mainly by oxidative deterioration. The oxidation of lipids lead to some drawbacks comprising rancid odours and flavours, decreased nutritional quality and safety which may be harmful to health by creating health issues (Bako et al.2017).

The conventional techniques like wet pressing and solvent extraction as well as modern procedures such as supercritical fluid extraction were employed for fish oil extraction. Also enzymes from fish or other sources (e.g.:Alcalase) were employed for extraction. The habit and habitat of fish has influence on amount of fish oil (Adeoti and Hawboldt 2014).

At industrial level wet pressing is commonly utilized technology for extraction of fish oil and it has four steps: - fish cooking, pressing, decantation and centrifugation (FAO 1986). Another traditional method is, use of solvents for extraction. This method is useful for analytical purposes but not for industrial production because some solvents which are restricted in food industry were utilized for extraction. The basic of this method depends on solubility of lipids in organic solvents and their insolubility in water. The soluble components such as proteins, carbohydrates and minerals can be separated from water. There are numerous methods depending upon type of solvent. The general used methods are Soxhlet and Bligh-Dyer as well as include McGill-Moffatt and Randall-Folch methods were assessed (Mendez and Concha 2018).

In current approach, gelatin was extracted from skin and omega-3 fatty acid were extracted from head waste of marine fish gethar (*Sarda orientalis*), also called as striped

bonito. It is tuna like fish having length 102 cm and belongs to species of marine perciform and family Scombridae along with tuna and mackerel. But it contains fewer amounts of lipids than other two fishes. The enzymatic concentration of fish oil was done to determine amount of omega-3 fatty acid in it. Thus, extraction of gelatin and omega-3 fatty acid were carried out and studied for its structural as well as functional properties and may be useful in different applications in various fields.

4.2. Experimental Methodology

4.2.1. Chemicals

Lactic acid, sodium hydroxide (NaOH), hydrochloric acid (HCl), butyl alcohol, chloroform, Bovine serum albumin (BSA,100% pure), leucine, L- hydroxyproline, sodium dodecyl sulphate (SDS), acrylamide, polyacrylamide, agarose, coomassaie brilliant blue and other chemicals required to perform experiments were purchased from Hi-Media and Sisco Research Laboratory, India. Chloroform, methanol, glacial acetic acid, sodium hydroxide (NaOH), potassium hydroxide (KOH), sodium thiosulphate ($Na_2S_2O_3$), sodium sulphate, iso-octane, *p*-anisidine reagent, carbon tetrachloride (CCl_4), hydrochloric acid (HCl), iodine solution, potassium iodide (KI), phenolphthalein indicator and other chemicals employed for current study were procured from Himedia, India and Sisco Research Laboratory, India. Different chemicals and reagents utilized for this study were of analytical grade.

4.2.2. Collection and cleaning of fish waste

By maintaining suitable conditions skin and head waste of *Sarda orientalis* (gethar) were collected from fish market Ratnagiri (MS, India) and Gadre fish processing industry, Ratnagiri (MS, India) during the period January to April. It was washed with cold tap water and scrubbed with knife to bring out flesh. The cold demineralized water was used for final wash and cut into small pieces for further procedures.

4.2.3. Proximate analysis of fish waste and gelatin

The standard techniques sanctioned by AOAC (1980) were carried out to determine proximate composition of fish waste and extracted gelatin. The content of moisture, fat, ash and protein was evaluated on dry (DWB) and wet weight (WWB) basis.

4.2.4. Pre-treatment of waste for gelatin extraction

Pre-treatment was carried out according to procedure of Kumar and Nazeer (2013) with slight modifications. The whole process was conducted at 4ºC. Non-collagenous proteins were removed by soaking 10 gm skin in 0.1 M NaOH (1:30 w/v) for

24 hr. Treated waste was washed with cold demineralized distilled water (D/W) till pH 7.0 and subjected to defatting by 10% butyl alcohol (1:30 w/v) for 48 hr. After defatting, it was washed repeatedly with cold demineralized water till neutral pH and was subjected to collagen extraction by 0.2 M lactic acid for 40 hr. Also, this pre-treated waste was employed directly to gelatin extraction.

4.2.5. Gelatin extraction

Partial hydrolysis of collagen by acid, alkali or thermal treatment causes formation of gelatin. One method utilizes extracted collagen for gelatin preparation while in another method pre-treated waste material was directly used for gelatin extraction using organic acid. Following two methods were exploited for extraction.

4.2.5.1. By collagen hydrolysis

Method given by Du et al. (2014) was slightly modified for formation of gelatin from collagen. Extracted and purified collagen (10 gm) was mixed with D/W in 1:3 (w/v) ratio. It was kept for thermal treatment at 60ºC-80ºC for 7 hr after that gel like appearance was observed. The water soluble gelatin was recovered by centrifugation at 5,000 x g for 15 min at 20ºC (Remi centrifuge).

4.2.5.2. By acid treatment method

Gelatin was extracted directly from pre-treated waste by the method of Tinrat and Asna (2017) with slight modifications. About 10 gm pre-treated fish waste was mixed with 0.2 M lactic acid in 1:10 (w/v) proportion and placed under continuous stirring for 2 hr at RT. The acid treated waste was washed thoroughly with D/W till neutral pH. Final extraction period was optimized for gelatin extraction. Thus, optimized final extraction was carried out with D/W in a ratio of 1:10 (w/v) and kept for continuous stirring at 80-100ºC for 1, 1.5, 2, 2.5 and 3 hr. Viscous solution was obtained after extraction and was centrifuged at 5000 x g for 10 min to remove impurities then it was filtered through muslin cloth. The resultant filtrate was dried using hot air oven at 60ºC for 48 hr. Powder form of fish gelatin was stored for further characterization and applicatory studies.

4.2.5.3. Yield of gelatin and hydroxyproline analysis

Yield of gelatin extracted by two methods were calculated by following formula (Bordignon et al.2019);

$$\text{Yield (\%)} = \frac{\text{weight of dried gelatin (g)}}{\text{weight of dried collagen (g)}} \times 100$$

$$\text{Yield (\%)} = \frac{\text{weight of dried gelatin (g)}}{\text{weight of dried waste (g)}} \times 100$$

Hydroxyproline analysis of gelatin samples was accomplished by the method of Neuman and Logan (2019) using L-hydroxyproline (HyP) as standard. Percentage of hydroxyproline was calculated as:

$$\text{Hyp (\%)} = \frac{\text{conc.of Hyp (µg ml}-1)}{\text{conc.of protein (µg ml}-1)} \times 100$$

4.2.5.4. Structural characterization of gelatin

Extracted gelatin was characterized by UV visible spectroscopy by using 1 mg/ml sample (Shimadzu UV-1800 Japan) between 200-800 nm and characteristic peak was detected while D/W was used as reference. SDS-PAGE was performed to analyse gelatin on the basis of its molecular weight. For this purpose, 8% resolving gel and 5% stacking gel was used while Coomassie brilliant blue was used as staining agent. 50-200 KD pre-stained protein ladder (Himedia, India) was utilized for comparison of molecular weight. The Fourier transform infrared spectroscopy (FTIR) was carried out to determine different functional groups attached to gelatin. The Nicolet iS10 Mid FT-IR spectrometer (Thermo electron scientific, Madison, USA) in the range of 500-4000 cm^{-1} was utilized. X-ray diffraction pattern gives information about the distribution and orientation of gelatin. The samples were subjected to Cu-Kα radiation at 40 kV voltage and current of 40 mA with scanning range 10°-80° (2θ). X-ray diffraction (XRD) analysis of extracted gelatin was done by Bruker AXS analytical instrument (Germany).

The differential scanning colorimetry (DSC) was performed on DSC Q20 V24.11 calorimeter (Netzsch-Geratebau GmbH, Germany). The gelatin sample was eradicated with ultrahigh-purity nitrogen at 50 cm^3/min and scanned from 25ºC to 400ºC at a heating rate of 5ºC /min. The 1 mg/ml gelatin was dispersed in D/W and subjected to zeta potential and particle size analysis by using Malvern Zetasizer Ver. 7.11 instrument. The electrophoretic mobility of the gelatin dispersion was measured by the instrument and then converted into zeta-potential values. The gelatin sample was hydrolysed with 5 N HCl at 100ºC for 24 hr. The amino acid analysis of extracted gelatin was carried out on a Waters-PICOTAG amino acid auto analyser high performance liquid chromatography (Model: Waters 501) connected to the automatic amino acid evaluating software. The Waters-Pico Tag column (size:- 3.9×150 mm) was used for this purpose. Structural characterization was used to determine physical appearance of gelatin which helps to confirm that, extracted component was gelatin.

4.2.5.5. Functional characterization of gelatin

4.2.5.5.1. Solubility and viscosity of gelatin

The 6.67% (w/v) of gelatin was mixed with D/W and heated up to 40-60ºC for solubilisation. The clearance of water was observed for gelatin solubility.

The viscosity of gelatin (6.67%,w/v) was determined according to procedure of Kuan et al.(2016).The viscosity of sample was carried out by using a Brookfield digital viscometer (Model LV-DV-II, Brookfield Engineering, Middleboro, MA, USA) equipped with a No. 1 spindle (Model LV) at 60 rpm and 40±1ºC.

4.2.5.5.2. Turbidity and clarity of gelatin

Turbidity of gelatin was measured according to method of Khiari et al.(2011) with slight modification. 6.67% (w/v) gelatin was dissolved in D/W by heating up to 70ºC and sample was placed into transparent glass tube. The turbidity of the gelatin samples was measured as formazin turbidity units (FTU) using UV visible spectroscopy (Shimadzu UV-1800 Japan) at 450 nm.

The method of Avena et al. (2006) was used to determine gel clarity of gelatin with minor changes. The 6.67% (w/v) of gelatin was heated at 60ºC for 1 hr to form soluble component. Clarity was determined by measuring transmittance (%) at 620 nm (Shimadzu UV-1800 Japan).

4.2.5.5.3. Melting and gelling temperature

The melting temperature was studied according to the method of Shyni et al. (2014) with slight modifications. Gelatin sample, 6.67% (w/v) was formulated and about 5 ml of aliquot was removed into glass tube in triplicate. The tubes were sealed by using parafilm and heated for 15 min at 70ºC. The tubes were instantly chilled in ice cold water and matured at 10ºC for 20 hr. Five drops of blend of 75% chloroform and 25% red food colour (Asian Food products, Maharashtra, India) was kept on the surface of gel. The formed gel sample was placed in water bath at 10ºC. The temperature at which the dye began to enter into gel was recorded as the melting point.

Gelling temperature was determined according to method of Ratnasari and Firlianty (2016) with minor modifications. About 30 ml of 6.67% gelatin solution was taken in glass tube and it was kept in cool box along with thermometer. The crunched ice cube was added gently until gel nature of gelatin forms and gelling temperature was note down.

4.2.5.5.4. Emulsifying properties

The procedure of Bichukale et al. (2018) was used to study emulsifying stability index (ESI) and emulsion activity index (EAI) with minor modifications. With the help of homogenizer, emulsions were prepared with 1%, 3% and 5% of gelatin in 50 ml of soyabean oil. The emulsion was pipetted at 0 min and 10 min. 0.1% sodium dodecyl sulphate (SDS) was mixed with it and absorbance was measured at 500 nm (Shimadzu UV-1800 Japan).

$$\text{Emulsion activity index } (m^2g^{-1}) = \frac{2^{\times}2.303^{\times}A0min}{0.25^{\times}\text{protein weight in gm}}$$

$$\text{Emulsifying stability index (min)} = \frac{A0min}{A0\,min - A10min} \times \Delta t$$

(where; A_{0min}:- absorbance at 0 min, A_{10min}:- absorbance at 10 min, Δ t:-10 min)

4.2.5.5.5. Foaming properties

To determine foaming properties of gelatin method of Tkaczewska et al. (2019) was slightly modified. 1%, 3% and 5% gelatin sample was prepared in water and heated up to 70ºC to dissolve it. All samples were homogenized to generate foam and the foam capacity (FC %) and stability (FS %) was calculated by comparing the ratio of foam to liquid. The foam stability was recorded by comparing initial volume of foam to the volume of foam after 60 minutes.

$$\text{Foaming capacity (\%)} = \frac{VT}{V0} \times 100$$

$$\text{Foam stability (\%)} = \frac{Vt}{V0} \times 100$$

4.2.5.5.6. Water holding and fat binding capacity

The method of Hue et al. (2017) was slightly modified to determine water holding and fat binding capacities (WHC and FBC) of extracted gelatin. For assessing WHC, 1 gm of gelatin was taken in centrifuge tube and mixed with 50 ml D/W. The tube was held at RT for 1 hr. The solution was vortexes for 5 sec after every 15 min and then centrifuged at 4500 x g for 20 min. The supernatant was removed and tube was tilted on paper to drain out remaining liquid. The WHC was calculated as the weight of the pellet after withdrawing liquid divided by weight of the gelatin and expressed as % weight of dehydrated gelatin.

For FBC determination, 50 ml water was replaced by 10 ml of sunflower oil and same procedure was executed to calculate FBC of extracted gelatin. FBC was expressed as % weight of dehydrated gelatin.

4.2.5.5.7. Effect of pH on gelatin solubility

To study effect of pH on gelatin solubility method of Ratnasari and Firlianty (2016) was slightly revised. The 6.67% (w/v) of gelatin was prepared by mixing it with water and stirred at 60ºC. The gelatin solution was formulated from pH 2.0 to 14 by using HCl and NaOH (6 N). The solution was making up to 10 ml with distilled water and adjusted to previous pH. All samples were centrifuged at 7000 x g for 15 min at ambient temperature. The protein content of supernatant was determined according Lowery method by using bovine serum albumin as standard. Relative solubility at each pH was calculated by relating the solubility value at the pH with highest solubility.

4.2.6. Experimental methodology for omega-3 fatty acid

In first step, solvent extraction of fish oil was carried out while in second enzymatic concentration of omega-3 fatty acid by lipase enzyme was done.

4.2.6.1. Solvent extraction of fish oil

The methodology of Bligh and Dyer (1959) was slightly modified to extract fish oil. 10 gm of cleaned and dry fish head waste was crushed by using mortar and pestle for 5 min. The methanol: chloroform (2:1) was added to it and homogenized for 5 min. In next step 10 ml chloroform was added and homogenized for 90 sec. The final homogenization was carried out for 90 sec by using 10 ml D/W. The resultant mixture was centrifuged at 5000 x g for 15 min at 5ºC and filtered through double layered muslin cloth. The filtrate and solids separated from each other. To the remaining solids methanol: chloroform (1:1) was mixed and centrifuged at 5000 x g for 15 min at 5ºC. The waste material containing pellet was removed and supernatant was filtered out. The concentrated filtrate from both steps was mixed together and kept at stable condition to form biphasic layer of aqueous and organic phase. The lipid containing organic phase was collected and passed through anhydrous sodium sulphate then supernatant was collected through filtration. The solution was evaporated at 50ºC to obtain crude fish oil. Further it was subjected to enzymatic hydrolysis to concentrate omega-3 fatty acids.

4.2.6.2. Enzymatic concentration of fish oil by lipase (*S.sciuri*)

The procedure of Mohammad et al. (2018) was slightly changed to carry out enzymatic concentration of fish oil which helps to determine actual composition of omega-3 fatty acid in fish oil. 30 ml of extracted oil treated with lab isolate *Staphylococcus sciuri* was added together. In this reaction, the lipase activity of enzyme from S.sciuri was used to carry out enzymatic hydrolysis of sample. The reaction was carried out at 37ºC for 24 hr in shaking incubator (Remi incubator) at constant stirring

(95 rpm). The both sample and control with respect to enzyme were analysed by GCMS for determination of omega-3 fatty acid concentration in fish oil.

4.2.6.3. Yield of oil (%)

The yield of extracted oil was calculated by following formula with respect to mass of dry matter.

$$\text{Yield of oil (\%) (w/w)} = \frac{\text{weight of oil obtained (gm)}}{\text{mass of dry matter (gm)}} \times 100$$

4.2.6.4. Fatty acid analysis

The composition of fatty acid present in fish oil were analysed by using gas chromatography-mass spectroscopy (GCMS) technique (TQ 8050 plus with HS 20, Shimadzu, Japan). The amount was computed from the integrated peak area as the percentage of the total area of the entire peak. The fatty acids in sample were identified by comparing retention times with those of known standard.

4.2.6.4.1. GCMS instrumentation

For sample separation, DB-5 MS high resolution capillary column (thickness: 0.25 μm, length: 30 m, diameter: 0.25 mm) was used. For temperature control, the oven was maintained at 80°C for one min and then raised up to 250°C with interval of 10°C per min and kept for 5 min. The 10:1 ratio of split injection was conducted while 0.8 ml/min helium gas was used as carrier with 1 μl sample injection volume. The mass spectrometer was functioned in electron-impact (EI) manner. The other experimental conditions include; pre column pressure: 70 kPa, injection temperature: 250°C, ion source: EI (200°C), interface temperature: 280°C, electron energy: 70 eV and solvent delay: 5.5 min. For qualitative analysis, the full scan mode was carried out with 40–400 m/z scan range.

4.2.6.4.2. Preparation of sample

About 60 mg of enzymatically hydrolysed and un-hydrolysed oil sample was taken in a centrifuge tube. 0.5 M of potassium hydroxide methanol was added to it. The components were rigorously mixed, further tube was filled with argon gas and heated at 60ºC in water bath with intermediate shaking for 20 min to form transparent solution. Boron trifluoride methanol complex solution (3 ml) was mixed with it and resulting mixture was kept for cooling. The argon gas was filled in tube and kept in water bath for 5 min.2 ml of each saturated sodium chloride and n-hexane were added to it and mixed properly. The centrifugation was carried out at 4000 x g for 10 min and 1 μl of

supernatant was used as sample solution for GCMS analysis (Yi et al.2014; Mustafa et al.2015).

4.2.6.5. Analysis of fish oil characteristics

4.2.6.5.1. Free fatty acid (FFA) content

The free fatty acid content (%) of fish oil was determined according to method of Murage et al.(2021). 2 gm of extracted fish oil was taken in 125 ml flask and 10 ml of ethanol were added into it. After that 0.5 ml of phenolphthalein was added into it and titrated against 0.1N NaOH until pink colour was observed. The FFA was calculated using following formula;

$$\text{FFA (\%)} = \frac{\text{NaOH (ml)} \times \text{N} \times 28.2}{\text{mass (g)}}$$

Where;

N = normality of the NaOH

mass (g) = mass of sample used.

4.2.6.5.2. Determination of acid value

The acid value of fish oil was calculated according to method of Sirilun et al.(2016) with some changes. It determines triglyceride amount in oil mixture as well as it's an indicator of rancidity and degradation of oil. 1 gm of oil was taken in Erlenmeyer flask and 25 ml of fat solvent (95% ethanol: ether) was added along with few drops of phenolphthalein. The sample was titrated against 0.1 N KOH till colour of solution forms permanent pink colour. The blank was carried out without addition of oil.

The acid value (mg KOH/g of sample) was given by the following formula:

$$\text{Acid value} = \frac{\text{Y}-\text{X} \times \text{N} \times 56.1}{\text{W}}$$

Where;

X= ml of standard alkali used in the titrating the blank

Y= ml of standard alkali used in the titrating the sample

N= normality of standard alkali

W= gm of sample

56.1= molecular weight of KOH in gm

4.2.6.5.3. Determination of iodine value

The iodine value of oil was determined as per method of Hanus (1966) with minor modifications.1 gm of extracted oil was added along with 10 ml CCl_4 into conical flask. To this, 25 ml of Hane's iodine solution was added and flask was kept in dark for 1 hr with frequent stirring. After incubation 10 ml of 15% KI solution along with 50 ml

D/W was added. The resultant solution was vigorously titrated against 0.1 N $Na_2S_2O_3$. Starch was used as indicator until the blue colour disappears. A blank test was carried out without oil under same conditions. The iodine value was calculated using:

$$\text{Iodine value (mg)} = \frac{(Vb - Vs) \times 12.7 \times 100}{W \times 1000}$$

Where;

V_b = volume of standard $Na_2S_2O_3$ solution used for blank test

V_a = volume of standard $Na_2S_2O_3$ solution used for the test sample

W = weight of oil sample (gm)

4.2.6.5.4. Determination of saponification value

The saponification value of oil was calculated as per procedure of Alam and Uddin (2017) with minor modifications. It gives information about the mean molecular weight of the collective fatty acids. It is the number of mg of KOH required to complete saponify 1 g of fat or oil. 25 ml of 0.5 M alcoholic KOH solution was added into 1 gm of extracted fish oil. An inverted funnel was kept on flask and refluxed for 30-40 min. The resultant mixture was cooled at room temperature and few drops of phenolphthalein indicator were added. The solution was titrated against 0.5 M HCl until a pink endpoint was attained. A blank was performed without addition of oil under similar conditions. Saponification value is calculated as;

$$\text{Saponification value (mg)} = \frac{Vb - Va \times 28.05}{W}$$

Where;

V_b = volume of standardized HCl solution used for the blank

V_a = volume of standardized HCl solution used for the test sample

W = weight of sample (gm)

4.2.6.5.5. Determination of peroxide value

The peroxide value of extracted fish oil was calculated as per method of Bako et al.(2017). 5 gm of fish oil sample was taken into conical flask. About 30 ml of glacial acetic acid and chloroform (3:1) was added into it and mixed thoroughly to dissolve sample completely. 0.5 ml KI was added and the solution was allowed to stand in dark with occasional shaking for precisely 1 min further 30 ml D/W was added. The resulting mixture was titrated against 0.1 N sodium thiosulphate ($Na_2S_2O_3$). Few drops of 1% starch indicator were added and titration was continued with constant shaking till the blue colour vanished. A blank was prepared without addition of oil sample. The peroxide value (milli equivalents peroxide/gm sample) was given by the following formula:

$$\text{Peroxide value} = \frac{(S-B)\times N \times 1000}{W}$$

Where;

B= ml of standard $Na_2S_2O_3$ used for titration of blank

S= ml of standard $Na_2S_2O_3$ used for titration of sample

N= normality of $Na_2S_2O_3$

W= gm of sample

4.2.6.5.6. Determination of p-Anisidine value

The p-Anisidine value is used to assess the secondary oxidation of oil or fat. It is a reagent used for the detection of oxidation products such as aldehydes and ketones in oil or fat. The p-Anisidine value of gethar extracted fish oil was calculated as per method of Hiremath et al., (2018). 0.7 gm of oil was taken in 25 ml volumetric flask and final volume was making up with diluted iso-octane. The 5:1 gethar extracted oil and p-anisidine reagent was taken in test tube and analysed by UV after 10 min. For blank 5:1 ratio of iso-octane and p-anisidine reagent was used. Finally absorbance was measured at 350 nm using spectrophotometer. The p-anisidine value was calculated by using following formula;

$$\text{p-anisidine value} = \frac{25 \times (1.2 \times As - Ab)}{W}$$

Where;

A_S = absorbance of the oil solution: p-anisidine reagent

A_B= absorbance of the oil solution

W= weight of sample (gm)

25= size of volumetric flask used

1.2= correction factor

4.2.7. Sensory evaluation of gelatin and fish oil

The sensory evaluation of gelatin and fish oil from gethar was carried out as per protocol of Oladapo and Awojide (2015) and Sinthusamran et al. (2017) along with market gelatin and mackerel fish oil as control. The sensory characteristics of fish oil like appearance, color, fishy odour and overall acceptability were analysed by non-trained 7 member panel from Food Science and Technology department, Shivaji University, Kolhapur using 5 point hedonic scale (from 1:- dislike very much; 2:- dislike slightly; 3:- neither like nor dislike; 4:- like slightly and 5:- like very much). The sensory properties of gelatin like appearance, odour, colour, texture and overall acceptability were analysed by non-trained 9 member panel from same department of Shivaji University, Kolhapur

using 9 point hedonic scale (from 1:- dislike extremely; 2:- dislike very much; 3:- dislike moderately; 4:- dislike slightly; 5:- neither like nor dislike; 6:- like slightly; 7:- like moderately; 8:- like very much and 9:- like extremely).

4.2.8. Statistical analysis

Each experiment was carried out in triplicates and standard deviation was calculated by using basic statistical programme and graph pad prism software.

4.3. Result and discussion

4.3.1. Proximate composition of fish waste and gelatin

Proximate composition includes moisture, protein, lipid and ash content of fish skin and extracted gelatin. The analysis was tabulated in table no.4.1. According to Ward and Courts (1977), the pre-treatment carried out during extraction abolish some cross linked components present on waste and used to eliminate impurities as well as unwanted materials. Waste material of gethar contains 26.42±1.38% moisture, 42.48±1.70% protein, 7.85±0.05% lipid and 2.35±0.14% ash on DWB while 67.88±2.14% moisture, 50.03±2.72% protein, 8.97±0.13% lipid and 3.03±0.07% ash on WWB. Current waste material contains good amount of protein thus it can be suitable for gelatin extraction. Muyonga et al.(2004) described that, increase in collagen content of material causes maximum production of gelatin from waste. Proximate analysis showed that protein content of gelatin was much higher than waste material (table no.4.1). Other contents like moisture, lipid as well as ash was found to be lower than waste. Extracted gelatin contains 14.19±1.04% moisture, 85.08±2.93% protein, 1.50±0.14% lipid and 1.04±0.04% ash on DWB while 65.45±0.37% moisture, 90.30±1.76% protein, 1.49±0.12% lipid and 1.07±0.02% ash on WWB. Similar results were obtained for gelatin extracted from catfish skin (Ardekani et al.,2013), calf skin, carp scale and pork skin (Dincer et al.,2016).

The proximate composition of gethar fish head based on wet and dry weight were tabulated in table no.4.2. As per wet weight, it contains 82.58±0.20% moisture, 21.43±0.32% protein, 9.85±0.57% lipid and 1.29±0.53% ash. The moisture content was reduced up to 51.37±1.56% upon drying the sample. Based on dry weight, fish head waste comprises 24.48±0.11% protein, 11.73±0.44% lipid and 2.35±0.14% ash. The head part of the fish possesses more amount of lipid than any other body site (Mustfa et al.2015). Thus, more amount of lipid was found in gethar head waste. The results were analogues to the proximate analysis of catfish viscera (Adetuyi et al.2014), catfish and mackerel fish waste (Oladapo and Awojide 2015).

Sample	Moisture (%)	Protein (%)	Lipid (%)	Ash (%)
Fish waste (WWB)	67.88±2.14	50.03±2.72	8.97±0.13	3.03±0.07
Fish waste (DWB)	26.42±1.38	42.48±1.70	7.85±0.05	2.35±0.14
Gelatin (WWB)	65.45±0.37	90.30±1.76	1.49±0.12	1.07±0.02
Gelatin (DWB)	14.19±1.04	85.08±2.93	1.50±0.14	1.04±0.04

Table 4.1.: Proximate analysis of fish skin and gelatin

Sample	Moisture (%)	Protein (%)	Lipid (%)	Ash (%)
WWB	82.58±0.20	21.43±0.32	9.85±0.57	1.29±0.53
DWB	51.37±1.56	24.48±0.11	11.73±0.44	2.35±0.14

Values were means ± SD from triplicate determinations

Table 4.2: Proximate composition of fish head

4.3.2. Yield of gelatin (%) and oil (%)

Time required for final extraction of gelatin with D/W was optimized from 1 hr to 3 hr by using 1 gm of waste for each reaction. Figure no.4.1 showed effect of extraction time on gelatin yield. At 1 hr, very less gelatin yield (0.12±0.02 gm gelatin/gm waste) was obtained while maximum yield was attained at 2 hr (0.63±0.03 gm/gm). The decreased yield was observed after 2 hr extraction time and at 3 hr 0.52±0.04 gm/gm gelatin was obtained. Continual hydrolysis of collagen during its conversion into gelatin may decline the final yield and also changes its functional characteristics (Aberoumand 2011).

The decreased yield of gelatin was attained due to partially unstable cross associations present in collagen (Niveditha et al.2020). Extraction of gelatin from pre-treated waste sample of fish by using acid followed by heat generates economically feasible yield of gelatin with good gel strength (Roy et al.2017). The gelatin recovery from waste sample is mainly depends on type of fish skin, acid concentration, solvent pH, breaking of inter linkages in collagen molecule during washing and swelling procedure (Chavan et al.2018). About 14.33% and 9.7% gelatin was produced from pre-treated waste material of gethar and collagen hydrolysis respectively. Figure no.4.2 depicted nature of gelatin before and after drying. The obtained gelatin yield was less

than gelatin extracted from fish hoof skin (20.85%) and shark (18.65%) (Aberoumand 2011), iridescent shark catfish (17.29%) and black spotted croaker (17.52%) (Chavan et al.2018), pangas catfish (22%), asian red tail catfish 21.28%, striped snakehead 20.25% and nile tilapia 21.93% (Ratnasari et al.2013) but higher than that of tilapia skin (12.24%) (Boulahsen et al.2018) and milkfish (12.93%) (Masirah et al.2017). Gelatin yield from collagen hydrolysis was lower than pre-treated waste method. Thus, mass production of gelatin from pre-treated waste was carried out and utilized for further characterization and applicatory studies.

The chloroform, methanol and water were used as effective solvent for extraction of fish oil from gethar head waste by solvent extraction method (figure no.4.3). The variation in PUFA content was occurred due to changes in environmental conditions around the fish species. Also it is related to seasonal variations, alterations in plankton species in their diet and also in the post-spawning time (Khoddami et al.2009). The yield of oil (w/w) was found to be 27.63±0.24%. It is related to the fish oil from bigeye tuna (27.7-31.5%) (Ahmed et al.2017) and head waste of sardine (26.39%) (Khoddami et al.2009). The yield was more than oil from cobia liver (21.15%) (Santos et al.,2016) and intestine as well as liver of sardine (24.90% and 22.67%) (Khoddami et al.2009).

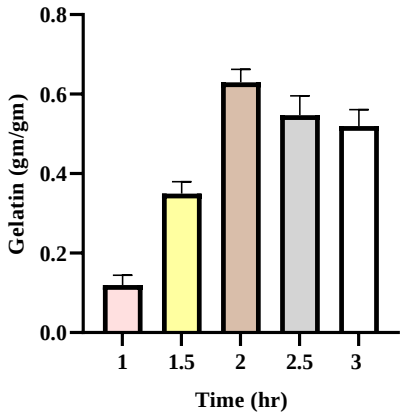

Figure 4.1.:Effect of extraction time on gelatin yield

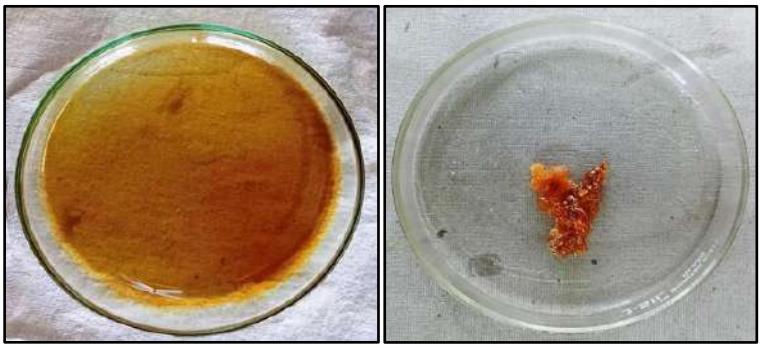

Figure 4.2.:Gelatin a) before drying; b) after drying

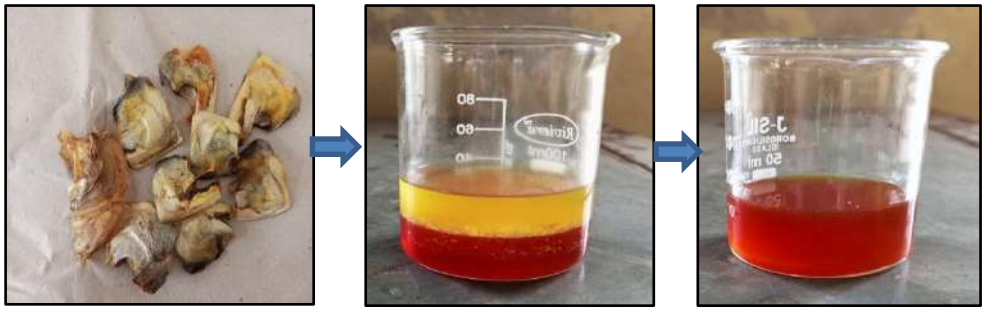

Figure 4.3.: Solvent extraction of fish oil

4.3.3. Yield of Hydroxyproline

Hydroxyproline determination is useful parameter for determination of collagen or gelatin concentration. Hydroxyproline content of gethar skin gelatin was found to be 22.46±0.61%. The yield was less than red tilapia skin gelatin which was 26.84±1.92% (Tinrat and Asna 2017) but higher than 18.5% hydroxyproline in nile tilapia skin gelatin (Zeng et al.2010).

4.3.4. Structural properties of gelatin

4.3.4.1. UV visible spectral analysis

UV visible spectroscopic analysis of extracted gelatin was carried out in the range of 200-800 nm. Chromophore groups present in gelatin exhibited characteristic absorption peak in UV analysis (Hermanto et al.2013). UV absorption spectrum of gelatin was given in figure no.4.4 and it has characteristic spectra at 280 nm. Species of fish, type of raw material used and extraction condition has effect on UV spectra. The n → π* transition of aromatic side chains gives characteristic spectra at 280 nm. The sensitive chromophoric components in the gelatin molecular structure may losses during extraction and therefore it shifts spectra to aromatic region (Xu et al.2017; Maharana and Misra 2018).

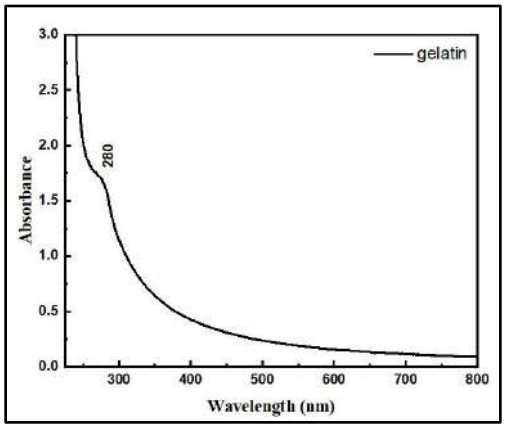

Figure 4.4.:UV spectra of gelatin

4.3.4.2. SDS-PAGE

The SDS-PAGE analysis of extracted gelatin was depicted in figure no.4.5 in which three bands were observed on gel that corresponds to two chains of gelatin. β chain with ~ 200 kD molecular weight and two α-chains (α1 upper and α2 lower) with 116 kD and 97 kD molecular weight respectively. Organic acid used in extraction did not effect on comparative movement as well as molecular weight distribution of α and β chains. The heat treatment during extraction process may cause partial breaking of protein chain into low molecular weight fragments also excess heat causes loss of component of gelatin (Gimenez et al.2005; Shyni et al.2014). It was reported that gelatin with more amount of crosslinking constituents (β- or γ-components) easily forms random coil and generates stable triple helical structure with high gel strength and good viscosity than other gelatins. The generation of degradation fragments is related with decreased viscosity, less melting point, reduced bloom strength and greater foaming properties (Kuan et al.2016). Similar electrophoresis results were obtained for mackerel head, duck feet and bovine gelatin (Khiari et al.2011; Kuan et al.2016).

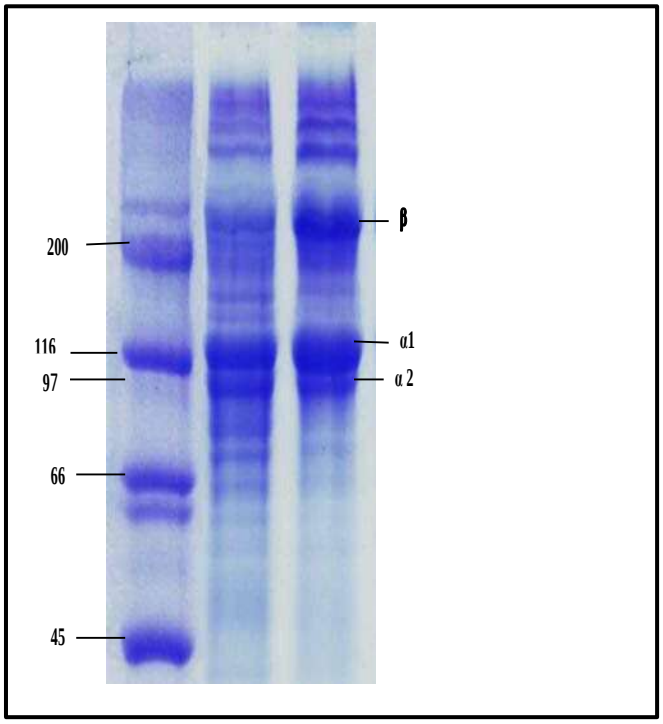

Figure 4.5.: SDS-PAGE analysis of gelatin. Lane 1: protein ladder; lane 2 and 3: extracted gelatin from gethar skin

4.3.4.3. Fourier transform infrared spectroscopy analysis (FTIR)

The various functional groups attached or inserted in gelatin structure during extraction process were analysed by Fourier transform infrared spectroscopy. The FTIR

spectrum of gethar skin gelatin was depicted in figure no.4.6 and it was slightly similar to the FTIR spectrum of catfish skin gelatin (Sai-Ut et al.2012) and black tilapia scale gelatin (Sockalingam and Abdullah 2015). The stretching at 3300 cm^{-1} was due to N-H bond (amide-A band) and it's a characteristic pattern of gelatin (Silva et al.2014). Peaks present at 1384 cm^{-1} and 1448 cm^{-1} were assigned due to methyl group which exhibit symmetric and asymmetric bending vibrations (Das et al.2017).The stretching occurred at 1211 cm^{-1} expresses C-N and N-H in-plane bending while bending at 1625 cm^{-1} occurred due to C=O (Mureithi et al.2017). The peak region present at 1034 cm^{-1} and 1116 cm^{-1} corresponding to Amide I, II and III (Arsyanti et al.2018). The reaction temperature, period of extraction and pH, material used for extraction, cross-linking bonds present in collagen peptide structure have impact on bond formation between gelatin molecule and thus, it exhibited variation in FTIR spectrum (Ahmad et al.2011).

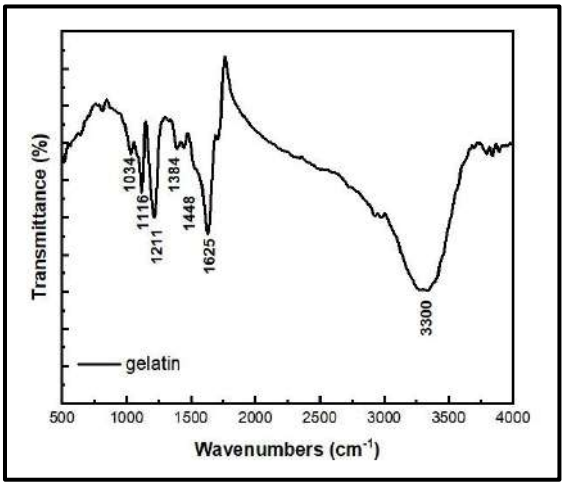

Figure 4.6.: FTIR spectrum of extracted gelatin

4.3.4.4. X-ray diffraction studies (XRD)

The X-ray diffraction analysis was studied to determine crystalline nature of several particles as well as biological structures. The X-ray diffractogram of extracted gelatin were given in figure no.4.7. The graph described partially crystalline nature of gelatin with a broad peak positioned at $2\theta = \sim 19^{o}$. This characteristics peak is occurred due to the triple helical crystalline nature of gelatin (Das et al.2017).The similar diffraction patterns were given by Yakimets et al.(2005) and Pena et al.(2010).

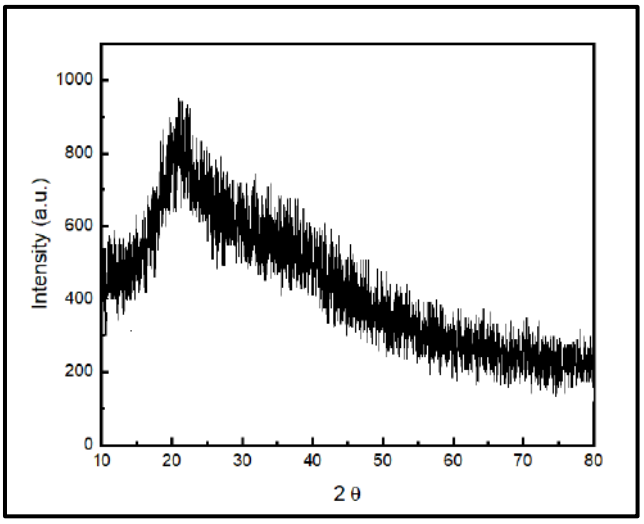

Figure 4.7.: XRD analysis of extracted gelatin

4.3.4.5. Differential Scanning Colorimetry (DSC)

Heat treatment of gelatin during DSC analysis beyond glass transition (Tg) and melting (Tm) temperature results in formation of large endothermic transition peak (Mukherjee and Rosolen 2013). The DSC analysis of gethar extracted gelatin was depicted in figure no.4.8 which exhibited single broad endothermic peak at 119.27ºC and three smaller peaks. Every peak on the heating curve associated to a helix–coil conversion while other number of peaks represents several fractions. The molecular weight distribution data indicates that there are four fractions in gelatin sample. The first small peak at 55.82ºC and broad peak at 119.27ºC is associated with melting temperature of low and high molecular weight fraction respectively (Derkach et al.2019). The breakage of peptide linkages due to polymer decomposition results in weak transition at 226.51ºC (Ghorani et al.2020). The smaller peak at 335.99ºC is occurred due to the isomerization of gelatin sample. The similar results were observed for tailoring cod and calf skin gelatin (Derkach et al.2019; Mukherjee and Rosolen 2013).

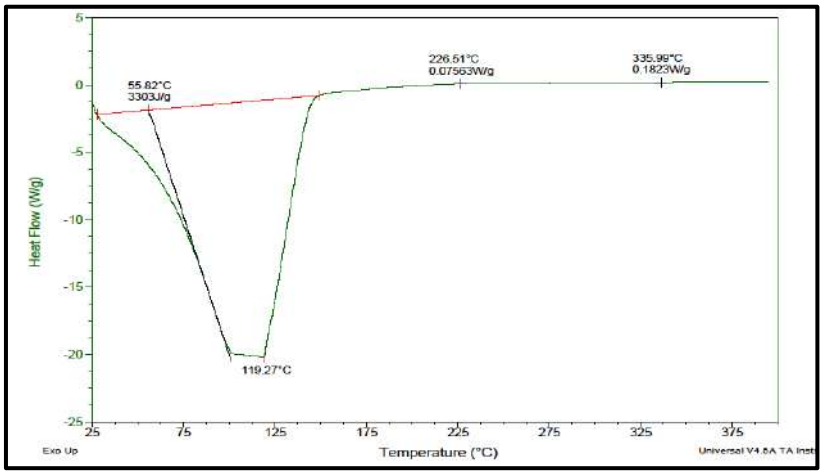

Figure 4.8.: DSC analysis of extracted gelatin

4.3.4.6. Zeta potential analysis

The zeta potential analysis of gethar extracted gelatin was depicted in figure no.4.9 and it was found to be 2.24 mV which is less than 2.56 mV of standard gelatin powder (Ghorani et al.2020). The isoelectric point (pI) of type A gelatin ranges from ~8–9, therefore it results in zwitter ion formation. The solution having pH above pI changes surface charge of the protein into negative and forms more negative carboxylic groups of the amide functional linkages. Nevertheless, acidic environment causes protonation of the amino acid groups thus gives positive charge to the gelatin backbone (Ghorani et al.2020). Usually, gelatin samples exhibited positive charge at acidic pH while negative at alkaline condition. At isoelectric point positive and negative charges were balanced by each other. Therefore, the difference in pI of total gelatin samples is occurred due to difference in their amino acid compositions and distribution. The unequal distribution of amino acid residues may be influenced by size or age of raw material utilized (Sinthusamran et al.2015). The pI of gethar skin gelatin was 8 which is less than pI of young nile perch (8.8) and adult nile perch (9.4) (Muyonga et al.2004).

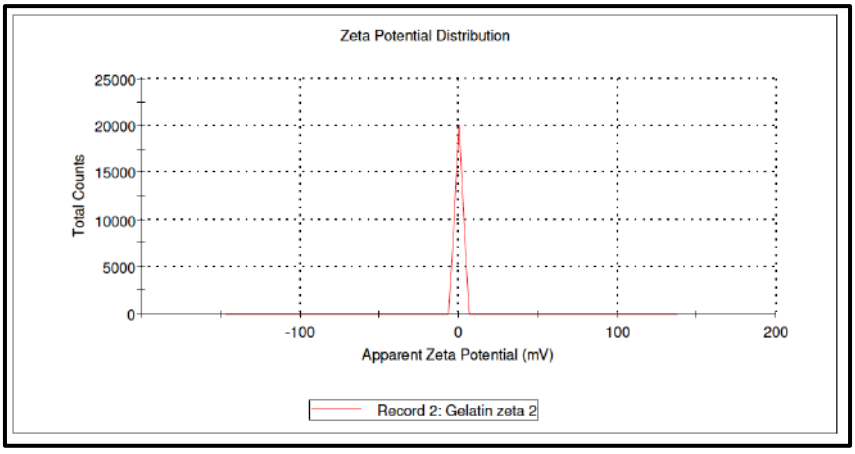

Figure 4.9.: Zeta potential of extracted gelatin

4.3.4.7. Particle size analysis

Particle size analysis is employed to characterize the size distribution of particles in a particular sample. The size distribution of gethar extracted gelatin sample based on its intensity and mass was depicted in figure no.4.10. The mean particle size of gelatin varies from 30-600 r.nm (radius values in nanometres). The yellowish brown colour of gelatin altered to yellowish orange and then to brown when extraction period of was increased (Dinarvand et al.2005).

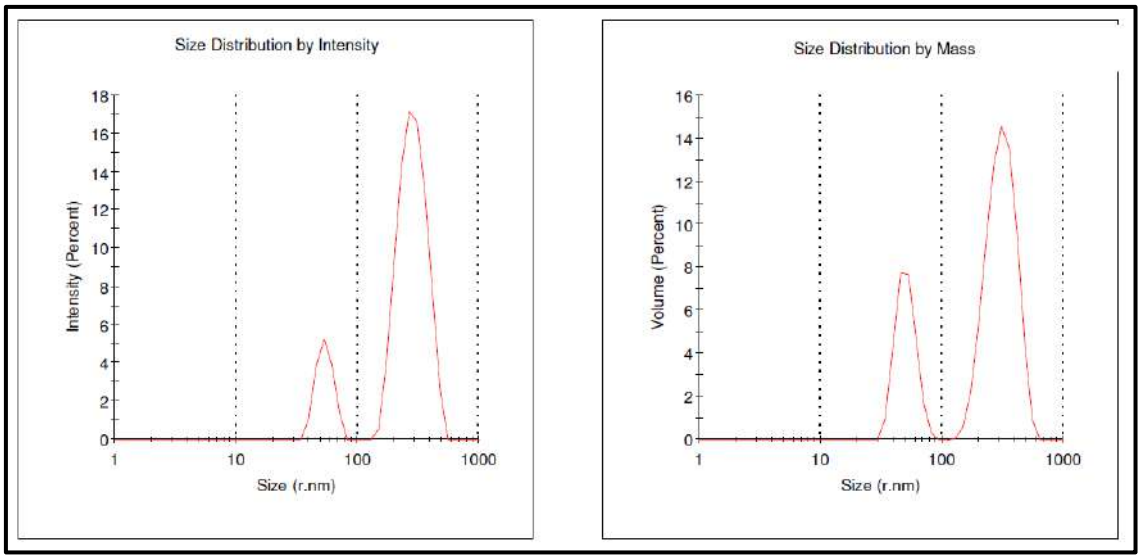

Figure 4.10.: Particle size analysis of extracted gelatin

4.3.4.8. Amino acid analysis

The distinctiveness of the amino acid sequence imparts characteristic coiled coil nature to the collagen molecule. The triple helical structure was steadied by inter chain hydrogen bonds as well as covalent cross linkages (Brodskey and Ramshaw 1997). The arrangement of amino acid determines the physical characteristics of gelatin. These physical characteristics influenced by amino acid arrangement, comparative amount of components as well as aggregation of higher and of lower molecular weight protein fragments. Also, the preservation of raw material is the key factor in determining properties of gelatin (Kala and Mathew 2017). Thermal degradation as well as physical or chemical denaturation causes the breaking of collagen triple helical polypeptide into gelatin (Bigi et al.2004). At temperature of about 40ºC, the aqueous solution of gelatin is in sol state and converts into physical thermo reversible gels upon cooling. During gelling the chains undergoes conformational changes, order alteration and slightly reorganize the triple helical collagen structure (Pezron et al.1991). The less amount of imino acid forms the less sterically hindered helix and may disturb the specific properties of gelatin. The HyP along with proline has important role in the stabilization of the triple helical collagen due to its hydrogen bonding capacity through its –OH group (Gormez-Guillen 2005).

The configuration of amino acids has main importance in determining gelatin gel strength and melting point. The significant amount of imino acid (proline and HyP) and glycine gives better strength to gelatin gel. The composition and alignment of the protein and amino acid differs in every fish species (Hossain et al.2016). The amount of proline and HyP was found to be 115.66±0.94/1000 residues and 92±0.86/1000 residues

respectively. The content of glycine of gethar gelatin (313.66±2.05/1000 residues) was more than bigeye snapper (246.57-259.38 residues/1000 residues; Benjakul et al.2009) but it was less than amur sturgeon skin gelatin (336 residues/1000 residues; Nikoo et al.2011) and shark species (321-322 residues/1000 residues; Kittiphattanabawon et al.,2010).

The acid hydrolysis of gelatin converts some amount of glutamine and asparagine into its acidic forms; i.e. glutamic acid and aspartic acid, respectively, and the associated removal of ammonia (Irwandi et al.2009). The aspartic and glutamic acid was found to be 49.66±1.69/1000 residues and 69.33±1.24/1000 residues respectively. The amino acid analysis of gethar extracted gelatin was tabulated in table no.4.3. The similar results were obtained for gelatin from the skin of farmed amur sturgeon and swim bladder of yellowfin tuna (Nikoo et al.2011; Kaewdang et al.2015).

Amino acid	**No.of amino acid per 1000 residues**	**% of amino acid**
Aspartic acid	49.66±1.69	4.96
Glutamic acid	69.33±1.24	6.93
Serine	44±1.63	4.4
Glycine	313.66±2.05	31.36
Histidine	3.66±0.47	0.36
Arginine	52.66±1.24	5.26
Threonine	27±0.86	2.7
Tyrosine	8.66±0.43	0.86
Alanine	108.33±0.94	10.83
Valine	22±0.81	2.2
Methionine	8.66±0.47	0.86

Cysteine	1	0.1
Isoleucine	17.66±1.24	1.76
Leucine	23.66±0.45	2.36
Phenylalanine	17±0.82	1.7
Lysine	26.66±0.44	2.666
Proline	115.66±0.94	11.56
Hydroxyproline	92±0.86	9.2

Table 4.3.: Amino acid analysis of extracted gelatin

4.3.5. Functional properties of gelatin

4.3.5.1. Gelatin solubility and viscosity

Generally commercial gelatin (eg: Hi-Media gelatin) used for laboratory experiment is hot water soluble while food grade gelatin in market is soluble in warm water. The gethar extracted gelatin was found to be soluble in hot water at 40ºC temperature and this is analogues to solubility of commercial gelatin.

Viscosity is the resistance of a fluid to change in shape or movement of neighbouring portions relative to one another. As per the British standard, gelatin viscosity values range from 1.5 to 7 cP. Figure no.4.11 showed viscous solution of gelatin. The viscosity of gethar skin extracted gelatin was reported as 6.67±0.19 cP. When compared with bovine skin gelatin, the obtained value is much more (1.74±0.03 cP; Khoirunnisa et al.2018) but less than 13.53±0.23 cP of black kingfish gelatin (Koli 2019). The viscosity was influenced by various factors which include temperature, pH, concentration as well as molecular weight and size distribution of proteins. Instrumental value of high gel strength does not give more viscosity value. The more viscosity of gelatin is occurred due to insoluble components present in it and less foam formation capacity (Lin et al.2015).

Figure 4.11.: Viscous solution of gelatin

4.3.5.2. Turbidity and clarity of gelatin

The decreased transparency of sample is generally referred as turbidity. Gel turbidity and clarity are complementary to each other. The effectiveness of purification procedure causes increased or decreased transparency of gelatin gel (Alfaro et al.2013). Gel clarity of sample is depends on temperature during extraction process. The figure no.4.12 illustrated the slightly turbid gelatin solution after clarification. The gelatin turbidity and clarity was influenced by inorganic and muco-substance contaminants which are not removed during purification as well as type of fish and raw material used (Eastoe and Leach 1977).

Clarity of gethar extracted gelatin was found to be 41.1±1.31 while turbidity value was 257.66±6.54 FTU. The gel clarity value of gethar skin gelatin was less than pacu skin and squid skin gelatin which ranges from 49.40±0.06 to 55.62±0.07 (Sahoo et al.2015; Nagarajan et al.,2012). The resulted turbidity value was higher than wami tilapia skin gelatin and mackerel head gelatin which was found to be 67 ± 4.3 NTU (Nephelometric turbidity unit) (Alfaro et al.2013) and 176±3.2 FTU (Khiari et al.2011) respectively. Gimenez et al. (2005) stated that, lactic acid employed for solubilisation of material causes more amounts of aggregation and thus results in greater turbidity value.

Figure 4.12.: Solution of gelatin after clarification

4.3.5.3. Melting and gelling temperature

It was reported that, melting and gelling temperature of fish gelatin was less than mammalian gelatin. Fish gelatin with less melting temperature releases enhanced odour and offered stronger aroma during preparation of food product also, control the consistency and flavour release during chewing of products. Gethar extracted gelatin possesses 10.75±0.65ºC gelling and 29.88±0.41ºC melting temperature. The results were less than gelling (28.69±0.67ºC) and melting (36.40±0.53ºC) temperature of bovine lung gelatin (Roy et al.2016) while more than cod skin extracted gelatin (gelling 4ºC and melting 13ºC; Haug et al.2004). Maturation time of gelatin results in rise of melting temperature (Koli et al.2013). The gelling and melting temperature is influenced by type and composition of raw material used, concentration of amino acid as well as molecular mass distribution of peptides in the gelatin solution. It has been reported that amount of imino acids (hydoxyproline+proline) is important parameter to study gelling and melting temperature (Pavan kumar et al.2017).

4.3.5.4. Emulsifying properties

Oil droplets were surrounded by longer chain so as to form sturdy and firm films and therefore increase the steadiness towards emulsion breakdown (Devi et al.2016). The attraction of protein towards oil droplets depends on surface hydrophobicity of proteins. The fundamental characteristics of protein, methods of formation and solubility have effect on emulsion capacity of protein (Damodaran 1997 and Sikorski 2001). Emulsifying characteristics of food proteins describe its capacity to stabilize newly generated emulsion and also provide strength to emulsion against stress conditions. Gelatin is a good surface active agent and has better emulsifying activity in oil-water emulsion (Zakaria and Bakar 2015). Table no.4.4 gives information about emulsifying activity index (EAI) and emulsion stability index (ESI) of 1%, 3% and 5% gelatin. EAI of gethar skin extracted gelatin ranges from 21.78±0.06 m^2g^{-1} to 29.24±0.06 m^2g^{-1} and it was highest for 3% gelatin with 37.17±0.12 m^2g^{-1}. ESI of gelatin varies from 12.63±0.29 min to 28.95±0.09 min and it was more for 3% gelatin with 28.95±0.09 min. The ESI and EAI of control gelatin were much higher than extracted gelatin. EAI was more than EAI of gelatin from skin of striped cat fish (11.21±0.72 to 16.89±0.05 m^2g^{-1}, Singh and Benjakul 2016). The value of ESI was analogues to ESI of marine iguana and great barracuda (Devi et al.2016).

4.3.5.5. Foaming properties

A protein with good foaming properties has capacity to migrate quickly to the air water boundary as well as have ability of unfolding and rearrangement (Devi et al.2016). Foaming capacity (FC) and stability (FS) are important functional properties of protein. Gelatin is generally employed as protein foaming agent. Due to this property, it may stabilize foams speedily and efficiently at less concentration and therefore utilized as an essential foaming agent over the wide pH range in different food applications (Zakaria and Bakar 2015). Table no.4.4 depicted FC and FS (%) of 1%, 3% and 5% gelatin. FC was increases from 1.22±0.08% to 1.40±0.21% and it was higher for 5% gelatin while FS rises from 1.06±0.01% to 1.09±0.01% and more for 5% gelatin. The values were slightly less than control gelatin.

Interaction of protein molecule with air-water surface generates foam and it was controlled by transportation, dispersion and reformation of protein component. More concentration of protein generates thick and steady foam, thus FC and FS increases with increase in gelatin percentage. Foaming properties were influenced by different factors such as protein source, protein water interaction, protein surface characteristics, equilibrium rate of surface tension, bulk and superficial viscosities, steric steadiness and electrical repulsion between the two edges of foam lamella (Pavan kumar et al.2017). The results were analogues to gelatin extracted from cobia (2.28% and 1.93%) and cynoglossus (2.35% and 1.86%) (Kala and Mathew 2017) but was much less than gelatin from skin of striped cat fish (Singh and Benjakul 2016).

Sample	Emulsifying activity index	Emulsion stability index	Foaming capacity	Foam stability
	($m^2 g^{-1}$)	(min)	(%)	(%)
1 % gelatin	21.78±0.06	12.63±0.29	1.22±0.08	1.06±0.01
3 % gelatin	37.17±0.12	28.95±0.09	1.34±0.07	1.03±0.02
5 % gelatin	29.24±0.06	19.22±0.32	1.40±0.21	1.09±0.01
Control	49.25±0.08	47.20±0.38	3.77±0.09	3.41±0.13

Table 4.4.: Emulsifying and foaming properties of gelatin

4.3.5.6. Water holding and fat binding capacity

Water holding (WHC) and fat binding capacity (FBC) are important functional characteristics that are resembled to interaction between gelatin surface with the water, oil and other constituents. Number of hydrophilic amino acids has positive effect on WHC while high value of FBC was attained due to more amount of tyrosine present in gelatin (Ninan et al.2009). The WHC of gethar extracted gelatin was found to be 262.33±13.88% which is higher than cobia skin (179.26±3.30%) and cynoglossus skin (169.34±4.01%) (Kala and Mathew 2017) as well as rohu skin (184.33±3.30%) and common carp skin (176.00±4.90%) (Ninan et al.2009). The FBC of extracted gelatin was recorded as 407.66±14.83% which is lower than rohu skin (457.33±6.55%) but was higher than common carp skin (333.00±5.10%), cynoglossus (389.90±5.12%) and cobia (369.78±4.67%) (Kala and Mathew 2017; Ninan et al.2009).

The WHC of gelatin is a useful property for the preparation of sausages, custards and dough because these products include gelatin dispersed in water which is used as thickening agent as well as increases viscosity of product. More value of FBC is required to preserve flavour, increases tastiness and extends shelf life of meat products. FBC is assumed to be associated with the percentage of available hydrophobic, electrostatic and hydrogen linkages as well as mainly with hydrophobic amino acids like tyrosine, leucine, valine and isoleucine (Roy et al.2016).

4.3.5.7. Effect of pH on gelatin solubility

The effect of pH on gelatin solubility was observed at broad pH range of 2-14 as showed in figure no.4.13 and it was increased from pH 2.0 to 6.0 and reached to maximum solubility (99.55%) at pH 6 while very low solubility was obtained at pH 8 (81.36%). However, commercial gelatin has maximum solubility at pH 6 (99.60%). This difference in solubility may be due to alteration in molecular weight and unequal distribution of polar and non-polar group concentration in amino acid. The pH solubility is essential requirement for functional food used as a protein supplement in food industry (Ratnasari and Firlianty 2016). The results were closely resembled with gelatin of pangas catfish (pH 5.8) and walking catfish (pH 5.9) (Cheow et al.2007) but higher than gelatin from nile tilapia (pH 3.05) and black tilapia (pH 3.91) (Bakar and Harvinder 2002). The reduction in solubility at pH 8.0 is adjacent to its isoelectric point and it is due to greater electrostatic interaction. Gelatin is an amphoteric protein with an isoelectric point between pH 5.0 to 9.0 depending on raw material and method of extraction. At pH values below and above the isoelectric point, proteins possess more net charges, thus improving

hydration (Haddar et al.2011). Increased viscosity and higher pH were positively associated with each other, thus increasing viscosity results in rise of gelatin pH. The increasing pH from 2.0 to 14 causes decrease in viscosity of gelatin at pH range of 6.0-8.0 (Stainsby 1987b).

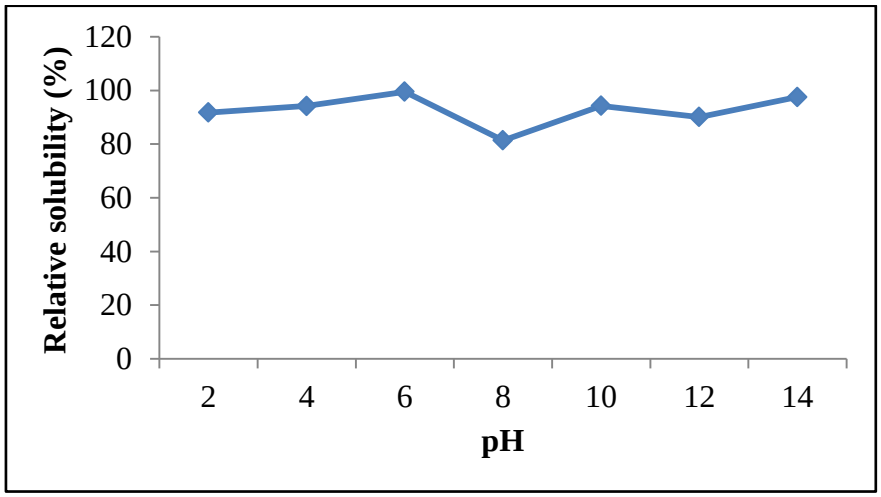

Figure 4.13.: Effect of pH on relative solubility of gelatin

4.3.6. Fatty acid profiling of fish oil

Gas chromatography followed by mass spectrometry was employed to detect and quantify the composition of fatty acids present in fish oils. Figure no.4.14 (a) and (b) elucidates the chromatogram of gethar fish head extracted oil before and after enzymatic action respectively. The triglycerides structure in gethar oil undergoes saponification to produce glycerol and free fatty acids. Then, methanol causes methylation of free fatty acids and derivatization in the methyl ester form and produces fatty acid methyl ester (FAME) (Iberahim et al.2018). The composition percentages polyunsaturated fatty acid is 3.18% EPA and 14.52% DHA before concentrated by enzyme while 1.74% EPA and 14.19% DHA was obtained after enzyme action which showed significant change and quantity of polyunsaturated fatty acid was reduced. This spontaneous change was observed due to auto-oxidation and photo-oxidation of fish oil.

It was studied that, polyunsaturated fatty acids more prone to oxidation than other. The oxidation of PUFA may release hydroperoxide and it will further oxidized into ketones or aldehydes compound (Iberahim et al.2018). The obtained results are in accordance with fish oil extracted from head of tropical little tuna in which 1.48% EPA and 15.7% DHA while in skipjack tuna 0.1% EPA and 18.8% DHA was found (Khoddami et al.2012; Chantachum et al.2000). The alteration in fatty acid compositions was occurred due to variations in the raw material utilized which has a lesser content of EPA and DHA than the cold water. The fatty acid composition of marine fishes varied on

the basis of lipid content of catch, water temperature, breeding season, age, geographical indices and nature of species (Ferdosh et al.2016).

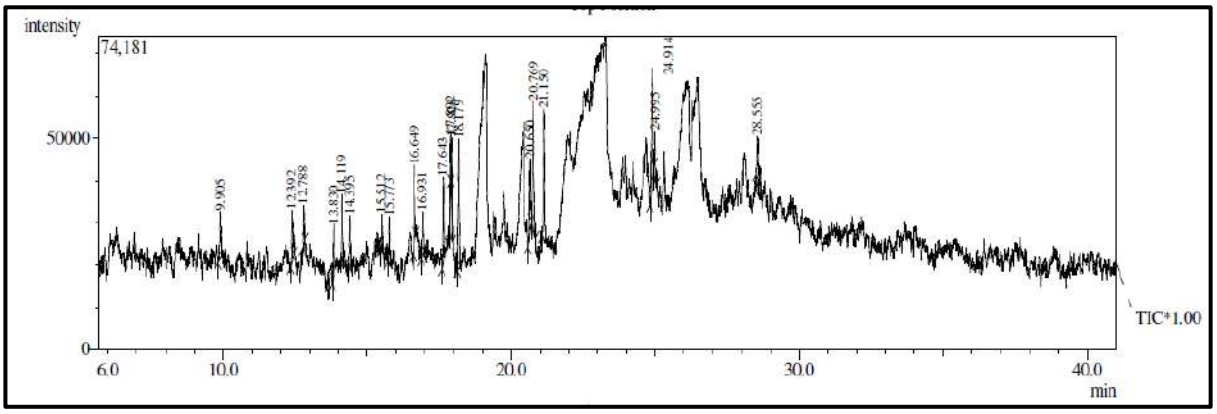

Figure 4.14 (a).: GCMS analysis of oil before enzymatic action

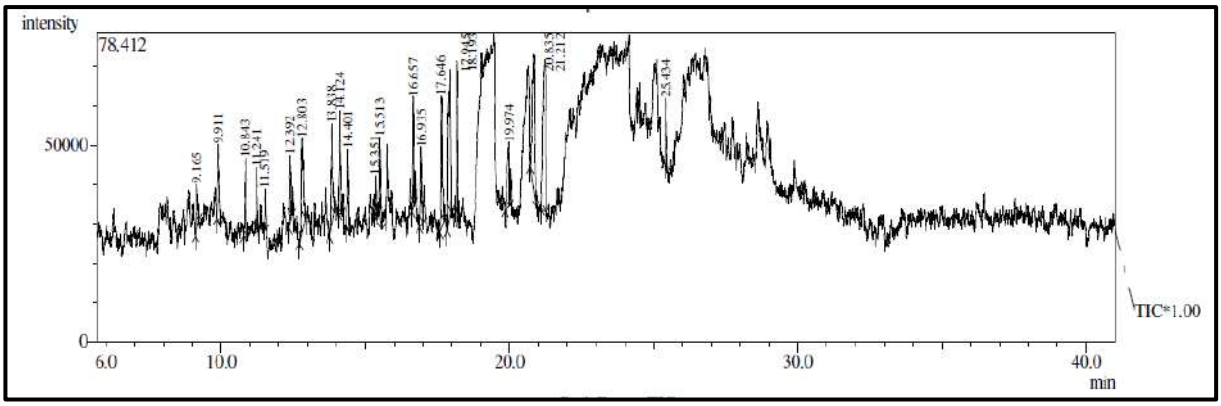

Figure 4.14 (b).: GCMS analysis of oil after enzymatic action

4.3.7. Functional properties of fish oil

4.3.7.1. Free fatty acid (FFA) content

The free fatty acid content determines the degree of disintegration of lipase activity and it was enhanced by heat as well as light. The lower value of FFA means oil is better quality because it has low lipase concentration (Aryee and Simpson 2009). About 2.25±0.20% FFA was present in extracted fish oil. The amount was higher than 0.7-1.7% of bigeye tuna (Ahmed et al.2017) but lower than 3.4% of tuna canning waste oil (Suriani and Komansilan 2019). Free fatty acids are vital component of hydrolytic degradation related to bad flavour and textural alterations. As per the International Association of Fish Meal and Oil Manufacturers (IFOMA 1981) the standard FFA value of fish oil ranges from 1%-7%. Above 3.5% FFA content of oil is inappropriate for edible purpose. The extracted oil has less than 3.5% FFA thus it may be suitable for edible purposes (Bako et al.2017).

4.3.7.2.Acid value of oil

The acid value determines the quantity of potassium hydroxide in milligram required to counterbalance the free fatty acid per one gm of oil. According to research, the lesser acid value means better the quality of oil while higher value is accompanying with the rancidity produced by hydrolysis of ester bonds as well as oxidation of double bonds (Ahmed et al.2017). The appropriate acid value of oil that can consume by human was lower than 5mg KOH/gm. The acid value was influenced by some factors including extraction method, freshness of raw resources and oil composition (Iberahim et al.2018). The acid value of gethar fish extracted oil was 2.57±0.36 mg KOH/gm of sample which was higher than 2.24mg KOH /gm of catfish oil (Iberahim et al., 2018) and lower than 13.63-16.94 KOH/gm of cobia liver oil (Santos et al.2016).

4.3.7.3. Iodine value

The quantity of unsaturated fatty acids present in fish oil was calculated using iodine value. The double bonds exhibited in unsaturated fatty acids would interact with iodine. More iodine value of oil indicated more number of unsaturated fatty acids. So it can easily bind with the iodine in large proportion (Pandiangan et al.2018). The iodine value of extracted oil was found to be 107.88±0.53 gm iodine/gm oil. The obtained value is related to the iodine value of crude, refined and hydrolyzed oil from different fish species and was reported as 117.7±0.2, 119±1.2 and 93.92±0.7 gm iodine/gm oil respectively (Nascimento et al.2015).

4.3.7.4. Saponification value

Saponification is the breakdown of neutral fat due to by alkali treatment into glycerol and fatty acids. The standard sap value for fish oil was 180-200 mg KOH/gm given by AOAC. The sap value of extracted oil was recorded as 78.53±0.49 mg KOH/gm oil which was much less than 295.4 mg KOH/gm oil of leaching fish waste as well as 177.8 mg KOH/gm of tilapia visceral oil (Norziah et al.2010; Oliveira et al.2013). It was observed that, crude oil comprises slight quantity of non-triglyceride constituents. Thus, higher sap value may be due to impurities that is unsaponifiable matter including sterols, glycerol ethers, hydrocarbons, fatty alcohols as well as less quantities of pigments and vitamins exhibited in crude fish oil (Bimbo and Crowther 1991; Norziah et al.2010).

4.3.7.5. Peroxide value

During the oxidation of oil hydrogen peroxides were generated and their quantity was determined by peroxide value. According to research, less peroxide value indicates good quality of oil. The gethar fish extracted oil has 25.72±0.45 Meq/gm oil peroxide

value which was higher than 2.5-5.5 Meq/gm oil of salmon waste (Jayasinghe et al.2013) and 20 Meq/gm of catfish oil (Iberahim et al.2018). There are various components that increases oxidation rate of oil. It includes moisture content, fatty acid concentration, light intensity, oxygen accessibility and temperature. Also greater amount of polyunsaturated fatty acid will cause fish oil enormously liable to oxidation (Iberahim et al.2018).

4.3.7.6. p-anisidine value

Anisidine value is an experimentally tested value which decides the progressive oxidative rancidity of oils and fats. The measure of α and β unsaturation due to secondary oxidation were determined by using p-anisidine value (Lee et al.2017). The p-anisidine value of gethar fish oil was recorded as 17.29±0.48 Meq/gm oil which was more than sardine fish (7.48 Meq/gm oil) and oyster oil (6.52-12.14 Meq/gm oil) (Hiremath et al.2018; Lee et al.2017).

4.3.8. Sensory analysis of gelatin and fish oil

The sensory analysis like appearance, odour, colour, texture and overall acceptability of pure and extracted gelatin were monitored using 9 point hedonic scale. The sensory analysis of extracted gelatin along with control was given in figure no.4.15. The gethar skin extracted gelatin has slight fishy odour but not easily perceivable. It has jelly like appearance with sticky nature and brownish yellow in colour. The colour and odour can be improved by utilizing more advanced technology for clarification of gelatin. Hence, good sensory characteristics of gelatin can be acceptable for various applications. The disparity in sensory characteristics of extracted gelatin was may be influenced by environmental living conditions of fish such as sea depth of fish habitat and habits, materials used for extraction, pollution level as well as variety of planktons around it (Jamilah and Harvinder 2002; Irwandi et al.2009).The sensory analysis was correlated to gelatin extracted from rohu, common carp and kerisi (white snapper) (Irwandi et al.2009; Ninan et al.2009). The sensory assessment of gethar fish extracted oil along with control (mackerel oil) was depicted in figure no. 4.16. The appearance, colour, fishy odour and overall acceptability were analysed using 5 point hedonic scale. The oil has slight fishy odour than control with reddish brown in colour as well as appeared same as control oil. Thus, due to good sensory properties it can be suitable for various applications. The similar analysis was observed for catfish and mackerel oil (Oladapo and Awojide 2015).

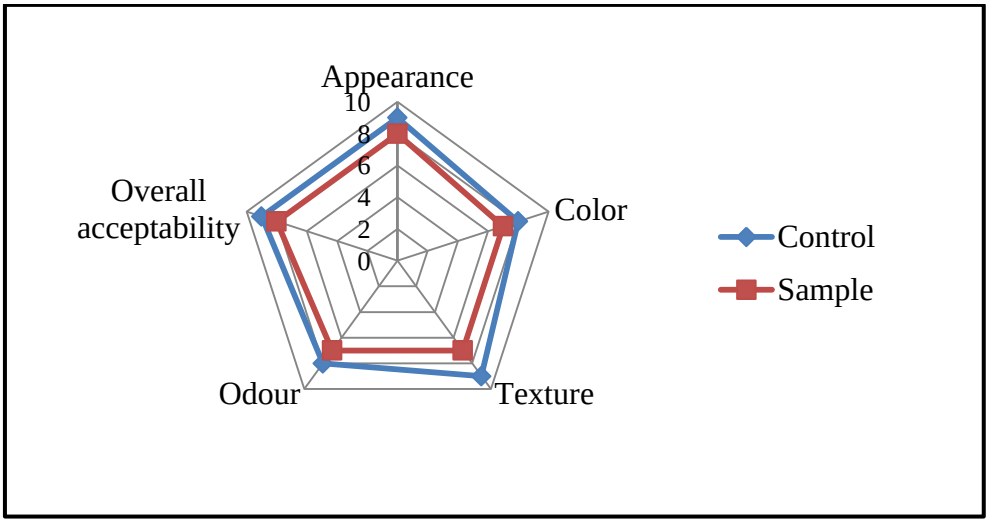

Figure 4.15.: Sensory analysis of extracted gelatin

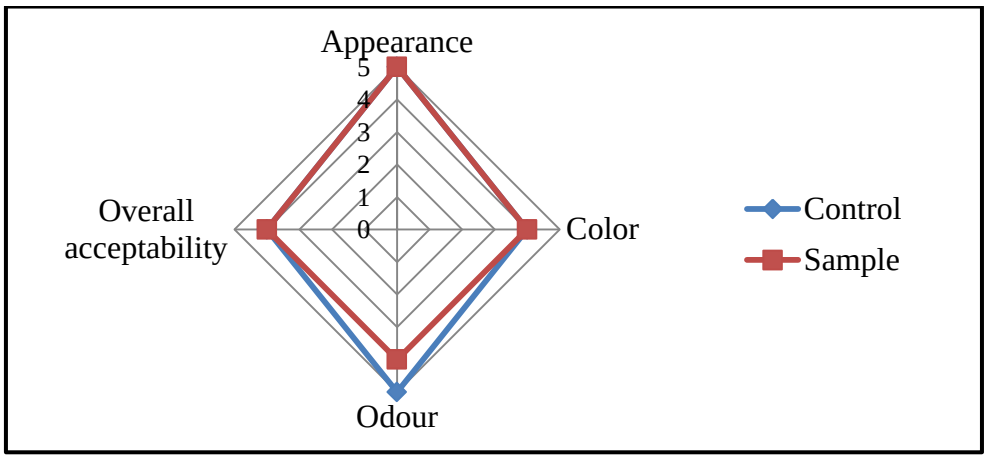

Figure 4.16.: Sensory analysis of gethar fish oil

4.4. Conclusion

In current study, proficient minimization and utilization of fish waste into value added products provides an attractive way to reduce some environmental problems by sustainable development. The fish waste which may acts as major environmental pollutant to disturb ecological balance was effectively utilized for extraction of gelatin and fish oil containing polyunsaturated fatty acids. Gelatin extracted from skin of gethar (*Sarda orientalis*) exhibited better yield with lactic acid treatment in less extraction time. It gives 0.63±0.03 gm/gm gelatin within 2 hr. The structural properties like UV-visible spectra, SDS-PAGE analysis, FTIR, XRD, DSC, zeta potential and particle size analysis confirmed that extracted gelatin was type A with pH 8.0 as isoelectric point. Extracted gelatin exhibited significant water holding capacity, fat binding ability, emulsifying and

foaming properties as well as good gelling and melting temperature. The good amount of lipid was found in proximate analysis of fish head so it can be useful for oil extraction. The methanol, chloroform and water are the best solvent system for oil extraction with 27.63±0.24% yield. The free fatty acid 2.25±0.20% was noted. The acid, iodine and saponification value of oil was found to be 2.57±0.36 mg KOH/gm of oil, 107.88±0.53 gm iodine/gm oil and 78.53±0.49 mg KOH/gm oil respectively. The oil possesses 25.72±0.45 Meq/gm oil peroxide and 17.29±0.48 Meq/gm oil p-anisidine value. Extracted oil exhibited good sensory properties with slight fishy odour. Gas Chromatography-Mass Spectroscopy was used to analysis the fatty acid composition of gethar fish oil. The analysis shows that there are two types of omega-3 polyunsaturated fatty acid found in gethar oil which is EPA (1.74%) and DHA (14.19%). Due to better characteristics gethar fish extracted gelatin and omega-3 fatty acid can be suitable for potential applications in nutraceutical as well as pharmaceutical fields. Therefore, there is a potential for exploitation of processing waste for gelatin and omega-3 fatty acid extraction from these species and also provides best health benefits.

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CHAPTER V

MICROBIAL DEGRADATION OF RESIDUAL FISH WASTE AND USE OF HYDROLYSATE AS PLANT GROWTH STIMULATOR

5.1. Introduction

From many decades, aquaculture and capture fisheries have contributed to the international economy by supporting livelihood as well as nutritional status. The subsequent rise in this area generates more than 60% by-products as waste which comprises scales, skin, head, frames, tail, fins and viscera. These unutilised wastes of fish are simply discarded which produces hazardous pollution and threat to the environment or can be used to prepare low market value products like fish meal, animal feed and fertilizers (Wangkheirakpam et al.2019). The fish releases non faecal components such as ammonia, urea, ortho-phosphate, and carbon dioxide into the surrounding water body. These wastes are harmful to the fish, if they are not removed and threat to environment (eutrophication and greenhouse effect), if they are released outside.

According to the research, fish waste contains some value added products including proteins like collagen and gelatin, amino acids, bioactive peptides, enzymes, oil and minerals (Kim and Mendis 2006; Blanco et al.2007; Ghaly et al.2013). More quantity of organic content was present in solid waste of fish released from seafood processing industries. Conventionally, fish wastes can be employed as fertilizer due to presence of its nutritive components (mainly N and P) and their quick disintegration. By using bioconversion practises, this waste can be converted into eco-friendly compost. The manufacturing of compost containing fish debris mainly obtained from aquaculture have been carried out in different regions of the world to find out feasible and sustainable procedures to convert fish waste into beneficial agricultural products. The microorganisms were utilized in this bioconversion helpful to accelerate the composting process. The produced compost is rich in nutrients as well as free from harmful elements. Thus it can be useful as organic fertilizer instead of commercially available chemical fertilizers. Composting method eliminates disease-causing organisms and flies larvae. Therefore, composting of fish waste is comparatively new, economically feasible, partially odourless method, environmentally safe and biologically favourable operation of fish waste disposal as compared to other discarding options (Lopez-Mosquera et al. 2011; Rajeswari et al.2018).

Protein hydrolysates like feather and fish hydrolysates are smaller group of plant biostimulants having phyto hormones like ability. Protein hydrolysates are made up of free amino acids and polypeptides as well as may consist of macro and micro-nutrients, polysaccharides and lipids which are present in original resources. The protein containing hydrolysate have capacity to improve physiological properties of crops that causes

growth which includes enhancement of yield and product quality, creating tolerance against environmental conditions like salinity, drought, thermal as well as nutrient stress and elevated soil pH (Fitriyah et al.2022).

Exploitation of waste containing organic matter in soil, is an appropriate way which naturally maintain the organic content in soil and has impact on soil fertility as well as provide essential nutrients required for plant growth (Ellyzatul et al.2018).The nutrients in fish waste hydrolysate stimulate development of plants through growth enhancing rhizobacteria, atmospheric nitrogen fixation and improving the uptake of necessary nutrients. Fish waste comprises vital minerals like calcium, phosphorus, potassium, sodium, magnesium, zinc, manganese and copper comparable to the nutritive value as fish so, it can acts as raw material for making of various nutritive and non-nutritive components. The quantity of protein, amino acids, calcium and phosphorus were found to be raised after the microbial degradation of the fish waste. These constituents are mainly utilized by plants for their growth and development (Thankachan and Chitra 2021).

Radziemska et al.(2018) stated that fertilizer from fish waste effectively utilized in agriculture. The study was carried out on ice lettuce (*Lactuca sativa L.*) to evaluate yield as well as macro and micro elemental composition of plant. The fish pond effluent was employed as organic fertilizer on growth of cucumber (*Cucumis sativus*) and helps to improve chemical properties of soil (Ndubuisi 2019). The fertilizer generated from fish and fish waste was effectively employed in organic farming to increase the properties of crop and horticultural plants (Ahuja et al.2020). There was little information about effect of protein hydrolysate derived from chicken feather waste and fish waste remaining after processing in agricultural crops (Fitriyah et al.2022).

The current study was designed for microbial degradation of residual fish waste remaining after valuable collagen recovery. The fish waste degrading micro-organism was isolated from waste dumping site and utilized for microbial hydrolysis of waste into protein hydrolysate. The nutrient rich protein hydrolysate was studied for its plant growth promoting potential on *Vigna radiata* as well as soil nourishment properties of hydrolysate was studied.

5.2. Materials and method

5.2.1. Sample collection

The soil samples were collected from fish waste dumping site of the Ichalkaranji region, Kolhapur, Maharashtra, India. The samples were collected in sterile autoclavable plastic bags and stored at RT for further studies.

5.2.2. Bacterial isolation

Approx. 1 gm of soil sample was added into minimal broth which consists of (gm/l): KH_2PO_4, 3; Na_2HPO_4, 6; NaCl, 5; NH_4Cl, 2; $MgSO_4$, 0.1; glucose, 8 and enrichment was carried out for 48 hr at 30ºC. The enrichment was repeated for 3 times. At the end of day, enriched sample was subjected to primary screening for isolation of micro-organisms.

5.2.2.1. Primary screening

The primary screening of enriched soil sample was carried out on nutrient agar. Serially diluted enriched sample were spread on media and incubated at 30ºC for 24 hr. Then, distinct isolates were selected and spread on casein agar media having composition 125 ml/l skimmed milk, 875 ml/l nutrient broth, 15 gm/l agar and pH was adjusted at 7.2 then after, plates were incubated at 30ºC for 24 hr and observed for zone of hydrolysis. Protease positive isolates were selected and used for secondary screening.

5.2.2.2. Secondary screening

To check potency of fish waste degradation ability, fish waste containing minimal broth was used. It was composed of (gm/l): NaCl, 0.5; $MgCl_2.6H_2O$, 0.24; K_2HPO_4, 0.3; NH_4Cl, 0.5; KH_2PO_4, 0.4; yeast extract,0.1; fish waste,10; pH 7.5 (Sekar et al.2015). 1 ml suspension of selected strains were inoculated into both and kept for 30ºC under constant stirring in shaking incubator. The cultures were supervised for fish waste degradation which lasts for 7 days. Degradation proficiency of micro-organism was calculated by using following formula;

$$\frac{\text{Weight of degraded fish waste}}{\text{Weight of fish waste}} \times 100$$

5.2.3. Enzyme activity assay

For enzyme activity assay, 0.2% gelatin was used as a substrate. The reaction mixture was prepared by mixing 0.3 ml of 0.2% w/v substrate, 0.2 ml culture supernatant and 0.5 ml 100 mM Tris-HCl buffer (pH 7.5). Incubation was carried out at 37ºC for 30 min and reaction was ceased by 0.5 ml TCA. Ninhydrin method was carried out to assess

liberated free amino acids (Mahesha 2012). One unit enzyme activity was defined as 1 μmol leucine released per ml of enzyme per min.

5.2.4. Effect for carbon and nitrogen sources on enzyme activity

To study effect of various carbon and nitrogen sources on enzyme activity, procedure of Pustake et al. (2019) was used. Carbon and nitrogen sources were screened to analyse their efficiency on the fish waste degradation activity of potent isolate. 1% w/v of numerous carbon sources (dextrose, fructose, lactose, CM cellulose, starch) as well as inorganic nitrogen sources (ammonium chloride, ammonium sulphate, urea, glycine, sodium nitrate) and organic nitrogen sources (soya peptone, yeast extract, beef extract, peptone) were applied to examine their impact on degradation activity.

5.2.5. Biochemical characterization of potent strain

The strain having effective waste degradation activity was selected and explored for biochemical characterization such as Gram staining, IMVIC test (Indole, Methyl Red, Voges Proskauer and Citrate), starch hydrolysis, gelatin liquefaction, urease activity as well as effect of NaCl concentration, pH and temperature were studied.

5.2.5.1. Gram staining

Gram staining was executed according to method of Abiola and Oyetayo (2016) with slight alterations. On a clean grease free slide, a thin smear of 24 hr old bacterial culture was prepared and fixed it by passing through gentle flame. Firstly, smear was stained by crystal violet for 60 second and cleaned with water. Then, smear was flooded with Gram's iodine for 60 second and again rinsed with water. It was decolorized by using ethanol for 30 second and rinsed with water. At the last, safranin was applied as counter stain for 60 second and again rinsed with water. The smear was allowed to air dry and observed under oil immersion lens of microscope using cidar wood oil. Gram positive bacteria appeared as violet color while Gram negative showed pink colour.

5.2.5.2. IMVIC analysis

IMVIC test consist of indole (I), methyl red (MR), vogues proskauer (VP) and citrate utilization (C). The tests were performed according to method of Hussain et al. (2013) with some changes. In indole test bacteria were incubated in tryptophan broth at 30ºC for 24 hr after that Kovac's reagent (0.5 ml) were added to it and observed for pink red ring formation. For MR test, micro-organism were incubated in MRVP broth at 30ºC for 24 hr and after incubation 5 drops of methyl red were added to it and observed for formation of red colored ring. VP test was performed by using MRVP broth and bacteria were incubated in it at 30ºC for 24-48 hr. After incubation, 0.6 ml of alpha-naphthol

solution (VP-A reagent) and 0.2 ml of potassium hydroxide (VP-B reagent) were added to it and observed for pink red color. For citrate utilization, micro-organism were streaked on citrate agar and observed for color change of media from intense blue to deep green within 24-72 hr at 30ºC.

5.2.5.3. Catalase activity

Catalase activity of isolate was performed according to Syahri et al. (2019) using 3% hydrogen peroxide (H_2O_2). Bacterial suspension was taken on glass slide and 100 µl of 3% H_2O_2 were added onto it. Slide was observed for formation of bubbles which indicates positive catalase activity of bacteria.

5.2.5.4. Urease test

Urease test was performed to determine the ability of micro-organism to split urea through urease enzyme. For this purpose, (gm/l): urea, 20; NaCl, 5; KH_2PO_4, 2; peptone 1; dextrose, 1; phenol red, 0.012; agar 15 gm; and pH was adjusted to 6.7. Bacterial culture were spread on plate and incubated at 35-37ºC for 48 hr and observed for colour change. The formation of pink colour indicates positive urease activity (Isenberg 1992).

5.2.5.5. Starch hydrolysis

Starch hydrolysis test of micro-organisms was done to check their capability to degrade substrates with carbon composites and able to grow on it. It was performed as per method of Abiola and Oyetayo (2016) with slight modifications. 2 gm of starch was solubilized in nutrient agar and plates were prepared. Bacterial suspension was spread on it and incubated at 30ºC for 24 hr. After growth, plates were flooded with Gram's iodine. Clear zone around colony indicates hydrolysis of starch due to alpha amylase activity of bacteria while unhydrolyzed starch forms blue colour in presence of iodine.

5.2.5.6. Gelatin liquefaction

Gelatin hydrolysis test was carried on nutrient gelatin media. For this, 23 gm/l nutrient agar and 8 gm/lit gelatin were used. Nutrient gelatin plates were inoculated with bacteria and incubated at 30ºC for 24 hr. After adequate microbial growth, plates were flooded with saturated ammonium sulphate to precipitate unhydrolyzed gelatin and observed for clear zone around bacterial colony within 5-10 min (Isenberg 1992).

5.2.5.7. Phenylalanine deaminase

The aim of this experiment is to determine ability of bacteria to oxidatively deaminate phenylalanine to phenylpyruvic acid. The media containing yeast extract 3 gm; NaCl 5 gm; Phenylalanine 2 gm; Na_2HPO_4 1 gm; agar 15 gm and D/W 1000 ml were used. The plates were spread with bacterial suspension and incubate at 30ºC for 24

hr. After incubation, plates were flooded with 10% ferric chloride and observed for light to deep green colour (Tille and Forbes 2014).

5.2.5.8. Effect of NaCl concentration, pH and temperature

The influence of NaCl concentration, pH and temperature on growth of micro-organism was studied by using method of Hussain et al. (2013) with slight modification. For this purpose, salt concentration from 1% to 10%, pH ranging from 2.0 to 10 and temperature varying from 10 to 50ºC were studied.

5.2.6. Microbial strain identification and phylogenetic analysis

DNA was isolated from the potent bacterial strain. Its quality was evaluated on 1.0% Agarose Gel, a single band of high-molecular weight DNA has been observed. Fragment of 16S rDNA gene was amplified by 27F and 1492R primers. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyser. Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the database of NCBI GenBank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 7.

5.2.7. Estimation of liberated end compounds

Fish waste degradation by micro-organism results in discharge of some useful end products like proteins as well as amino acid. Assessment of these products was carried out till complete degradation was attained. Protein estimation was carried out by Lowery method using BSA as standard (Waterborg 1996) while amino acid content was calculated by Ninhydrin method using Leucine as standard (Mahesha 2012).

5.2.8. Antibiotic susceptibility test of micro-organism

After 16 S rDNA sequencing, identified isolate was tested for antibiotic susceptibility on nutrient agar according to method of Begum et al. (2017) with some modification. Disc diffusion method was performed and commercially available seven antibiotic discs of penicillin (P, 10 mcg), chloramphenicol (C, 10 mcg), streptomycin (S, 10 mcg), gentamycin (GEN, 10 mcg), azithromycin (AZM, 15 mcg), amoxicillin (AMC, 30 mcg) and clarithromycin (CLR, 15 mcg) was employed for this investigation. Above pre-treated antibiotic plates were incubated at 37ºC for 24 hr. Sensitivity of micro-

organism against each antibiotic was determined by measuring zone of inhibition (mm) on next day.

5.2.9. Plant growth promotion by fish waste hydrolysate

After degradation of fish waste by micro-organisms, the residual hydrolysate was tested for their capacity of plant growth stimulation. The mung seeds (*Vigna radiata*) were used for this study.

5.2.9.1. Plantation and dose application

To evaluate plant growth promotion, fish waste hydrolysate was utilized. The study was carried out during month of September to November using pot method. The mung seeds were surface sterilized by 70% ethanol and further washed appropriately with D/W and sowed at the start of (3^{rd} Sept.) September. 5ml of fish hydrolysate was supplemented to test plant; with 5 days of interval and continued for one month until germination was achieved. The water was supplied to both plants test and control while control was excluded of hydrolysate.

5.2.9.2. Evaluation of morphological parameters of plants

Both control and hydrolysate treated plant were monitored for above stipulated time and at the middle of November, plants were fully developed into mature plant and attained maximum growth. After that plants were analysed for its biochemical as well as morphological parameters including, shoot, root length, root hairs, number of leaves, flowers, both chlorophyll a and b pigment, protein, phenolic and flavonoid content were investigated.

5.2.9.3. Biochemical analysis of plants

5.2.9.3.1. Chlorophyll estimation

Chlorophyll content was estimated as per protocol of Arnon (1949) with few modifications. Thoroughly washed 1g fresh leaves of mung were crushed in (80 % v/v) acetone to prepare slurry and make the final volume 20ml. The extract was refrigerated overnight followed by centrifugation and absorbance of supernatant was measured spectrophotometrically at 645 nm and 663 nm (Shimadzu UV-1800, Japan).

$$\text{Chlorophyll a } (\mu g/L) = (12.7 \times A663) - (2.69 \times A645)$$

$$\text{Chlorophyll b } (\mu g/L) = (22.9 \times A645) - (4.68 \times A663)$$

$$\text{Total Chlorophyll } (\mu g/L) = (20.2 \times A645) + (8.02 \times A663)$$

5.2.9.3.2. Estimation of free proteins and amino acids

The procedure of Gurav and Jadhav (2013) was followed to determine the free proteins and amino acids. For sample preparation, 0.5gm of vegetative part of it

homogenised in 80% ethanol and 100 mM phosphate buffer (pH 7.0) respectively. Extracts were centrifuged and estimated for protein and amino acids by using Lowry (Lowry et al.1951) and Ninhydrin method respectively (Moore and Stein 1954).

5.2.9.3.3. Determination of total phenolics and flavonoid

The total phenolics content was calculated by Folin-Ciocalteu's reagent (Singleton and Rossi 1965). Reaction mixture were prepared and incubated for 90 min in dark. After incubation, the absorbance was measured at 765 nm and total phenolics were calculated by using standard gallic acid calibration curve. The total flavonoid was determined by measuring absorbance at 415 nm (Shimadzu UV-1800, Japan) by using standard quercetin curve (Chang et al.2002).

5.2.9.4. Chemical analysis of soil

Chemical analysis of sample soil was carried out to study the effect of fish waste hydrolysate on plant growth. The Kjeldahl's method and TOC analyser was used to determine total nitrogen and carbon content respectively. Similarly, samples were prepared to check its P, K, Ca, Mg and Zn content by the method of Hseu (2004). Diluted samples were analysed for the presence of P, K (flame photometry), Zn, Mn, Ca and Mg (atomic absorption spectrophotometer).

5.2.10. Statistical analysis

All the experimental sets were performed in triplicates (n=3). The statistical analysis was carried out on the basis of mean and standard derivation (SD).

5.3. Result and discussion

5.3.1. Isolation of potent bacterial strain from soil

In current work, it was observed that the enriched minimal media containing micro-organisms exhibiting fish waste degradation potential. Total 13 micro-organisms were potent and are selected on the basis of their growth on nutrient agar and named as PSD 1 to PSD 13.

5.3.2. Proteolytic activity

Isolates selected from serially diluted sample were listed in table no.5.1 and checked for their proteolytic activity on casein agar. Out of which, eight isolates displayed significant and prominent hydrolytic zone (figure no.5.1). Isolate PSD 11 showed remarkable casinolytic activity and screened for its degradation ability as well.

Figure 5.1.: Plates showing zone of casein hydrolysis by eight isolates during screening

No.	Strains	Growth on nutrient agar	Caseinase activity	Zone of hydrolysis (mm)	Fish waste degradation activity
01	PSD 1	+	+	11	+
02	PSD 2	+	+	12	+
03	PSD 3	+	+	8	-
04	PSD 4	+	-	-	-
05	PSD 5	+	+	7	+
06	PSD 6	+	-	-	-
07	PSD 7	+	-	-	-
08	PSD 8	+	+	5	-
09	PSD 9	+	+	2	-
10	PSD 10	+	+	10	-
11	PSD 11	+	+	15	+
12	PSD 12	+	-	-	-
13	PSD 13	+	-	-	-

Table 5.1.: Strains isolated using synthetic casein medium

5.3.3. Fish waste degradation activity

Isolates was selected on the basis of their ability to hydrolyse fish waste in minimum period. Figure no.5.2 represents the residual fish waste remaining after collagen recovery; utilized for degradation studies. This study was carried out by using a minimal broth containing fish waste as sole carbon and nitrogen source. Entire fish waste was accompanied in media to study waste degradation by visual identification (figure no.5.3). The maximum degradation of waste showed by PSD 11 than PSD 1, PSD 2 and PSD 5, this noticeable change was observed in 7 days while rest of three strains were delay in biochemical process.

The obtained results were closely related to feather waste degradation by keratinolytic bacteria (Nagarajan et al.2017; Bhange et al.2016) and fish waste degradation by *B. cereus* (Bhagwat et al.2018). The results were further assisted by their considerable increase in turbidity of media which indicates that fish waste was utilized for microbial growth (Reyes et al.2018) and therefore, PSD 11 was explored for further characterization. Fish waste degrading efficiency of bacterium under suitable conditions was found to be 64% (weight of initial fish waste: - 0.5 gm/50 ml and degraded one: - 0.32 gm).

Figure 5.2: Fish waste remaining after collagen extraction

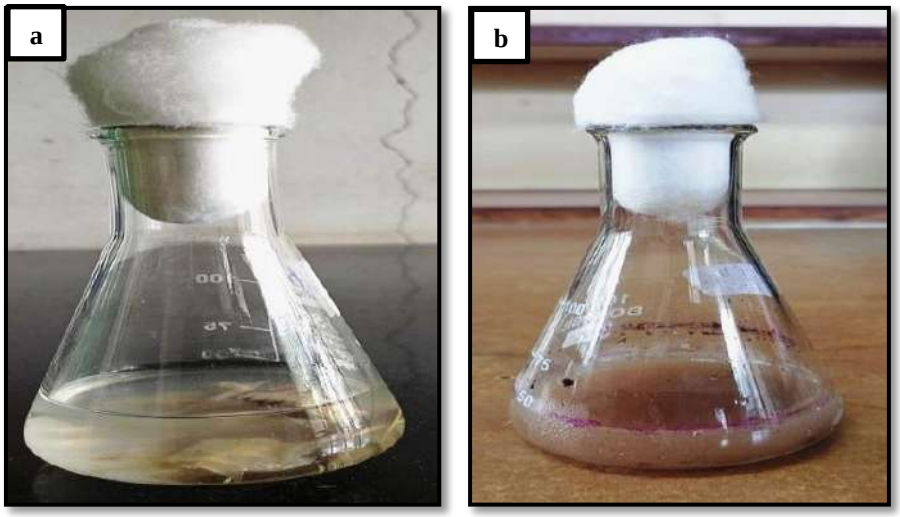

Figure 5.3.: (a) Control without micro-organism; (b) Test sample with micro-organism

5.3.4. Enzyme activity

Fish waste degradation capability of PSD 11 was continuously monitored during microbial growth. It was observed that, the activity was enhanced day by day (Govinden and Puchooa 2012) and the change was significant. Activity was minimum on zero day (8.24±0.27 µg/ml/min) while at the end of seventh day maximum activity was attained. The figure no. 5.4 indicated the increase in enzyme activity within seven days and after that it was ceases. In current study, it was observed that activity was increased up to 72.41±0.21 µg/ml/min in same period which was more significant in comparison of earlier studies.

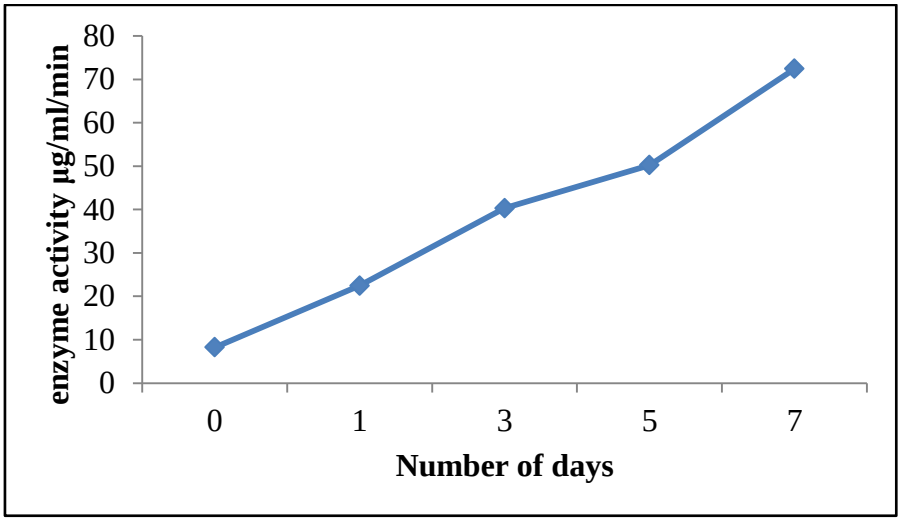

Figure 5.4.: Enzyme activity with respect to number of days

5.3.5. Enzyme activity in presence of carbon and nitrogen sources

Carbon and nitrogen source plays important role in any media formulation. Simple and easily available sources are usually preferred. Enzyme activity may be increased or decreased in presence of these sources and it depends on nature of sources used. Enzyme activities were analysed with respect to various simple to complex carbon and nitrogen sources (organic as well as inorganic). Among these different carbon sources used, enzyme activity was maximal in presence of dextrose (77.35±0.85 µg/ml/min) while least in starch (44.97±0.69 µg/ml/min). In comparison with organic and inorganic nitrogen sources; yeast extract (organic source) and ammonium chloride (inorganic source) exhibited 46.13±0.45 µg/ml/min and 63.06±0.25 µg/ml/min enzyme activity respectively. Thus, dextrose, ammonium chloride and yeast extract was employed as carbon and nitrogen source for efficient fish waste degradation and enzyme activity with respect to these components were illustrated in figure no.5.5 and 5.6.

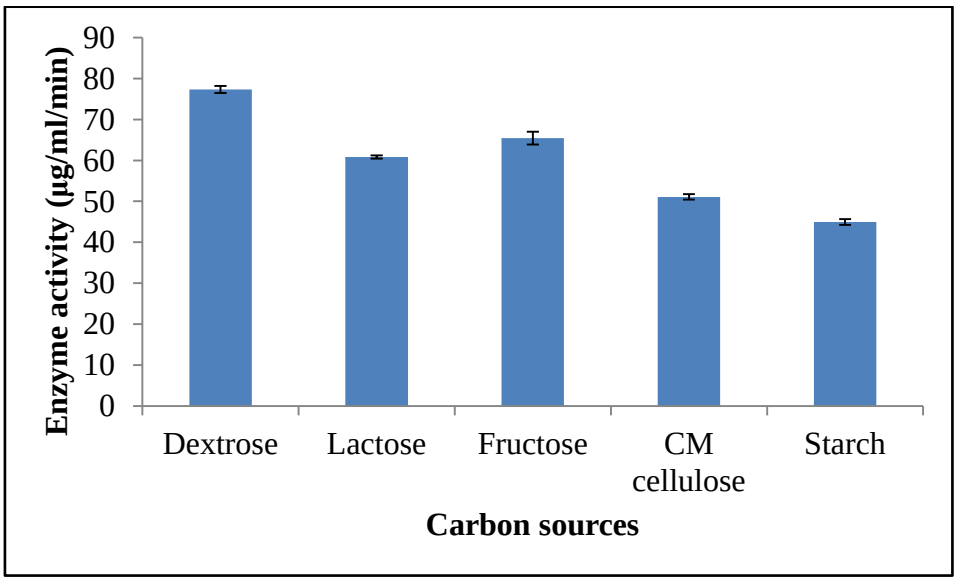

Figure 5.5.: Effect of carbon sources on enzyme activity

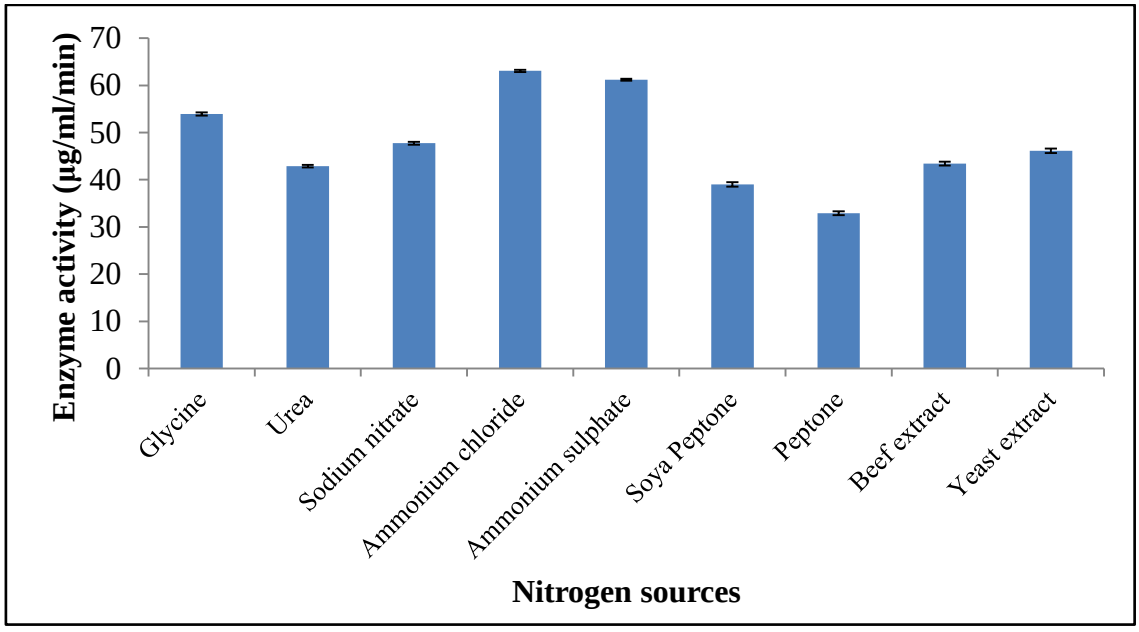

Figure 5.6.: Effect of nitrogen sources on enzyme activity

5.3.6. Microscopic and biochemical assay

The strain PSD 11 was characterized by biochemical and microscopic studies. The colony characterization and biochemical assays were listed in table no.5.2 and 5.3 respectively. Figure no.5.7 showed growth of PSD 11 on nutrient agar which was further identified by 16 S rDNA sequencing.

Size	Shape	Margin	Elevation	Color	Opacity	Consistency
3 mm	Circular	Regular	Convex	White	Opaque	Mucoid

Table 5.2.: Colony characteristics

No.	Test	Result
01	Gram nature	Gram positive
02	Morphology	Clustered Cocci
03	Motility	Motile
04	Indole	+
05	Methyl red	+
06	Voges-Proskuer	-
07	Citrate utilization	-
08	Catalase	+
09	Urease	+

10	Starch hydrolysis	+
11	Casein hydrolysis	+
12	Gelatin liquefaction	+
13	Phenylalanine deaminase	-
14	NaCl (%)	
	1.0	+
	2.0	+
	4.0	W
	6.0	W
	8.0	-
	10.0	-
15	Growth pH	
	2	-
	4	-
	6	-
	8	+
	10	W
16	Growth temperature (ºC)	
	10	+
	20	+
	30	+
	40	W
	50	-

Table 5.3.: Biochemical characterization of PSD 11 (+: positive; -: negative; W: weak)

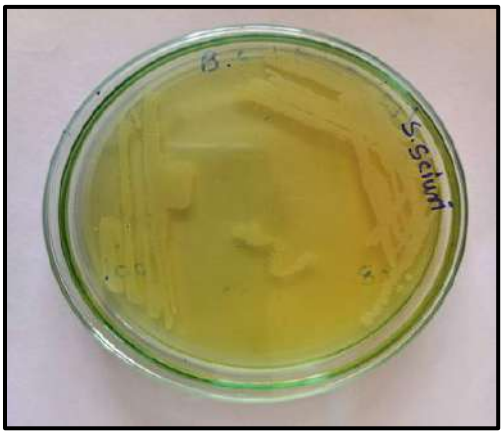

Figure 5.7.: Growth of *S. sciuri* on nutrient agar

5.3.7. Identification by 16S ribosomal DNA sequencing

The 16S ribosomal DNA sequencing of PSD 11 was carried out. From sequencing, it was revealed that isolate PSD11 showed highest similarity with *Staphylococcus sciuri* strain DSM 20345 based on nucleotide homology and phylogenetic studies. The PCR amplification of genomic DNA along with ladder was depicted in figure no.5.8.(a). The molecular phylogenetic analysis was carried out by using MEGA7 software and results were given in figure no.5.8.(b). BLAST similarity alignment of micro-organism with respect to NCBI GenBank was given in figure no. 5.9. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura 1980). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed (Felsenstein 1985).

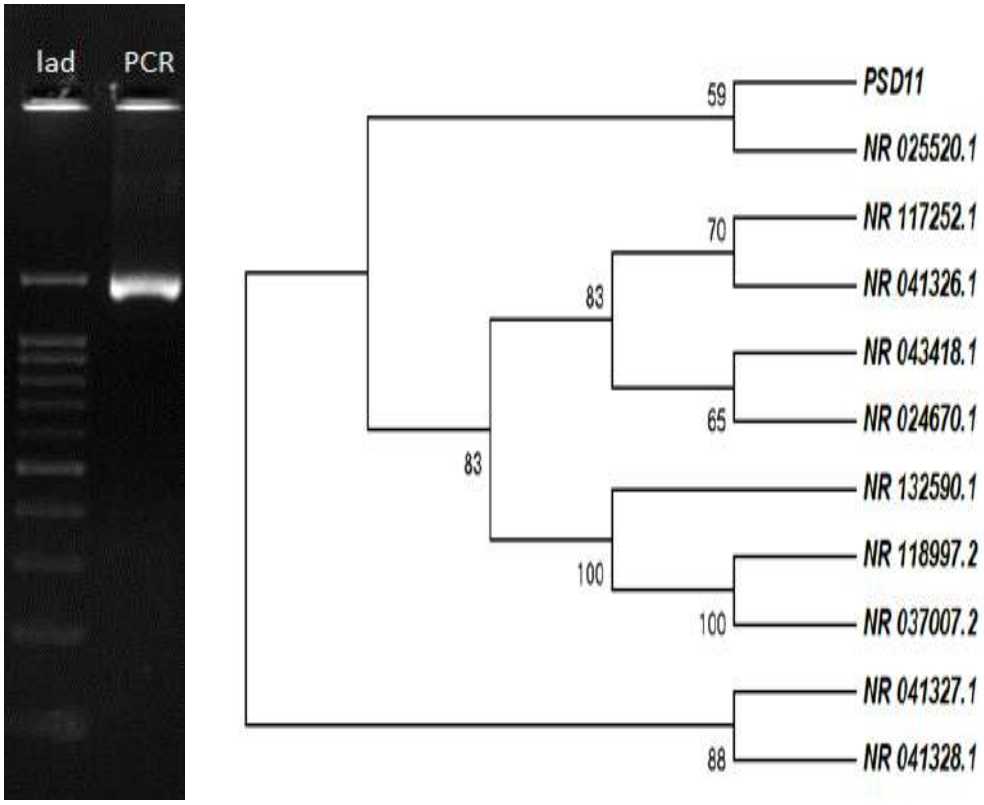

Figure 5.8.: (a) PCR amplification of DNA fragments with ladder; (b)Molecular phylogenetic analysis of PSD 11(*S. sciuri*) by Maximum Likelihood method

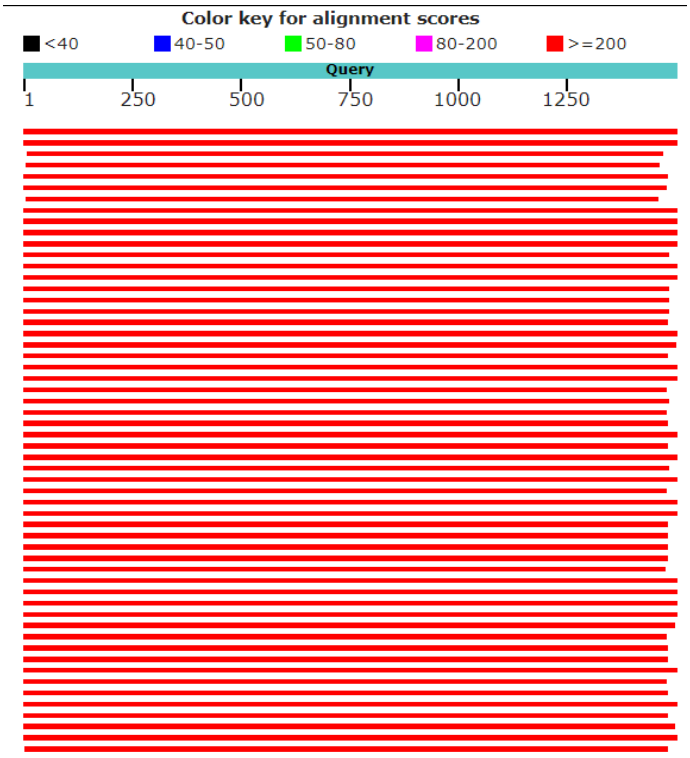

Figure 5.9.: Alignment view of *S.sciuri* using combination of NCBI GenBank

5.3.8. Analysis of liberated end products

It was found that *S.sciuri* isolated from fish waste dumping site has good capacity to degrade waste. It utilizes fish waste as whole carbon and nitrogen source therefore, results in generation of free proteins and amino acids. Figure no. 5.10 depicted, rise in amount of free amino acids and proteins during waste degradation by *S.sciuri* which indicated that fish waste was utilized by micro-organism for growth.

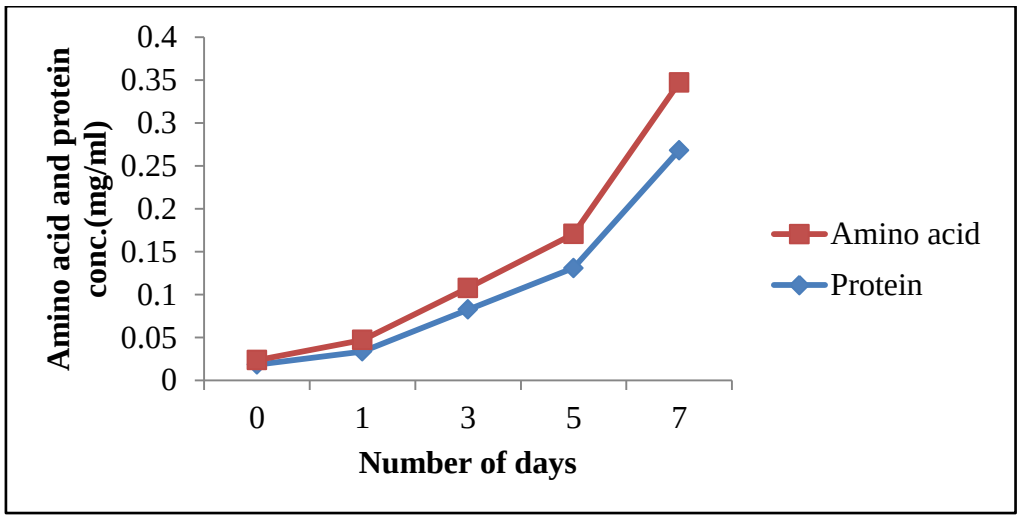

Figure 5.10.: Analysis of degraded end products during fish waste degradation by *S.sciuri*

5.3.9. Antibiotic susceptibility analysis

Antibiotics are the vital secondary metabolites synthesized by bacteria during stationary phase of growth. More than 80% antibiotics were extracted from soil micro-organisms. In various bacterial infections antibiotics perform an essential role but several bacteria were resistant to these antibiotics. This resistance was occurred due to impermeable cell membrane, genetic transformation, alteration of plasmids and the generation of beta- lactamases (Odusanya 2002). *S.sciuri* isolated from fish waste dumping site was tested for antibiotics sensitivity against commercially available antibiotics. It was observed that, isolate was susceptible to penicillin, streptomycin, gentamycin, chloramphenicol, azithromycin, amoxicillin and clarithromycin. *S.sciuri* was more sensitive to amoxicillin and less sensitive to streptomycin with zone of inhibition about 31 mm and 19 mm respectively (Begum et al.2017) while marginal changes was observed for other antibiotics. Antibiotics susceptibility of *S.sciuri* against commercial antibiotics was depicted in figure no. 5.11 and 5.12. Due to susceptibility against various antibiotics it can be concluded that, isolated micro-organism is non-pathogenic and can

be explored for different applications in agricultural field and as a probiotics in poultry as well as aquaculture feed.

Figure 5.11.: Zone of inhibition by *S.sciuri* against antibiotics

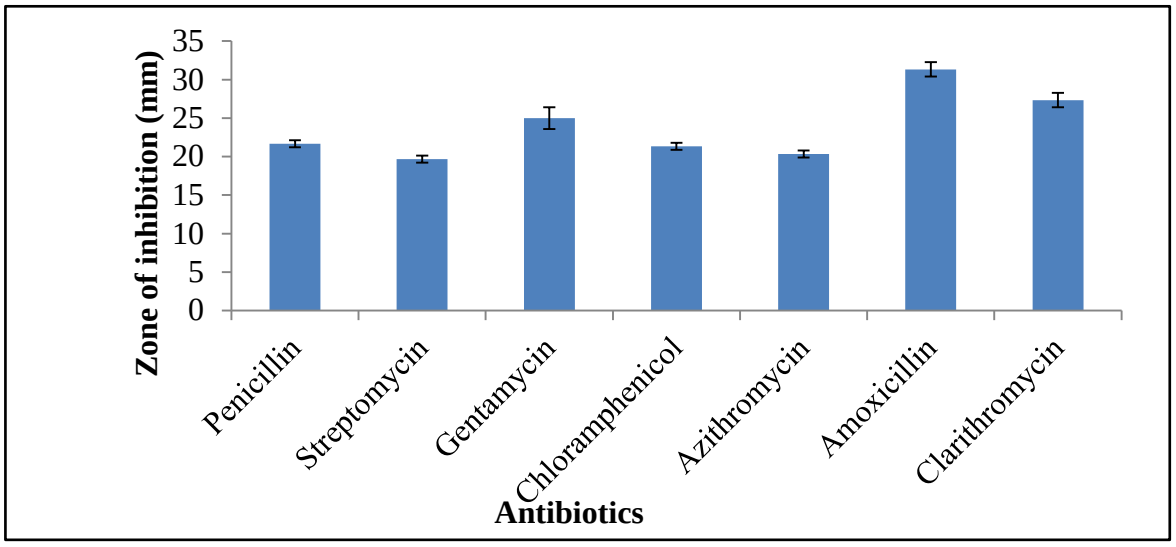

Figure 5.12.: Antibiotics susceptibility of *S.sciuri*

5.3.10. Effect of degraded feather waste hydrolysate on plant growth

The following morphological and biochemical analysis of plants and soil supplemented with fish waste hydrolysate were carried out.

5.3.10.1. Plant growth promotion study

It was observed that, there is significant enhancement of phytochemicals in case of test while marginal change was observed in control plant. This confirms, fish waste hydrolysate was rich in nutritional factors that act as effective plant growth stimulator so it has positive effect on test plants. Besides this, time duration study revealed that, mung plant nourished with fish waste hydrolysate showed germination after 2 days and healthy growth of all vegetative parts were observed after one week, but in control plant delayed in germination as well as all phytochemical parameters; no significant change was observed.

5.3.10.2. Effect of hydrolysate on morphological parameters of plants

The organic components of fish waste hydrolysate were utilized by mung plant. Figure no.5.13 depicted the results of waste hydrolysate on morphological parameters of plant. The hydrolysate treated plants exhibited better morphological characters than control one (table no.5.4). Test plant showed 36±0.84 cm and 2.9±0.24 cm shoot and root length respectively while in control it was decreased in shoot and root length (25±0.81 cm and 2.46±0.12 cm respectively). The increases in number of leaves, root hairs, root nodules, number pods as well as seeds were observed in treated plant as compared to control (figure no.5.14). Early fruiting was detected in test plant (2 days) while control (3 days) was delayed in fruiting. The pod colour of test plant was black while control showed brown colour, thus it indicated the good quality of seeds as well as weight of seeds also increases (figure no.5.15).

Other morphological parameters such as stem and leaf width as well as length of leaves and pod were increased in test plant than control plant. This significant change was detected due to microbial degraded fish waste hydrolysate. Similar results were observed after application of feather hydrolysate on *Vigna radiata* (Paul et al.2013; Bhange et al.2016) and fish waste hydrolysate (Bhagwat et al.,2018) on *C. arietinum* (Bhagwat et al.2018), *Amaranthus dubius* and *Trigonella foenum- graecum* (Thankachan and Chitra 2021).

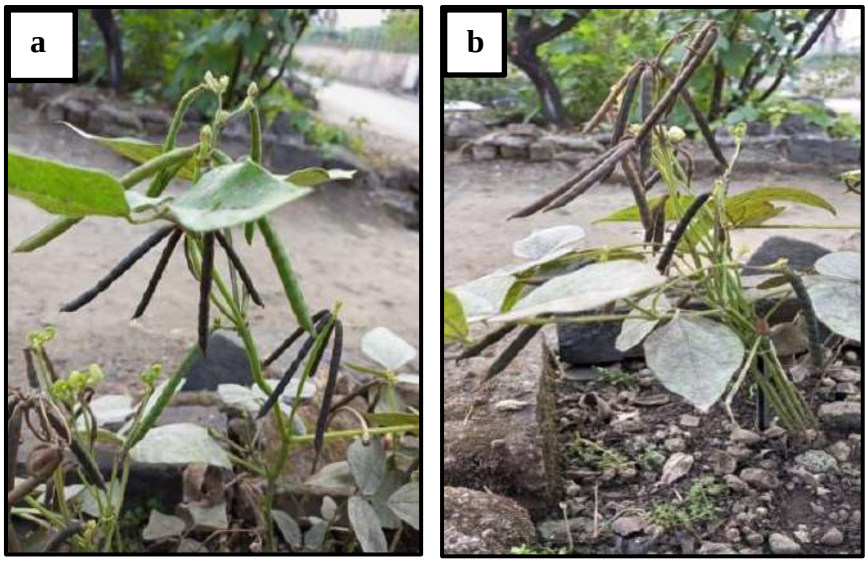

Figure 5.13.: Plant study of hydrolysate (a) Control; (b) Sample

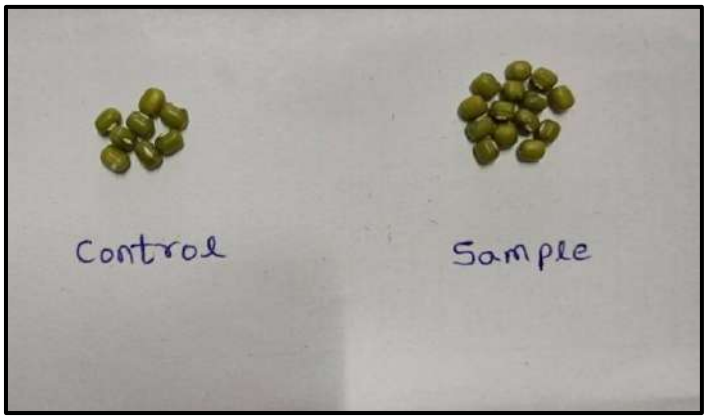

Figure 5.14.: Seeds of control and treated mung plant

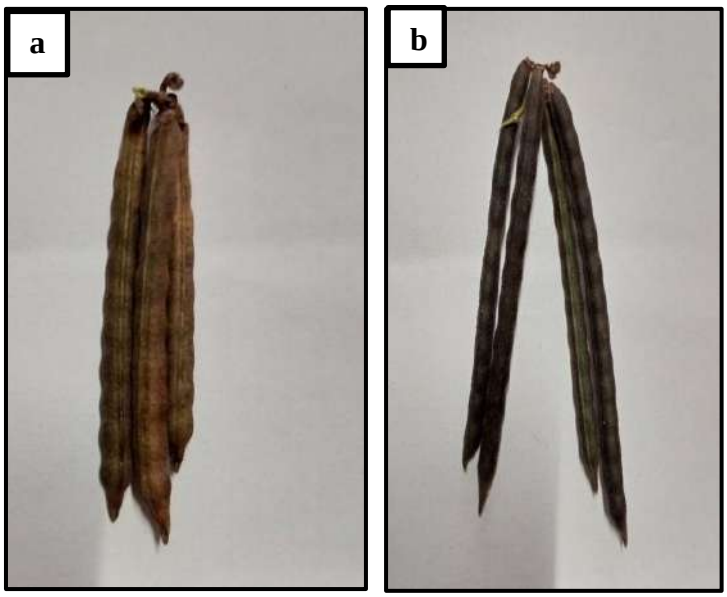

Figure 5.15.:Pod study (a) Control; (b) Sample

Parameters	Control	Sample
Germination time	3 days	2 days
Flowering time	10 days	7 days
Fruiting time	3 days	2 days
Stem length	25±0.81 cm	36±0.84 cm
Stem width	0.5±0.081 cm	0.6±0.04 cm
Leaf width	5.63±0.12 cm	7.06±0.12 cm
Leaf length	6.93±0.12 cm	7.3±0.16 cm
Root length	2.46±0.12 cm	2.9±0.24 cm
No.of leaves/plant	8±0.14	11±0.47
No.of root hairs	05±0.47	08±0.41
No.of root	03±0.47	04±0.86

nodules/plant		
No.of pods/plant	03	04
Colour of pod	brown	black
Length of pod	6.83±0.23 cm	7.5±0.40 cm
No.of seeds	05±0.47	08±0.94
Weight of seeds	0.047±0.14 gm	0.091±0.14 gm

Table 5.4.: Morphological characters of control and treated mung plant ((n=3)

Addition to that, development of root nodules was detected in *both control and test V.radiata* (figure no.5.16), representing that amendment of fish waste hydrolysate could help to colonize and elevate population of nitrogen fixing bacteria in soil. Nitrogen fixers assimilate nitrogen and show rapid invasion of root cortex that stimulates nodulation using protein rich hydrolysate (Paul et al.2013).

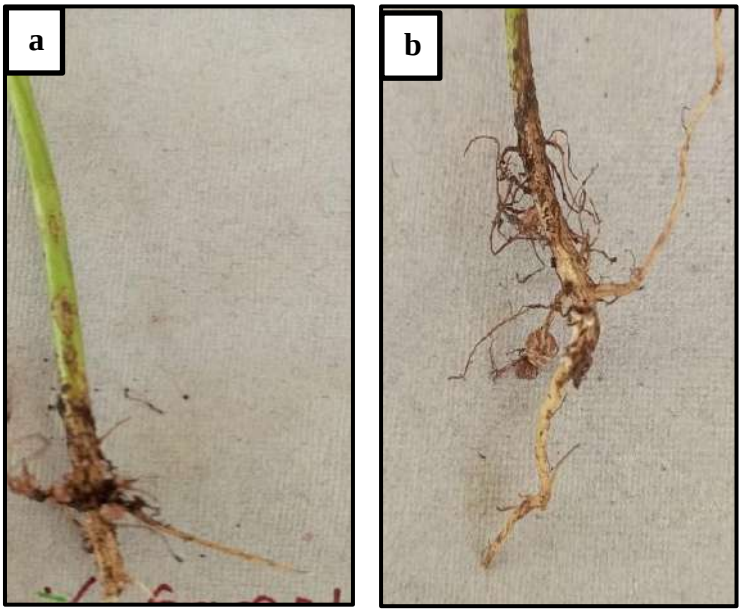

Figure 5.16.: Root study (a) Control; (b) Sample

5.3.10.3. Biochemical parameters of plants

The hydrolysate of fish waste degradation by microbes contains important nutrients which act as plant growth stimulant so it has potential applications in agriculture particularly horticulture field which has great commercial value. It is potent source of small peptides and amino acids that involved in protein synthesis, secondary metabolism and signalling mechanism of organism. Some amino acids in waste hydrolysate act as precursor of plant growth hormones thus; it may helpful to develop

tolerance in various stress conditions (Colla et al.2015). Hence, plants treated with fish waste organic hydrolysate exhibited effective increase in biochemical characters of plants like concentration of photosynthetic pigments, proteins, amino acids, flavonoid and phenolic. Some amino acids in waste hydrolysate may acts as precursor for plant growth hormone thus helpful to develop tolerance in various stress as well as atmospheric seasonal conditions.

5.3.10.3.1. Chlorophyll content

There was increase in chlorophyll a and b pigment concentration of treated plant than control. Table no.5.5 illustrated the change in chlorophyll content. This significant change was observed because along with soil macro and micronutrients, hydrolysate provides additional nutrients which enhance the growth, development and improve in chlorophyll content as well (Neales 1956). The rise in photosynthetic pigments may cause rapid synthesis of carbohydrates thus improve yield in plants (Paradikovic et al.2011). Chlorophyll a content in treated plant was found to be 3.481±0.49 µg/ml while 1.836±0.45 µg/ml for control. The chlorophyll b content was found to be 11.117±0.53 µg/ml and 15.294±0.49 µg/ml for control and treated mung plant respectively. The overall chlorophyll content was recorded as 17.123±0.53 µg/ml in treated 14.593±0.37 µg/ml in control. The results were analogous to *T. aestivum* and *V. radiata* treated with feather and fish waste hydrolysate respectively (Bhise et al.2017; Bhagwat et al.2018).

Variants	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Total Chlorophyll (µg/ml)
Control	1.836±0.45	11.117±0.53	14.593±0.37
Test	3.481±0.49	15.294±0.49	17.123±0.53

Table 5.5.: Concentration of chlorophyll

5.3.10.3.2. Free protein and amino acids

The absorption of macro and micronutrients in hydrolysate causes improvement in amino acid and protein synthesis in treated plants than control (table no.5.6) which may help to the plant for easy absorption, results in healthy growth of plants. Fish hydrolysate acts as rich source of L-amino acids and peptides which are simply absorbed by test plants. Similarly this improved uptake of macro and micronutrients increases protein as well as amino acid synthesis in plants (Colla et al.2015). The concentration of

free proteins and amino acids in test plant was significantly higher than control one. Hence, fish waste hydrolysate promotes rapid growth and development of treated plants.

Variants	Protein (mg/g fresh weight)	Amino acid (mg/g fresh weight)	Total phenolics (mg GAE /g fresh weight)	Total flavonoids (mg Que /g fresh weight)
Control	1.052±0.13	0.176±0.34	4.942±0.23	3.973±0.39
Test	1.885±0.23	0.256±0.37	6.105±0.27	5.635±0.40

(GAE- gallic acid equivalent; Que- quercetin equivalent; n=3±SD)

Table 5.6.: Biochemical analysis of vegetative tissue in control and treated plants

5.3.10.3.3. Total phenolics and flavonoid

The natural antioxidants, polyphenols are present in all plants and have vital function in defence mechanism of it. Besides this, polyphenols have effective health benefits in human beings also (Mahesha 2012). The vegetative parts of plants contains high amount of phenolic and flavonoid. The treatment of fish waste hydrolysate to test mung plant showed little bit increase in total phenolics and flavonoid content (table no.5.6) as compared to control plant. These results are in accordance with Gurav and Jadhav (2013) and Bhise et al. (2017) who reported effects of feather hydrolysate on banana and wheat.

5.3.10.4. Chemical analysis of soil

Soil composition and its chemical parameters are crucial factors for growth and development of any crop. The pre-treatment of hydrolysate on soil and observed its effect on plants and results showed that tremendous positive change was occurred in all constituents of soil tabulated in table no 5.7. The increased concentration of macro and micronutrients were observed in test soil. The current study showed enrichment of C and N percentage which indicates raise in soil fertility. With the supplement of hydrolysate, it was found that, other macro and micro components of soil drastically increased viz; Mg, P, Zn, K, Mn, Ca, Fe, N, K, Cu and proteins. Hydrolysate treated soil contains increasing amount of total nitrogen, phosphorus, potassium of about 22±0.02 %, 22±0.02 mg/l and 65±0.02 mg/l respectively. Thus, this micro-organism degraded fish waste can be considered as a best source of nutrients which improve soil in terms of soil fertility or

quality and crop productivity. The high amount of carbon and nitrogen causes increase in plant biomass. Therefore, fish waste hydrolysate efficiently employed as soil conditioner which elevates the water holding and buffering ability of soil (Barrow 1960; Haynes and Naidu 1998).

Sample	**P (mg/l)**	**K (mg/l)**	**Ca (mg/l)**	**Mg (mg/l)**	**Mn (mg/l)**	**Zn (mg/l)**	**Total C (%)**	**Total N (%) Kg/ hector**
Control	9.52± 0.02	52.5 ±0.02	197.5± 0.016	11.05± 0.01	BDL	4.892± 0.05	1.18± 2.07	235.2± 0.30
Sample	22± 0.02	65± 0.02	1050± 0.06	13.68± 0.01	1.971 ±0.28	1.971± 0.28	3.01± 0.05	282.2± 0.17

(Test: - 5ml hydrolysate; n=3±SD)

Table 5.7.: Chemical analysis of control and sample soil

5.4. Conclusion

In present study, complete utilization of fish waste including skin, fins and tail was carried out which was remaining after the recovery of valuable collagen. Previous study was restricted to collagen extraction only, but remaining waste has gained increasing attention for bioprocessing in agricultural field and could become vital factor for fish waste management. *Staphylococcus sciuri* (PSD 11) has potential to degrade fish waste within 24 hours hence successfully exploited for production of organic nutrient rich hydrolysate. Similarly, fish waste hydrolysate serve as an organic liquid fertilizer with significant plant growth stimulator. Amendment of liquid fertilizer results in improved morphological, biochemical parameters in mung (*Vigna radiata)* plant. Therefore, application of nutrient rich hydrolysate from *S.sciuri* PSD 11 has capability to degrade fish waste and could increase the yield of crops by ameliorating growth of plants. Hence, this work efficiently employed as novel, eco-friendly procedure to overcome environmental pollution problems as well as those arise from agriculture associated with chemical fertilizer.

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CHAPTER VI

APPLICATIONS OF MACROMOLECULES DERIVED FROM WASTE MATERIAL OF GETHAR (*Sarda orientalis*)

6.1. Introduction

Pollution due to waste is key negative factor produced by an industry and human being. The manufacturing processes carried out in all types of industries generate more or less amount of waste. The environmental impact caused by chemical wastes is immediately detected when it released beyond allowable limits. Generally, food processing trades creates organic wastes and its effect on environment is not easily identified until it reached to unmanageable proportion. One of the main food industry which has large impact on environment is seafood processing industry. It generates more pollution due to its coastal specific location (Sasidharan et al.2013). The retrieval of merchantable by-products from fish wastes is an important waste minimization approach. The waste material contains significant amount of bioactive constituents with varied pharmaceutical and biotechnological applications. It includes proteins and bioactives like (enzymes, collagen and gelatin), protein hydrolysates, lipids, astaxanthin, chitin. Also, oil from fish waste comprises more amount of polyunsaturated fatty acids (PUFAs) particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). It has increasing demand because of its great profitable value as well as probable applications in pharmaceutical and nutraceutical sectors. Fish waste and its hydrolysate can be employed as organic fertilizer to improve crop productivity. However, more research was carried out to convert waste from marine sources into beneficial products. Also it generates extra revenue as well as decreases the cost required for its proper disposal (Mathew 2010; Caruso 2015).

Inside the natural protein, bioactive peptides are in inactive state. The digestion of native protein by physical, chemical or enzymatic method cleaves peptides from it which has various beneficial effects. Collagen is one of the abundant source of biologically active peptides and these peptides possess different physiological properties which are important to maintain good health (Paul et al.2019). The gelatin is produced from triple helical collagen after thermal denaturation which changes molecular configuration of amino acids. Gelatin from mammalian sources is mostly utilized for industrial purpose. But, there are some ethical concerns behind the use of this gelatin. The fish waste is the most prominent alternative source for gelatin extraction. The extracted gelatin exhibits better functional properties so can be useful in biomedical sector (Yusof et al.2019). The EPA and DHA are mainly found oil from marine fishes and it has valuable health benefits as they are used in treatment of arteriosclerosis and hyperlipemia as well as

cardiovascular disease. Thus, it can be useful in prevention of various health related diseases and acts as potential therapeutic agent (Byun et al.2008).

The current research work was designed to synthesize collagen peptides from extracted collagen by enzymatic hydrolysis. The biological macromolecules derived from marine fish gethar (*Sarda orientalis*) were studied for its applications in biomedical and agriculture field as well as utilized for functional food preparations.

6.2. Experimental methodology

6.2.1. Chemicals and reagents

The chemicals including lactic acid, sodium hydroxide (NaOH), phosphate buffer, agarose, acrylamide, bisacrylamide, ammonium persulphate, coomassaie brilliant blue, sodium dodecyl sulphate (SDS), methanol, ascorbic acid and dimethyl sulfoxide (DMSO) were procured from Hi-Media and Sisco research laboratory, India. Also, egg albumin, standard acarbose, Diclofenac sodium and α-amylase enzyme from fungal diastase were purchased from Sigma-Aldrich, USA. The reagents like 3,5, dinitro salicyclic acid (DNSA), phosphate buffered saline (PBS), Dulbecco's modified eagle medium (DMEM), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 3-(4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide) (MTT) were obtained from Sigma-Aldrich, USA. All the chemicals and reagents utilized for current study were of analytical grade. The food grade components were used for functional food preparations.

6.2.2. Formation of collagen peptides

6.2.2.1. Extraction of collagen hydrolysate

For the extraction of collagen hydrolysate from gethar fish waste methodology of Kumar et al. (2018) was used with slight modification. The waste material was washed with cold tap water followed by cold D/W and cut into small pieces. Then, it was treated with 0.1 M NaOH for 24 hr at 4ºC to remove non-collagenous impurities. After that, it was washed with cold D/W till neutral pH attained. The pre-treated sample was placed in 0.2 M lactic acid (1:30, w/v) at 4ºC for swelling and then homogenized with 50 mM phosphate buffer (pH 7.0) for 5 min (1:2, w/v). The 1 ml suspension of *Staphylococcus sciuri* (*S.sciuri*) was mixed into mixture and kept for constant stirring (150 x g) at 4ºC for 24 hr. The protease enzyme from bacteria hydrolyses collagen; reaction was ceased by adding 4 ml of 6.0 N HCl and centrifuged at 6000 x g for 15 min at 4°C. The supernatant was collected and neutralized with the help of 1 N NaOH and passed through 0.2 μm millipore syringe filter to obtain collagen hydrolysates.

6.2.2.2. Fractionation of collagen peptides

Fractionation of collagen peptides was carried out on the basis of their molecular weight cut-offs (MWCOs). The 30 kD biopeptide fractions were acquired by using ultra-membrane filters with MWCO <30kD (Amicon membrane filters). These fractions were utilized to determine bioactive characteristics.

6.2.2.3. SDS-PAGE analysis of peptides

Enzymatic hydrolysis of collagen results into peptides was confirmed by SDS-PAGE analysis. The 3 mg/ml standard as well as test (30 µl each) was mixed with gel loading buffer (30 µl) and about 50 µl of each sample were loaded into wells. The separation was carried out by using 4% stacking and 8% separating gel. The gel was stained by coomassie brilliant blue to visualize separated fragments (Laemmli 1970).

6.2.3. Biomedical applications

6.2.3.1. In vitro anti-diabetic assay of peptide

An in vitro anti-diabetic potential of collagen peptides was determined by using α-amylase inhibition assay as per protocol of Mccue and Shetty (2004) with some modifications. For this activity, 1 mg/ml, 3 mg/ml and 5 mg/ml of collagen peptide was used from stock solution. The 500 µl of each sample was mixed with 500 µl of 0.1 M phosphate buffer (pH 6.9) containing α-amylase from fungal diastase (0.5%). The reaction was incubated at 25ºC for 10 min and 500 µl of 0.1 M phosphate buffer (pH 6.8) containing 1% starch was added to it. The resulting mixture was incubated at 25ºC for 10 min followed by addition of 1 ml dinitro salicylic acid (DNSA). The reaction mixture was kept in boiling water bath for 10 min and cooled. After cooling, absorbance was measured at 540 nm using UV-visible spectrophotometer (Shimadzu UV-1800, Japan). 0.2 M lactic acid was used as control while standard acarbose was taken as positive control. The percentage inhibition of α-amylase enzyme was calculated using following formula;

$$\text{Inhibition (\%)}=\frac{\text{A(control)}-\text{A(sample)}}{\text{A(control)}}\times 100$$

6.2.3.2. Cytotoxic activity of peptide

The cytotoxic activity of collagen peptide was studied by using MTT assay according to method of Chang et al. (2015) with slight modifications. The activity was checked on breast cancer cell line (MCF-7, NCCS Pune). Collagen peptides 1, 3, 5 mg/ml from stock solution were utilized. Then cells were incubated at a concentration of

1×10^4 cells /ml in DMEM culture medium for 24 hr at 37ºC and 5% CO_2. Cells were seeded at a concentration (70 µl) 10^4 cells /ml in 100 µl culture medium and 100 µl of each sample was added into 96 well micro plates. Control wells were incubated with DMSO (0.2% in PBS) and cell line. Control was maintained to determine the control cell survival and percentage of live cells after culture. Cell cultures were incubated for 24 hr at 37°C and 5% CO_2 in CO_2 incubator (Thermo scientific BB150, USA). After incubation, the medium was completely removed and 20 µl of MTT reagent (5mg/ml PBS) was added to it. After addition of MTT, cells were incubated at 37ºC for 4 hr in CO_2 incubator. The cells were observed for formazan crystal formation under electron microscope. The viable cells reduce yellowish MTT into dark coloured formazan. The medium was removed completely and 200 µl DMSO was added to it (kept for 10 min) and incubate at 37ºC (wrapped with aluminium foil). Triplicate samples were analysed by measuring the absorbance of each sample by microplate reader (BeneSphera E21, Avantor USA) at 550 nm wavelength.

6.2.3.3. Antioxidant activity

The antioxidant activity of fish waste extracted macromolecules was performed according to method of Chang et al. (2013) with some modification. The DPPH (2,2-diphenyl-1-picrylhydrazyl, Sigma-Aldrich, USA) was employed for this study. The DPPH (24 mg) was diluted in methanol until 1.1±0.02 absorbance was obtained at 517 nm. Ascorbic acid (vitamin C) was utilized as standard (100 µg/ml). 20-100 µl of standard vitamin C and 10, 50, 100 µl of each collagen peptide, gelatin and omega-3 fatty acid was added to test tube. The volume was adjusted to 100 µl with D/W. Into each tube 3 ml of DPPH was added and incubated in dark for 30 min. The absorbance at 517 nm was measured using UV-visible spectrophotometer (Shimadzu UV-1800, Japan). The free radical scavenging activity (%) of each sample was calculated using following formula;

$$\% \text{ inhibition} = \frac{A(\text{control}) - A(\text{sample})}{A(\text{control})} \times 100$$

Where;

A (control):- initial DPPH absorbance

A (sample):- absorbance of each sample

6.2.3.4. Antimicrobial activity

Antimicrobial efficacy of collagen peptide and gelatin was evaluated against Gram positive *Staphylococcus aureus* NCIM 2654 (*S.aureus*) and *Bacillus subtilis* NCIM

2635 (*B.subtilis*) as well as Gram negative *Escherichia coli* NCIM 2832 (*E.coli*) and *Proteus vulgaris* NCIM 2813 (*P.vulgaris*). Sterilized nutrient agar media plates were prepared by using standard procedure. 100 µl of each bacterial suspension was spread with the help of sterilized borer. The 10-100 µl of 1 mg/ml of collagen peptide and gelatin were added into respective well. The prepared plates were incubated at 37ºC for 24 hr and observed for zone of inhibition. The 0.2 M lactic acid was used as control and antimicrobial activity was studied as per same method.

6.2.3.5. Anti-inflammatory activity of omega-3 fatty acid

The in vitro anti-inflammatory activity of omega-3 fatty acid was investigated by using protein denaturation method as per method of Nayaka et al.(2021). From stock, 1.5 and 3 mM concentration of omega-3 fatty acid was used for this study. The reaction mixture contains, 0.4 ml egg albumin (from fresh hen's egg), 5.6 ml PBS (pH 6.4) and 100 µl of sample. The double distilled water was employed as control. The incubation of resulting mixture was carried out at 37ºC in incubator for 15 min and further heated for 5 min at 70ºC. The absorbance of both control and sample was recorded at 660 nm. Diclofenac sodium was utilized as reference and its absorbance was determined as per same protocol. The percentage inhibition (IC 50) of protein denaturation was calculated by using following equation,

$$\% \text{ inhibition} = \frac{C - T}{C} \times 100$$

Where,

T:- absorbance of test

C:- absorbance of control

6.2.4. Agriculture applications

6.2.4.1. Seed germination by collagen

The extracted collagen was tested for its efficacy to stimulate seed germination.The seeds of mung (*Vigna radiata*), masoor (*Lens culinaris*) and mataki (*Vigna aconitifolia*) were surface sterilized with 70% ethanol and washed thoroughly with D/W. The seeds were placed in petri plate and various concentrations of collagen (1%, 3% and 5%) were supplemented to it. The radicle emergence of all three types of seed was recorded after 24 and 48 hr. The deionized water was used as control. The experiment was carried out in triplicate on 30 seeds with 10 ml of each solution.

6.2.5. Preparation of functional foods supplemented with collagen and gelatin

6.2.5.1. Raggi balls

For preparation of raggi balls incorporated with collagen, 100 gm fine raggi flour, 50 gm jaggary, 75 gm ghee and 2% (w/w) collagen was used. Raggi flour and jaggary were taken in a bowl and mixed properly. The mixture was poured into ghee containing pan and continued with further addition of remaining ghee on low gas flame. Mix all components appropriately to avoid clumping and continue it till flour attains brown colour. At last, collagen was added to it and balls were prepared while control balls were made without addition of collagen. Physicochemical and nutritional characteristics were studied according to method of Kim et al., (2012).

6.2.5.2. Gelatin jelly

The jelly incorporated with gelatin was prepared as per protocol of Choi and Regenstein (2000) with some changes. The 2.5 gm of extracted gelatin was soaked in hot water. The sugar syrup was prepared by keeping it on low gas flame followed by addition of 2 ml lemon juice. The soaked gelatin was poured into sugar syrup and boiled for 5 min for proper mixing. The prepared mix was transferred into large plate and 15 ml mango juice as well as few drops of yellow food colour (Asian Food products, Maharashtra, India) were mixed together. The resulting solution was kept for maturation at 4ºC for 4 hr. After incubation, the gelatin jelly was cut into pieces for its nutritional and physicochemical analysis. The market jelly was used as control (Choi and Regenstein 2000).

6.2.6. Sensory analysis of functional foods

The sensory properties of functional foods prepared from collagen and gelatin were carried out. The characteristics like food appearance, odour, colour, texture and overall acceptability were examined by non-trained 9 participants from Food Science and Technology department, Shivaji University, Kolhapur by using 9 point hedonic scale (from 1:- dislike extremely; 2:- dislike very much; 3:- dislike moderately; 4:- dislike slightly; 5:- neither like nor dislike; 6:- like slightly; 7:- like moderately; 8:- like very much and 9:- like extremely).

6.3. Result and discussion

6.3.1. Enzymatic hydrolysis of peptide

Use of proteolytic enzymes for the enzymatic hydrolysis of proteins is commonly used in food sector. As compare to acids and alkalis, enzymes hydrolyse proteins more softly. They do not need high temperature and generally breakdown the specific peptide

linkages, so it was easy to predict the peptide profile of protein. Collagen peptides with low molecular weight are expected to possess improved bioactivities than their larger parts (Hong et al.2019). The various commercial enzymes were employed for collagen peptide or hydrolysate preparation. It includes papain (Hong et al.2017; Sasaoka et al.2017), alcalase, pepsin (Cheung and Chan 2017), α-chymotrypsin (Ngo et al.2011), neutrase, flavourzyme (Chen et al.2016), trypsin (Abdollahi et al.2018), pronase E (Kim et al.2001) and collagenase (Liu et al.2011).

6.3.2. Molecular weight distribution of collagen peptide

The collagen peptides of extracted collagen were generated by enzymatic hydrolysis from protease of *S.sciuri* PSD 11 and it was depicted in figure no.6.1. It was observed that, the fractional breaking of β chain was occurred due to enzyme within 24 hr. The both α chains cleave into fragments of 10-60 kD. The similar peptide pattern was observed for standard calf skin collagen. However, complete hydrolysis of collagen was not happened because it was resistant to enzymatic hydrolysis.

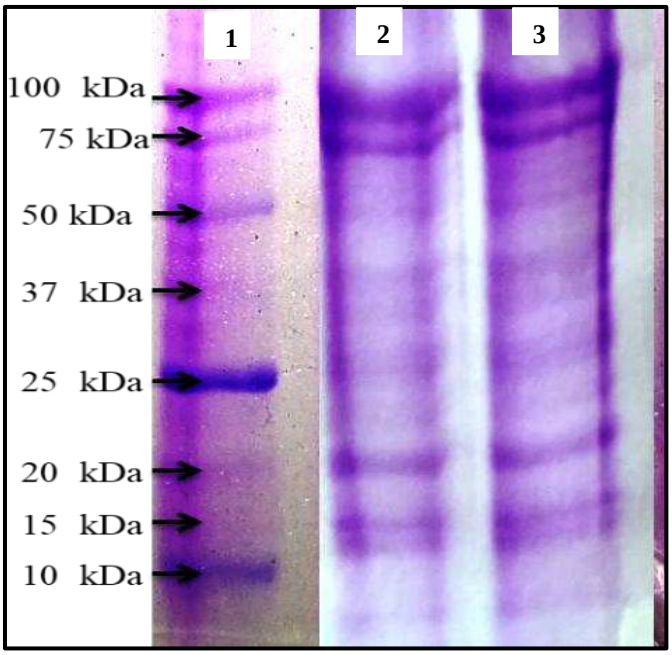

Figure 6.1.: Peptide mapping of collagen: lane 1:-molecular weight marker; lane 2:-peptides of waste collagen; lane 3:-peptide of standard calf skin collagen

6.3.3. Biomedical applications

6.3.3.1. Anti-diabetic activity of peptide

The interaction between genetic and environmental elements is the main reason of diabetes and is identified by absence of insulin secretion and resistance leads to metabolic illnesses of fat, protein and carbohydrates (Kumanan et al.2010). In presence

of calcium, α-amylases carry out hydrolysis of α-1,4 glycosidic bond. Due to more α-amylase action and insulin scarcity, the blood glucose level raises results in type II diabetes (Agarwal and Gupta 2016). In current study, the α-amylase inhibition assay was implemented to evaluate in vitro anti-diabetic ability of collagen peptides. The percentage (%) inhibition of α-amylase is increased as the concentration of peptide increases from 1 to 5 mg/ml (figure no.6.2;a).The maximum inhibition was recorded for 5 mg/ml (65.59±0.10%).

The standard acarbose exhibited 73.64±0.53% inhibition which is more than synthesized collagen peptide. The 1, 3 and 5 mg/ml collagen peptides displayed 6.51, 6.78 and 7.13 mg/ml IC50 values respectively while acarbose has 7.75 mg/ml (figure no.6.2;b). The anti-diabetic capability was absent in 0.2 M lactic acid. The obtained % inhibition and IC50 of collagen peptides were more or less similar to the red porgy, common pandora, annular seabream and unicorn leatherjacket (Fernandez et al.2001; Kumar et al.2018). The potency of α-amylase inhibition by collagen peptides is influenced by substrate, peptide configuration as well as hydrolysis temperature (Kumar et al.2018).

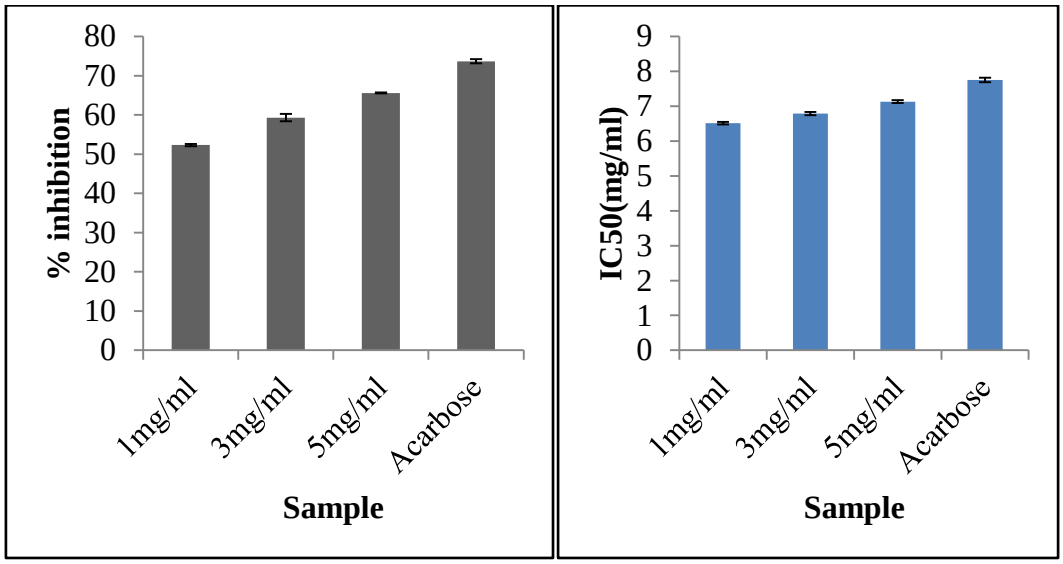

Figure 6.2.: Anti-diabetic activity of collagen peptide (a) % inhibition; (b)IC50

6.3.3.2. Cytotoxic activity of peptide

The cytotoxic activity was performed by membranolytic and non-membranolytic mechanism. In membranolytic action, initially peptide binds parallel to the membrane at low concentration while connects perpendicularly as concentration increases. Besides this, higher peptide to lipid ratio causes peptide insertion into bilayer results in formation of trans membrane pores. The non-membranolytic mechanism involves stimulation of

apoptosis, activation of extrinsic apoptotic pathways and inhibition of angiogenesis (Oelkrug et al.2015).

MTT assay was performed to study the influence of collagen peptides on growth, and viability of MCF-7 (Breast cancer cell line). The 1-5 mg/ml collagen peptides were used and from above stock solutions, 10, 40 and 100 µg/ml concentration was used. The cytotoxic activity of peptides ranges from 24.63 to 80.46% (figure no.6.3;a). It was found that, 1 mg/ml displayed 24.57±0.44%, 49.19±0.57% and 65.47±0.49% inhibition while 3 mg/ml showed 35.59±0.40%, 40.02±0.549% and 54.56±0.57% inhibition at 10, 40 and 100 µg/ml concentration respectively. Also, 5 mg/ml exhibited 70.80±0.49%, 71.97±0.65% and 80.40±0.46% inhibition at 10, 40 and 100 µg/ml concentration respectively. The positive control cyclophosphamide exhibited 75.29±0.51, 83.81±0.42 and 86.54±0.49% inhibition at same concentration. The observed values are very close to standard cyclophosphamide. The cytotoxic activity of peptides increases subsequently with increase in sample concentration from 1 mg/ml to 5 mg/ml. IC50 value is the measure of half maximal inhibitory concentration of a drug.

The figure no.6.3 (b) depicted the improvement in IC50 with respect to increase in concentration. The IC50 for 1, 3 and 5 mg/ml collagen peptide was reported as 37.0, 47.0 and 58.59 mg/ml respectively. Comparatively less IC50 was detected for cyclophosphamide (32.91 mg/ml). The control 0.2 M lactic acid does not show cytotoxic effect. In some cases, only collagen from bluefin tuna skin may reduce the growth of HepG2 and HeLa cells (Han et al.2011). The obtained results were analogues with the collagen peptides from salmon, milk fish, tilapia and unicorn leatherjacket which showed anticancer activity against HepG2, HeLa and HCT-166 cells, human fibro sarcoma and COLO320 cancer cells Han et al.2011; Baehaki et al.2016; Chen et al.2009; Kumar et al.2018). This results indicated that, the decline in cell growth by collagen or collagen peptides was influenced by type of cancer cell, fish species used and sometimes degree of hydrolysis. The enzymatic hydrolysis converts triple helical collagen into its disintegrated structure with low molecular weight. So, peptides can easily adhere to the surface of cancer cells resulting in inhibition of their growth and proliferation (Kumar et al.2018).

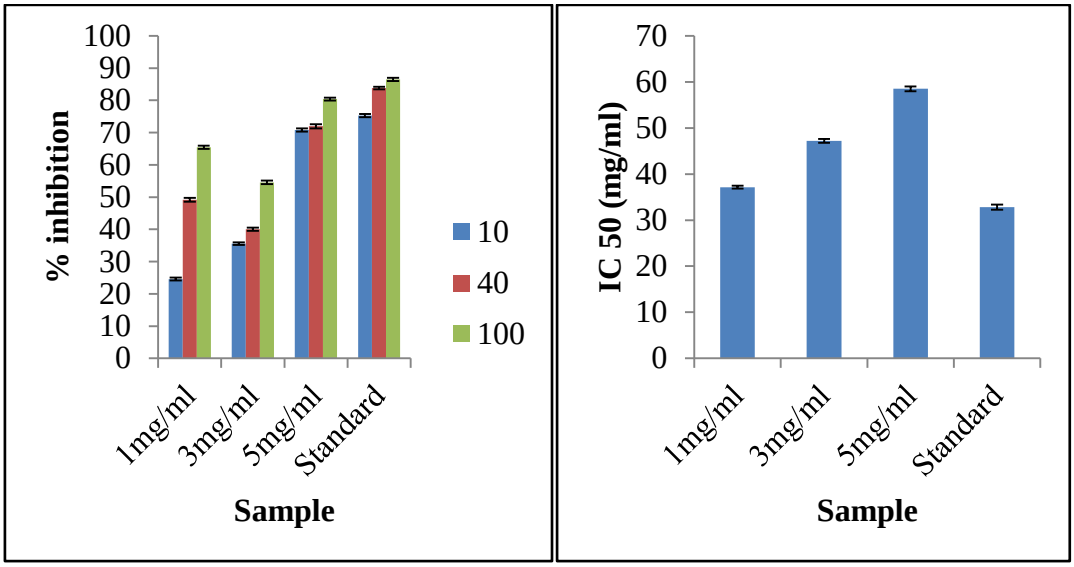

Figure 6.3.: Cytotoxic activity of collagen peptide (a) % inhibition; (b)IC 50

6.3.3.3. Antimicrobial activity of collagen peptide

The collagen peptide was assessed for its antimicrobial activity against Gram positive (*B. subtilis* and *S. aureus*) and Gram negative (*E.coli* and *P. vulgaris*) bacteria by agar well diffusion technique. The observation of activity was given in figure no.6.4. The highest 32.66 mm zone of inhibition was observed against *S.aureus* for 100 µl and 24.33mm zone was detected against *B.subtilis*. In case of Gram negative bacteria, *E.coli* and *P.vulgaris* showed 23.83mm and 27.83mm zone of inhibition respectively. The collagen peptide from marine fish gethar has good antimicrobial activity against Gram negative than Gram positive micro-organism. Collagen from marine sources exhibited improved quantity of peptides having antimicrobial potential. These peptides mainly synthesized by enzymatic hydrolysis so safe for various applications related to human health. The similar activity was observed by Ennaas et al. (2016).

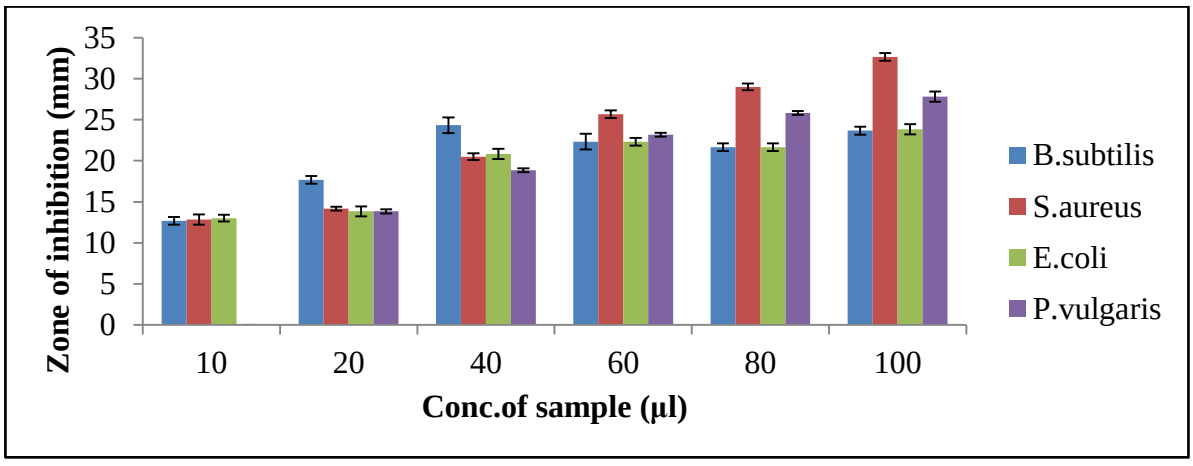

Figure 6.4.: Antimicrobial activity of collagen peptide

6.3.3.4. Antimicrobial activity of gelatin

The antimicrobial compound has ability to damage cell wall which leads to disruption of cell membrane, cytoplasmic leakage, cell lysis and ultimately causes cell death. The damage of cell membrane leads to reduction in pH results in loss of regulation of cellular metabolism and other activities such as ATP biosynthesis, DNA transcription as well as protein synthesis (Kavoosi et al.2013). Antimicrobial activity of extracted gelatin was studied by agar well diffusion method. The results were given in figure no.6.5. In case of Gram positive bacteria, 21mm zone of inhibition against *S.aureus* was observed for 100 µl while 19mm zone was detected against *B.subtilis* for same concentration. Both Gram negative bacteria *E.coli* and *P.vulgaris* showed 18mm zone of inhibition for 100µl concentration. The 10µl sample was ineffective against all four micro-organisms.

It was noticed that, 60µl concentration of gelatin effectively inhibits the growth of micro-organisms by generating 14, 19, 14 and 15 mm zone of inhibition against *B.subtilis*, *S.aureus*, *E.coli* and *P.vulgaris* respectively. It was found that; gelatin from skin of marine fish gethar exhibited good antimicrobial potential against Gram positive and negative micro-organisms while it was more useful in inhibiting growth of Gram positive than Gram negative bacteria. The results were similar to the gelatin from skin of unicorn leatherjacket incorporated with essential oils (Ahmad et al.2012). Due to good antimicrobial potential gelatin can be acts as a suitable food packaging material.

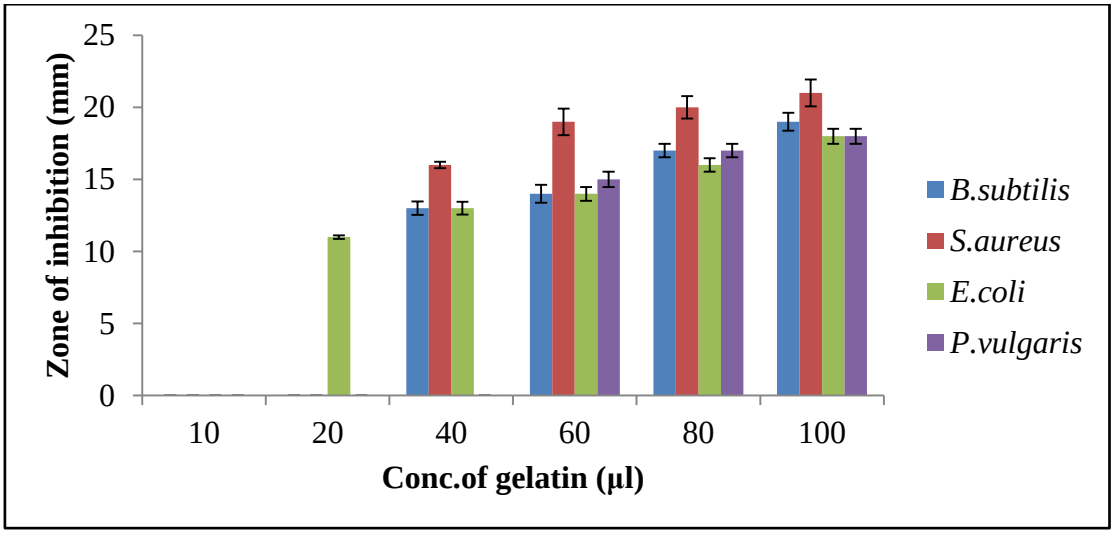

Figure 6.5.: Antimicrobial activity of gelatin

6.3.3.5. Antioxidant activity of collagen peptide, gelatin and omega-3 fatty acid

The radical scavenging capacity of collagen peptides, gelatin and omega-3 fatty acid was examined to determine their hydrogen atom or electron transfer property. The amount and sequence of amino acid, degree of hydrolysis and molecular weight of peptides has effect on antioxidant activity of peptides. The low molecular weight hydrolysate has greater antioxidant ability than high molecular weight. (Hong et al.2019; Sionkowska et al.2021). DPPH is a cell permeable stable radical and therefore usually utilized for rapid assessment of antioxidant properties of compounds. The reaction between DPPH and antioxidant molecules generates analogous hydrazine while purple colour of solution gets reduced (absorbance at 517 nm) (Yang et al.2018).

Gomez-Guillen et al.(2010) reported that, repeating motif Gly-Pro-Hyp of peptides is the prime sequence responsible for antioxidative property of peptides. The antioxidant activity of studied collagen peptide and gelatin was related to peptide from porcine and bighead carp skin (Hong et al.2019; Sionkowska et al.2021) as well as hoki skin and giant squid muscle gelatin respectively (Mendis et al.2005a; Rajapakse et al.2005b). The obtained antioxidant activity of omega-3 fatty acid was in accordance with EPA and DHA of fish oil capsules (Kotue et al.2019). The antioxidant activity of collagen peptide, gelatin and omega-3 fatty acid was depicted in figure no.6.6. The results showed that, increase in sample concentration from 10-100 µl improve the antioxidant potential of each sample. The maximum % inhibition was obtained at 100 µl. For collagen peptide, inhibition was 23.51±0.58% while 13.51±0.76% and 6.27±0.31% was obtained for gelatin and omega-3 fatty acid respectively.

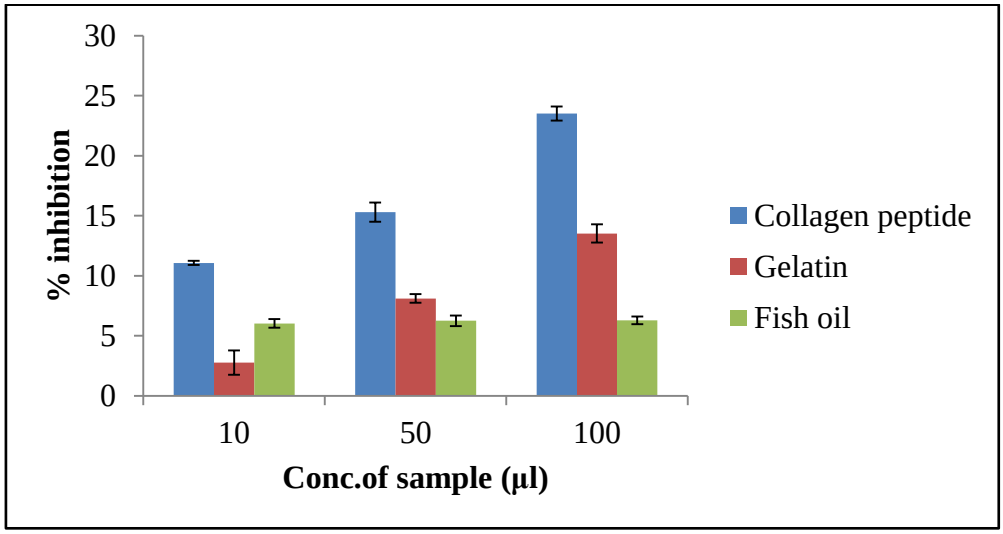

Figure 6.6.: Antioxidant activity

6.3.3.6. Anti-inflammatory activity of omega-3 fatty acid

Protein denaturation involves damages of protein's secondary and tertiary structure due to the interference of external pressure or compounds, including strong acid or base, organic solvents, concentrated inorganic salts or heat. Numerous biological proteins fail to perform their biological action after its degradation. The disintegration of tissue proteins is one of the important causes of inflammation. Hence, protein denaturation is useful screening test for the determination of anti-inflammatory potential of compounds without exploitation of animals. The anti-inflammatory activity of polyunsaturated fatty acids was confirmed by various studies. PUFA supplemented diet has been advantageous in treatment of irritable bowel disease (IBD), psoriasis, eczema, rheumatoid arthritis, ulcerative colitis as well as helps to reduce mucosal damage (Fratianni et al.2021).

The n-3 fatty acids especially EPA and DHA from fish oil found to inhibit different inflammation processes like leucocyte chemotaxis, adhesion molecule communication and leucocyte-endothelial adhesive connections. The anti-inflammatory mechanism of n-3 fatty acid is associated with the variation in phospholipid concentration of cell membrane, interruption of lipid rafts and reduced expression of inflammatory genes by inhibiting activation of the pro-inflammatory transcription factor nuclear factor kappa B (Calder 2012). The EPA and DHA provide protection to egg albumin against heat induced denaturation. The gethar fish oil exhibited less % inhibition than pure fish oil and Diclofenac sodium which was taken as control. The % inhibition of 1.5mM and 3mM of oil was recorded as 25.62±0.47% and 57.70±0.34% respectively (figure no.6.7). The obtained results were related to the % inhibition by n-3 fatty acids from seeds of apricot, peach, cherry and plum (Fratianni et al.2021).

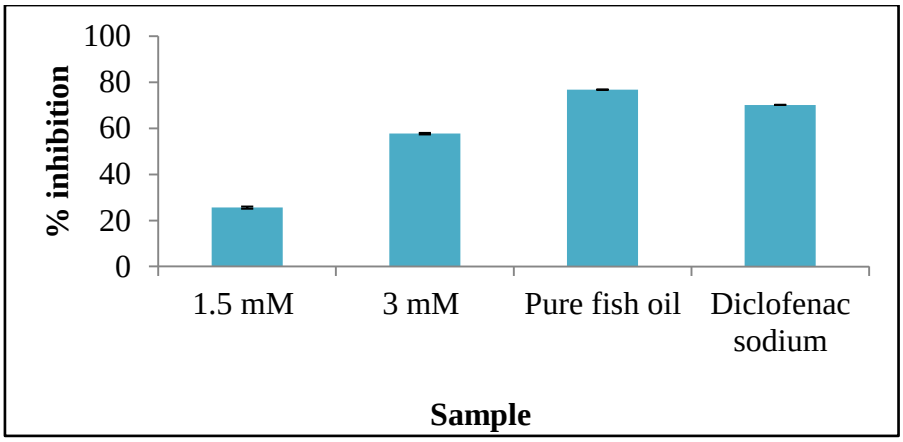

Figure 6.7.: Anti-inflammatory activity of fish oil containing omega-3 fatty acid

6.3.4. Agriculture applications

6.3.4.1. Seed germination by collagen

Collagen and its hydrolysate widely applied as plant growth stimulator because they have ability to promote plant growth by increasing shoot and root length, plant biomass as well as its productivity (Luziatelli et al.2016). The good amount of amino acids in collagen supplies energy during germination phase and hence enhances germination index (Niculescu et al.2017). In present work, 1 to 5% collagen was tested for seed germination enhancement against mung (*Vigna radiata*), masoor (*Lens culinaris*) and mataki (*Vigna aconitifolia*) for 24 and 48 hr.

The results were depicted in figure no.6.8 (a), (b) and (c) respectively. It was observed that, maximum sprout length was detected for 3% collagen in all seeds mentioned above. The *V.radiata* possessed highest sprout length as 22.33±2.62mm for 48 hr as compared to other seeds. The similar results was reported for chicken feather and protein hydrolysate which promotes the growth of wheat and lettuce respectively (Bhise et al.2017; Luziatelli et al.2016). All three concentrations have good germination effect as compared to control (D/W). Thus, collagen extracted from fish waste can be employed as alternative to chemical biofertilizer for various agricultural applications.

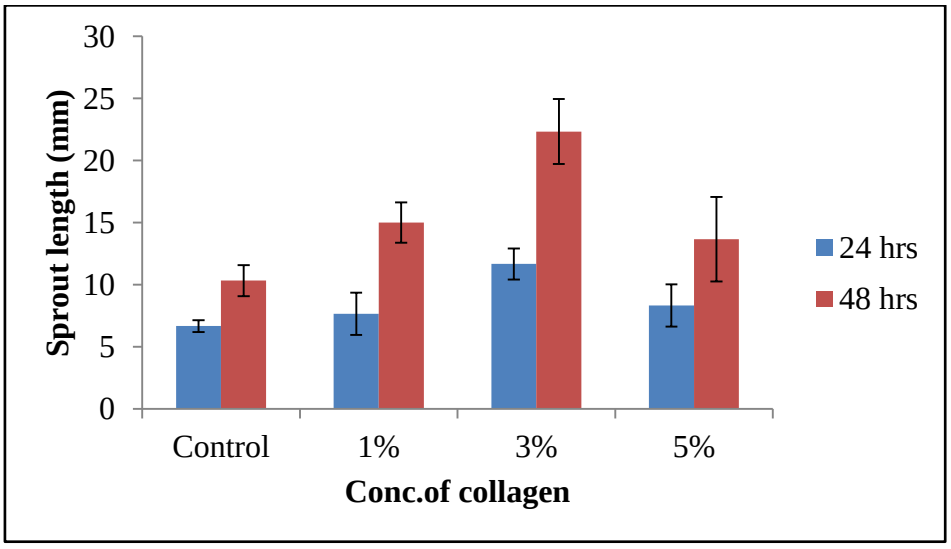

Figure 6.8 (a).: Seed germination analysis of *V.radiata*

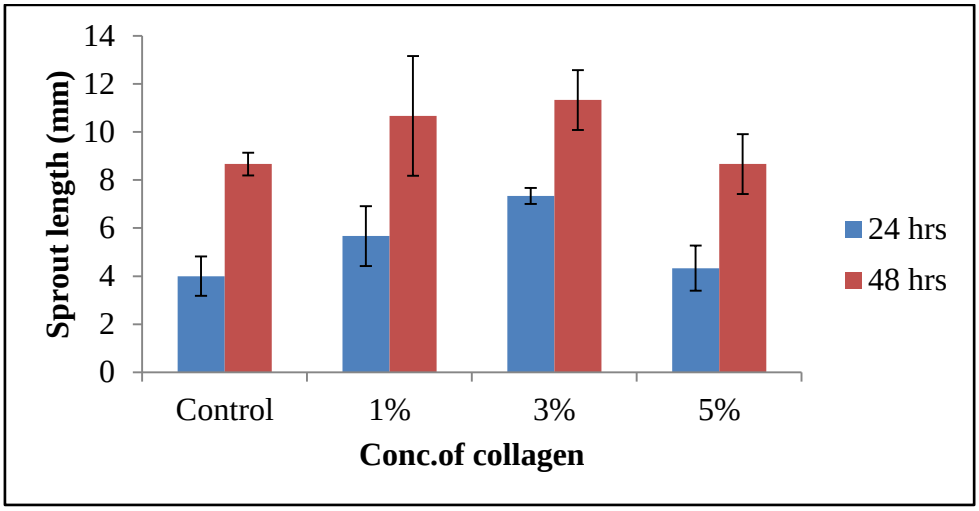

Figure 6.8 (b).:Seed germination analysis of *L.culinaris*

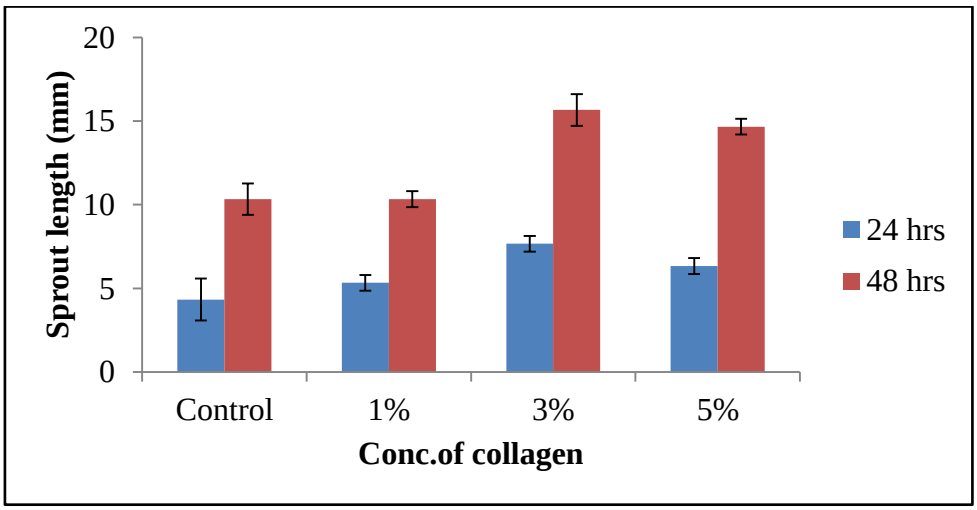

Figure 6.8 (c).: Seed germination analysis of *V.aconitifolia*

6.3.5. Functional food preparations

In this modern era, human beings are very conscious about health, so development of nutritious diet has increasing demand to fulfil the body need. The collagen content in body decreases due to aging so collagen supplementation in diet maintains its level. So, collagen becomes an important component for the preparation of functional foods by combining it with other nutritional components. Gelatin is the hydrolysed form of collagen. It has good sensory characteristics thus, useful in the preparation of jelly and other food products.

6.3.5.1. Raggi balls incorporated with collagen

Raggi is useful in natural weight loss. It is rich source of iron so, its consumption minimizes problem of anaemia. Also, it has good amount of protein so raggi containing

supplements along with collagen provides essential health benefits. The nutritional and physicochemical analysis of raggi balls was tabulated in table no.6.1. As per analysis, it was detected that, very less moisture (%) and fat content was present in both control and test. The amount of protein was increases in test (8.33±0.61 gm) than control (6.99±0.80 gm) due to incorporation of 2% collagen. Nearly same quantity of carbohydrates was noticed in both control and test while small difference was observed in energy (533.43±0.53 Kcal for control and 533.89±0.54 for test). The amount of calcium, iron and potassium was slightly reduced in test than control one.

The figure no.6.9 depicted the raggi balls incorporated with collagen while sensory analysis of it by using 9 point hedonic scale was given in figure no.6.10. The analysis was carried out with respect to appearance, colour, odour, texture and overall acceptability. The sample raggi ball possesses slight fishy odour but not easily perceivable, this is due to marine source of collagen which can be removed by utilizing more effective purification techniques. This is the first report on preparation of raggi balls incorporated with collagen. Therefore, the overall nutritional and physicochemical characteristics of raggi balls were increased due to supplementation of collagen.

Parameters/100gm	Control	Test
Moisture (%)	5.04±0.90	5.13±0.44
Fat (gm)	1.92±0.48	1.77±0.28
Protein (gm)	6.99±0.80	8.33±0.61
Total ash (gm)	1.12±0.41	1.13±0.65
Carbohydrates (gm)	64.89±0.31	64.91±0.89
Energy (Kcal)	533.43±0.53	533.89±0.54
Salt as NaCl	Nil	Nil
Calcium (mg)	279.48±0.57	273.56±0.69
Iron (mg)	5.36±0.44	5.43±0.19
Potassium(mg)	17.59±0.50	17.52±0.99

Table 6.1.: Nutritional and physicochemical analysis of raggi balls

Figure 6.9.: Raggi balls incorporated with collagen

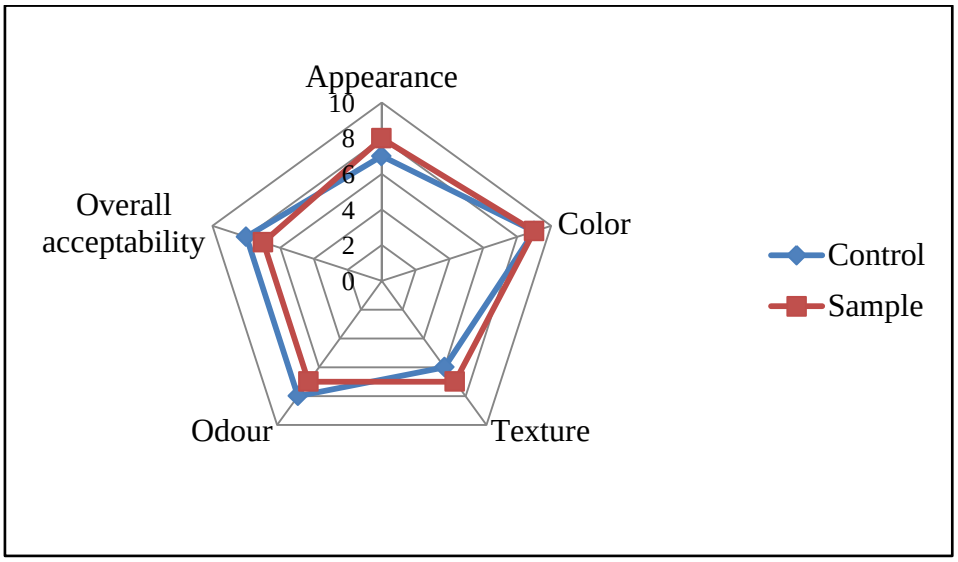

Figure 6.10.: Sensory analysis of raggi balls

6.3.5.2. Jelly incorporated with gelatin

Jelly is the common product that used for decoration of food components like cake and ice-cream. It is a soft elastic component made up from gelatin, boiled sugar and fruit juice. Gelatin is extensively used in confectionery because of its gel like nature and solidifies into small pieces that dissolve slowly in mouth to create sweet sensation. The nutritional and physicochemical analysis of jelly incorporated with gelatin was presented in table no.6.2. Jelly is one of the high moisture containing food and it was prime factor to define shelf lifespan and pureness of protein. The moisture content of control and test gelatin was recorded as 84.89±0.23% and 86.24±0.53% respectively. The ash content has no significant effect on jelly quality (Chukwu and Abdullahi 2015). The slight variation was detected in carbohydrate and fat concentration. The quantity of calcium, iron and

potassium was detected as 6.13±0.41 mg, 0.168±0.97 mg and 52.11±0.25 mg respectively in test which was more than control. The calorie obtained from control was 266.25±0.18 Kcal but in test it was enhanced as 271.14±0.07 Kcal.

The figure no.6.11 showed the jelly incorporated with gethar skin extracted gelatin. The sensory evaluation of jelly was carried out using 9 point hedonic scale with respect to appearance, colour, odour, texture and overall acceptability was given in figure no.6.12. The jelly incorporated with gelatin has slightly rough texture than control because of crystallization of sugar. The colour alteration is due to use of different food colours. Thus, jelly integrated with fish gelatin is acceptable along with control for various applications in nutraceutical. The similar results were reported by Yusof et al. (2019) for jelly combined with halal gelatin.

Parameters/100gm	Control	Test
Calories (Kcal)	266.25±0.18	271.14±0.07
Fat (gm)	0.023±0.04	0.024±0.003
Carbohydrates (gm)	69.95±0.41	68.73±0.82
Protein (gm)	0.157±0.48	0.175±0.32
Calcium (mg)	7.04±0.36	6.13±0.41
iron (mg)	0.191±0.03	0.168±0.97
Potassium (mg)	54.01±0.08	52.11±0.25
Ash (%)	0.25±0.08	0.29±0.01
Moisture (%)	84.89±0.23	86.24±0.53

Table 6.2.: Nutritional and physicochemical analysis of jelly

Figure 6.11.: Jelly incorporated with gelatin

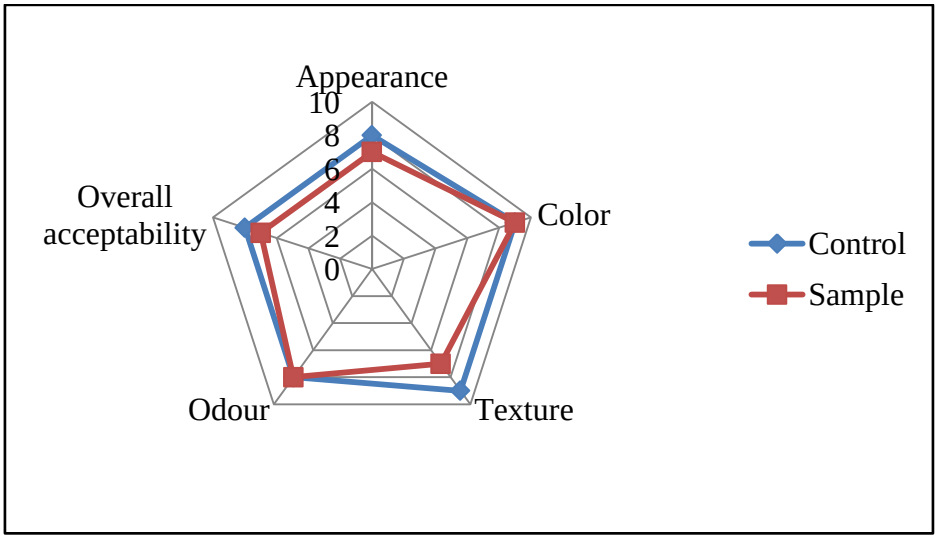

Figure 6.12.: Sensory analysis of jelly

6.4. Conclusion

The biological macromolecules and peptide from fish waste material of gethar (*Sarda orientalis*) was extracted and evaluated for various applications. The enzymatic hydrolysis of collagen generates peptide of less than 30 KDa. This peptide was effectively prevent growth of MCF-7 cancer cells and also has good anti-diabetic as well as antioxidant potential. It also inhibits growth of Gram positive and negative micro-organisms. The amino acids present in collagen provide nutrition during seed germination of *Vigna radiata, Lens culinaris and Vigna aconitifolia*. The extracted gelatin has ability to scavenge free radicals as well as good antimicrobial ability. The EPA and DHA present in fish oil have anti-inflammatory property. As collagen and gelatin are proteins, they incorporated in functional food preparations such as raggi balls and jelly, so this the major avenue to fulfil the need of protein as protein supplement in sports, bodybuilders and malnourished peoples in all age groups. Thus, total exploitation of fish waste was carried out which helps to diminish environmental pollution caused by these waste. The compounds derived from it have potential applications in pharmaceutical, nutraceutical and agricultural industries.

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CHAPTER VII

SUMMARY AND CONCLUSION

7.1. Summary

The continuous development in fishing industries from last few years produces more amount of waste. But till date, there is no proper disposal strategy was available for this. The improper disposal causes hazardous impact on environment as well as on health. The fish waste comprises scale, skin, fins, tail and head. Fish and its waste contains good amount of biomolecules such as proteins, lipids, enzymes, vitamins, macro and micronutrients. Among these biomolecules, the valuable collagen, gelatin and omega-3 fatty acid containing oil has increasing demand. Thus, marine species provides an alternative and safe resource for the extraction of these components with minimum ethical and health issues. The fish waste remaining after recovery of these valuable components was degraded by micro-organism. The formed organic liquid hydrolysate was applied as plant growth stimulator as well as soil conditioner to improve crop productivity and soil fertility. This study provides a creative idea for the utilization of fish waste in a proper technological way which helps in management of fish waste. Henceforth, this research work compromises stepwise and complete exploitation of fish waste. It will help to decrease environmental pollution problems and small scale industry can be developed by utilizing these techniques to help the entrepreneurs. The collagen, gelatin and omega-3 fatty acid from this waste has diverse applications in pharmaceutical, nutraceutical and agriculture sector.

7.2. Conclusion

- The waste material of marine fish gethar (*Sarda orientalis*) was utilized for extraction of collagen, gelatin and omega-3 fatty acid.
- For collagen extraction, the protocol was optimized to find out suitable organic acid for maximum production of collagen.
- The 0.2 M lactic acid gives 34% collagen yield within 40 hr and further it was utilized to study its different structural and functional properties.
- The collagen showed UV absorption spectra at 269 nm. The SDS-PAGE revealed that extracted collagen was type I with two α and one β chain.
- The various functional groups attached to collagen were detected by FTIR spectroscopy while fibrous nature of it was observed in SEM analysis.
- The good thermal stability of collagen was determined using DSC analysis. The zeta potential was found as -0.345 mV and the mean particle size ranges from 3.12-14.51 d.nm.
- The collagen contains high amount of glycine, proline and alanine amino acid. Also good amount of hydroxyproline was detected.
- The extracted collagen possesses good functional properties. It has 0.229±2 turbidity, 57.23±0.37% solubility and 2.18±0.04 cP viscosity. Also 19.7µl/mg water holding capacity and 12.2±0.21 gm/gm oil absorption capacity was detected. It exhibits better emulsifying and foaming characteristics.
- The collagen has maximum solubility at pH 2.0 (99.36%) while forms more precipitation at 2 M NaCl (99.96%).
- The sensory analysis score of colour, appearance and overall acceptability of extracted ASC was more than 3 hedonic points. It has slight fishy odour but is not easily detected and can be removed by using more specific purification techniques.
- The skin and head part of gethar was utilized for extraction of gelatin and fish oil containing omega-3 fatty acids respectively.
- The gelatin was extracted using lactic acid solubilisation method and chloroform: methanol: water solvent system was utilized for extraction of omega-3 fatty acid.
- The 0.63±0.03 gm/gm gelatin was obtained within 2 hr from skin of gethar.
- The gelatin showed UV absorption spectra at 280 nm and its molecular distribution was studied using SDS-PAGE.

- The FTIR spectroscopy was used to analyse different functional groups attached to gelatin. It exhibits 2θ = ~19º in XRD analysis which is occurred mainly due to gelatin.
- In DSC studies, gelatin forms single broad endothermic peak and three smaller peaks. It has 2.24 mV zeta potential. The particle size of gelatin rages from 30-600 r.nm.
- Gelatin contains more amounts of glycine and alanine followed by imino acids (proline and hydroxyproline).
- The extracted gelatin possesses goof functional properties. It is hot water soluble and has 6.67±0.19 cP viscosity. Clarity and turbidity value of gelatin was found to be 41.1±1.31 and 257.66±6.54 FTU respectively. It exhibits 10.75±0.65ºC gelling and 29.88±0.41ºC melting temperature.
- The 3% gelatin showed good emulsifying properties while 5% gelatin has good foaming characteristics. The water holding and fat binding capacity was recorded as 262.33±13.88% and 407.66±14.83% respectively.
- The extracted gelatin from gethar skin was type A with pH 8.0 as isoelectric point but it has maximum solubility at pH 6.0 (99.55%).
- The 27.63±0.24% oil was extracted from gethar fish head waste.
- The fatty acid profiling of fish oil was carried out using gas chromatography-mass spectroscopy. The analysis shows that there are two types of omega-3 polyunsaturated fatty acid found in gethar oil which is EPA (1.74%) and DHA (14.19%).
- The functional characteristics of fish oil were evaluated to study its quality. It contains 2.25±0.20% free fatty acids. The acid, iodine and saponification value was recorded as 2.57±0.36 mg KOH/gm of oil, 107.88±0.53 gm iodine/gm oil and 78.53±0.49 mg KOH/gm oil respectively. The peroxide and p-anisidine value was found to be 25.72±0.45 Meq/gm and 17.29±0.48 Meq/gm.
- The isolation and characterization of fish waste degrading micro-organism from fish waste dumping site was carried out and named as *Staphylococcus sciuri* PSD 11.
- The microbial hydrolysis of waste remaining after extraction was carried out by *S.sciuri* and degrades waste within 7 days with liberation of amino acids and proteins.

- The organic liquid hydrolysate was generated after degradation was studied for its plant growth promotion ability on *Vigna radiata*.
- The improvement in physical and biochemical characteristics of *V.radiata* was observed. Thus, fish waste hydrolysate effectively acts as plant growth stimulator.
- The collagen, gelatin and omega-3 fatty acids were extracted from various waste materials of gethar (*Sarda orientalis*) using suitable extraction procedures.
- The collagen peptides of molecular weight less than 30 KDa were synthesized using enzymatic hydrolysis technique. These peptides were explored to study its bioactive properties like anticancer, anti-diabetic, antioxidant and antimicrobial.
- These peptides inhibit growth of MCF-7 cancer cells with 58.59 mg/ml IC50. It also has anti-diabetic potential and IC50 value ranges from 6.51 to 7.13 mg/ml. It has ability to scavenge free radicals with 23.51±0.58% inhibition. Also acts as antimicrobial agent against Gram positive and negative micro-organisms.
- The different amino acids present in collagen supplies nutrition during seed germination of *Vigna radiata, Lens culinaris and Vigna aconitifolia*. All seeds form good sprout length within 24 and 48 hr.
- The extracted gelatin was tested for its antioxidant and antimicrobial potential. It has capacity to scavenge free radicals with 13.51±0.76% inhibition. Also has antimicrobial activity against Gram positive and negative micro-organisms.
- The EPA and DHA present in fish oil have better anti-inflammatory and antioxidant property with 57.70±0.34% and 6.27±0.31% inhibition respectively.
- The extracted collagen and gelatin was utilized in functional food preparations like raggi balls and jelly respectively. The sensory analysis of these foods showed that, incorporation of collagen and gelatin from fish source is moderately acceptable. Both functional foods have better nutritional and physicochemical characteristics. Thus, helps to provide potential health benefits.
- This was the first report in which waste material of marine fish gethar was utilized for extraction of collagen, gelatin and omega-3 fatty acid.
- Collagen is a rich source of amino acids. It's supplementation in functional foods helps to fulfil the protein demand of health. Also provides protection against some diseases. It was the first report in which collagen was incorporated into raggi balls.
- The complete utilization of fish waste was carried out to extract biologically important macromolecules. The residual waste remaining after extraction was

degraded by micro-organism to generate organic liquid fertilizer which is used to improve crop productivity. This will helps to reduce use of chemical fertilizers in agriculture.

- This research work successfully employed as innovative and sustainable technology to minimize the environmental pollution caused by improper disposal of fish waste.
- The efficient extraction of collagen, gelatin and omega-3 fatty acid from waste material decreases the cost of waste management and the extracted macromolecules have increasing demand in food, pharmaceutical and agricultural sectors.

RESEARCH PUBLICATIONS

Research publications:-

- **Kirdat P.N.**, Dandge P.B. (Dec 2021).Structural properties of gelatin extracted from marine fish Gethar (*Sarda orientalis*). International Journal of Innovative Science, Engineering and technology, volume 8, issue 12.ISSN:- 2348-7968.
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- **Pranoti N. Kirdat**, Sandip S.Kale, Padma B. Dandge. Structural and Functional properties of gelatin extracted from fish waste. International E-conference on sustainable development in chemistry and scientific applications (SDCSA-2021) organized by Sadguru Gadage Maharaj College, Karad, 16-17 December 2021.
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- **Pranoti N. Kirdat**, Padma B. Dandge, Priyanka S. Pawar, Amruta C. Tate. Antibacterial activity of chemically synthesized cadmium sulphide nanoparticles and its characterization. National conference on Frontiers in biopesticides and biofertilizers organized by P.E.S.R.S.N collage of Arts and science Farmagudi. Goa, 6-7th December 2019.
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1) University Name : Shivaji University,Kolhapur

2) Department of : Biochemistry

3) Name of Researcher : Miss. Kirdat Pranoti Nagesh

4) Name of Guide : Dr.(Mrs.). Padma B. Dandge

5) Type of Degree : Doctor of Philosophy

6) Registration Date : 01/07/2018

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SCIENCE AND TECHNOLOGY

BY

Miss. PRANOTI NAGESH KIRDAT
M. Sc.

UNDER THE GUIDANCE OF

Dr. (Mrs.) PADMA B. DANDGE
M.Sc. Ph.D.

ASSOCIATE PROFESSOR
DEPARTMENT OF BIOCHEMISTRY,
SHIVAJI UNIVERSITY, KOLHAPUR,
MAHARASHTRA, INDIA

2022

Department of Biochemistry
Shivaji University, Kolhapur

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I hereby declare that the thesis entitled **"Studies On Biochemical Analysis Of Fish Waste And Its Applications**," completed and written by me and have not previously formed the basis for the award of any degree or diploma or other similar title of this or any other university or examining body. Further, I declare that I have not violated any of the provisions under Copyright and piracy/Cyber/IPR Act amended from time to time.

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Department of Biochemistry
Shivaji University, Kolhapur

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Place: Kolhapur

Date:

Prof.K.D.Sonawane
Professor & Head
Department of Biochemistry,
Shivaji University, Kolhapur

Dr. (Mrs.) Padma B. Dandge
Research Guide
Department of Biochemistry
Shivaji University, Kolhapur

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PRANU.....

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ABBERATIONS

Symbol	Long form
%	Percentage
nm	Nanometre
ml	Millimetre
Mg	Microgram
Meq	Miliequivalent
kD	Kilo Dalton
Sec	Second
kPa	Kilopascal
eV	Electron volt
d.nm	Diameter in nanometre
r.nm	Radius in nanometre
mV	Millivolt
ASC	Acid soluble Collagen
ASE	Acid soluble extract
Conc.	Concentration
LA	Lactic acid
FA	Formic acid
OA	Oxalic acid
AA	Acetic acid
PA	Phosphoric acid
cP	Centipoise
FTU	Formazin turbidity unit
HyP	Hydroxyproline
IC	Inhibition concentration
w/w	Weight by weight ratio
w/v	Weight volume ratio
D/W	Distilled water
v/v	Volume by volume ratio
m/z	Mass to charge ratio

gm	Gram
Kg	Kilogram
m	Molar
mg	Milligram
µm	Microgram
mm	Millimetre
hr	Hour
sec	Seconds
mM	Millimolar
A	Absorbance
µl	Microliter
Min.	Minutes
No.	Number
N	Normal
RT	Room temperature
SD	Standard deviation
bp	Base pair
TNF	Tumor necrosis factor
IL	Interleukin
OFAT	One factor at a time method
pH	Hydrogen ion concentration
PUFA	Poly unsaturated fatty acid
SDS	Sodium dodecyl sulphate
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
NCCS	National centre for cell science

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1.Introduction

Fish is multipurpose and diverse commodity covering a wide domain of world. Fish is an intensive source of protein, micronutrients and numerous essential fatty acids, so it play distinct role by providing advantageous and nutritious contribution to variegated and healthy diet. The management policy and implementation actively impact on the global extent of fish production including both aquaculture and capture fisheries. Dissimilarity in fish utilization, processing and consumption within and between continents, regions and countries also influence on fish productivity. From last several years, problem due to fishery waste has increased and becoming a worldwide concern. It causes negative impact on some biological, technical and operational components as well as socio-economic factors. About 50% of fish tissues including tail, fins, head and viscera are discarded as 'waste'. The amount of waste produced through processing depends upon nature of species used, fishing areas and the product formed (Caruso 2015). The fish industry engenders remarkable quantity of waste at several phases of manufacturing. Majority of fish processing industries discard such waste in rigid mode, by incineration, disposal in sea or by landfilling manner. According to recent scenario, these conventional practices are affecting aquatic and terrestrial ecosystem with drastic environmental issues.

The fisheries and aquaculture sector contributes significantly to food security and nutrition, particularly in some of the world's most food-limited regions, while concurrently supporting lifestyle of millions of people throughout the world. Global capture fisheries production in 2018 reached up to 96.4 million tonnes, an increase of 5.4% from the average of earlier three years. Marine captured fish production was increased from 81.2 million tonnes in 2017 to 84.4 million tonnes in 2018, but less than in the year 1996 (86.4 million tonnes). Further, in 2018 global fish production was reached about 179 million tonnes of which 82 million tonnes derived from aquaculture production (table no.1.1 and figure no.1.1). Aquaculture accounted for 46% of the total production and 52% of fish for human consumption (FAO 2020).

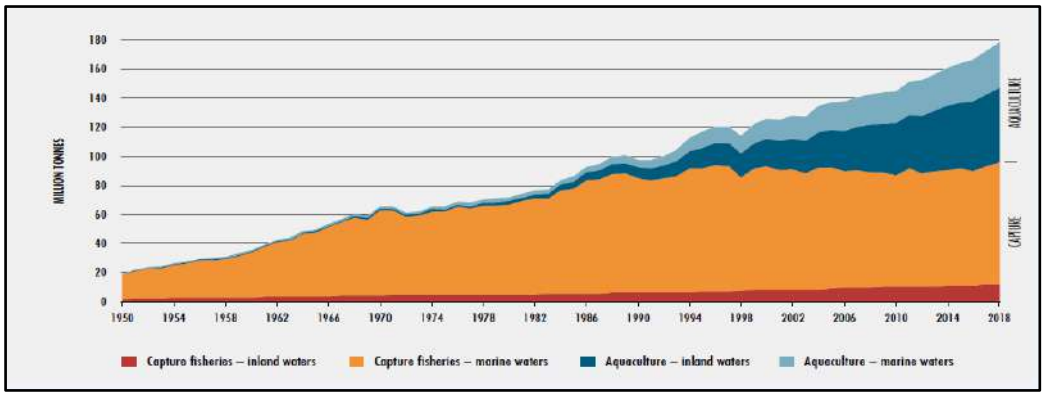

Figure 1.1.: World capture fisheries and aquaculture production (Source: FAO 2020)

Capture	1986–1995	1996–2005	2006–2015	2016	2017	2018	2019
Production(Million tonnes)							
Inland	6.4	8.3	10.6	11.4	11.9	12.0	12.08
Marine	80.5	83.0	79.3	78.3	81.2	84.4	80.40
Total capture	86.9	91.4	89.8	89.6	93.1	96.4	92.48
Aquaculture							
Inland	8.6	19.8	36.8	48.0	49.6	51.3	53.3
Marine	6.3	14.4	22.8	28.5	30.0	30.8	34.6
Total aquaculture	14.9	34.2	59.7	76.5	79.5	82.1	87.9
Total world fisheries and aquaculture	101.8	125.6	149.5	166.1	172.7	178.5	180.38
(Source: FAO 2020)							

Table 1.1.: Summary of worldwide aquaculture and fisheries production

Fish and fishery products remain most important food commodities in the world. In 2018, 67 million tonnes, or 38% of total fisheries and aquaculture production, were traded internationally (figure no.1.2). Of the whole total, 156 million tonnes were used

for human consumption, equivalent to an estimated annual supply of 20.5 kg per capita. The remaining 22 million tonnes were intended for non-food uses, generally to produce fishmeal and fish oil. Global food fish consumption increased at an average annual rate of 3.1% from 1961 to 2017. Per capita food fish consumption grew from 9.0 kg (live weight equivalent) in 1961 to 20.5 kg in 2018, by about 1.5% per year (table no.1.2). The top 20 producing countries accounted for about 74% of the total capture fisheries production. China has continued a major fish producer, accounting for 35% of overall fish production in 2018. Excluding China, a substantial share of production in 2018 came from Asia (34%), followed by the Americas (14%), Europe (10 %), Africa (7 %) and Oceania (1%). A growing share of fishmeal and fish oil, estimated at 25–35%, is produced from by-products of fish processing, which previously were often discarded or used as direct feed, in silage or in fertilizers (FAO 2020).

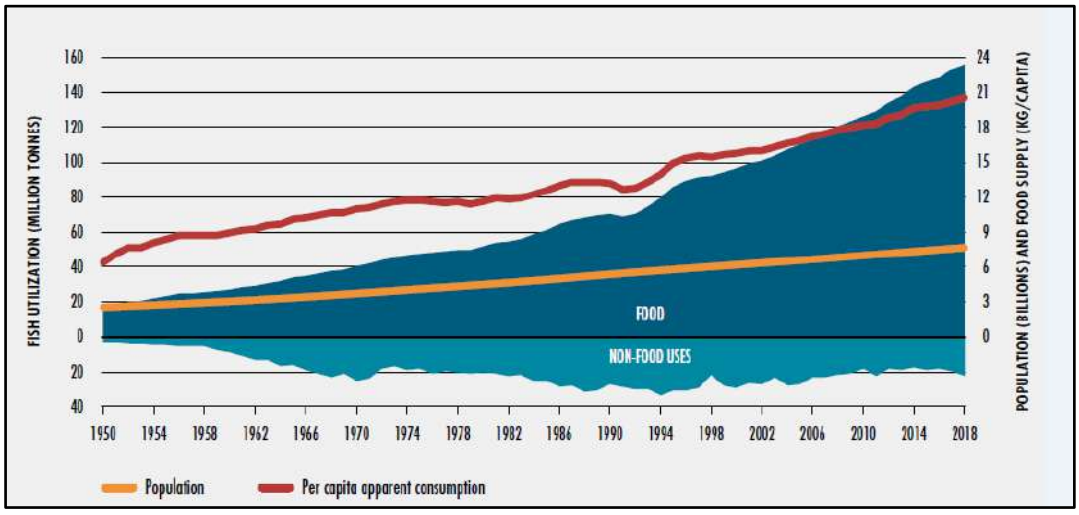

Figure 1.2.: World fish utilization and apparent consumption

(Source: FAO 2020)

	1986–1995	1996–2005	2006–2015	2016	2017	2018
Production (Million tonnes)						
Human consumption	71.8	98.5	129.2	148.2	152.9	156.4
Non-food uses	29.9	27.1	20.3	17.9	19.7	22.2
Population (billions)	5.4	6.2	7.0	7.5	7.5	7.6

Per-capita apparent consumption (kg)	13.4	15.9	18.4	19.9	20.3	20.5

Table 1.2.: Summary of worldwide fish utilization and consumption (Source: FAO 2020)

Excluding aquatic plants and other aquatic animals, the worldwide production of fish, crustaceans and molluscs was 177.8 million tonnes in 2019 and it was decreased by 1% than 2018. So, sum of capture production accounted for 92.5 million tonnes in 2019, a decrease of 4.3% compared with the earlier year. Aquaculture production was 85.3 million tonnes in 2019, an increase in 2018 up to 3.7 %. The involvement of aquaculture in total production of aquatic animals from capture and aquaculture collectively has grown gradually 39.9 to 48.0 % from year 2010 to 2019. The decrease in catches causes reduction in capture fisheries up to 80.4 million tonnes in 2019 which is less than 5% as compared to 2018. Global capture fisheries production in inland waters in 2019 reached the highest levels ever recorded at 12.1 million tonnes and it is continued. Its share in total global capture production remains around 13 %. Also, rise in catches can partially be attributed to enhanced reporting and assessment at the country level rather than entirely due to increased production (OECD/FAO 2019).

The majority of fishes are utilized to produce food products for human consumption and this share will lead to increase at 91% (178 million tonnes) by 2028. This represents in general increase of 16% compared to the average for 2016-18. The entire quantity of fish produced at the world level is estimated to be 196.3 million tonnes by 2028, an increase of 14% relative to the base period (average of 2016-18) and an additional 24.1 million tonnes of fish with seafood in absolute terms. Overall fish production increased at about 171 million tonnes in 2016, with aquaculture representing 47 % of the total and 53 % for non-food uses (including reduction to fishmeal and fish oil). The quantity of fish produced at the global level is projected to continue growing (1.1% p.a.), but at a slower rate than observed over previous decade (2.4% p.a.). Altogether, Asia is predicted to consume 71% (or 126 Mt), of the total food fish, while lowest quantities will be consumed in Oceania and Latin America (figure no.1.3). The proportion of fish meal being produced from waste is projected to increase from 25% in 2018 to 31% by 2028, while for fish oil it is projected to increase from 35% to 40%. Climate change, weather

variability and changes in the frequency and extent of extreme weather events are anticipated to have a major impact on availability, trade of fish and fish products mainly through habitat destruction, changes in fish migration patterns and natural productivity of fish stocks (OECD/FAO 2019).

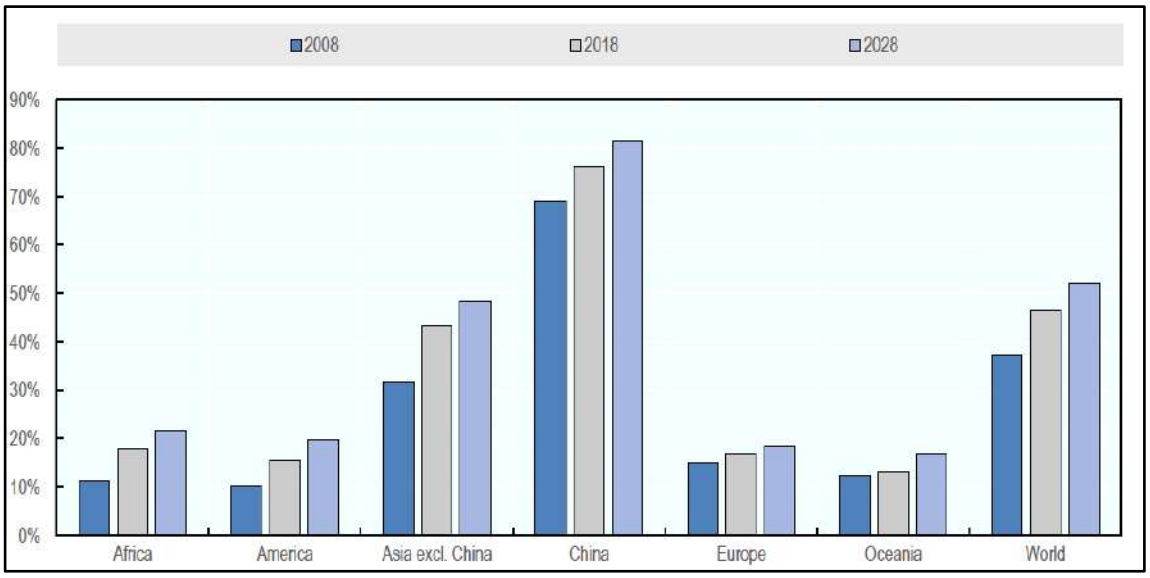

Figure 1.3.: Contribution of aquaculture to regional fish and seafood production (OECD/FAO 2019)

Fish is a vastly perishable food matter because of its moisture and nutrient contents. Seafood generated from wild or cultivated species causes formation of more amount of by-products upon processing or during capturing of targeted fisheries that may not be fully utilized. These by-products may serve as rich source of various biomolecules having significant health benefits. The conventional fishery by-products include fish meal, fish liver oils, fish maw, isinglass etc. Some other by-products derived from fish and its waste material include fish protein concentrate, glue, gelatin, pearl essence, amino acids, protamine, fish skin leather etc. (Pagarkar et al.2014).

Fish waste is abundant in potentially valuable oils, minerals, enzymes, pigments and flavours etc. that have multiple applications in food, pharmaceutical, agricultural and aquaculture industries. Organic fertilizers and composts produced from fish waste have significant advantages over chemical based products. Fish by-products can offer number of biomolecules involving proteins, lipids, oil, enzymes, micronutrients (riboflavin, niacin and vitamin A, D) as well as minerals (iron, iodine, selenium and zinc). In addition to fishmeal and oil production, there is potential in silage production, fertiliser, composting, fish protein hydrolysate and concentrate. Non-nutritional applications

include chitin and chitosan, carotenoid pigments, enzyme extraction, leather, glue, pharmaceuticals, cosmetics, fine chemicals, collagen and gelatin. There are countless other uses for this material and new uses are emerging all the time.

Collagen is present in almost all organs of vertebrates and is the main structural component of connective tissues like skin, bone, tendon, cartilage, blood vessels and teeth. Collagen is accommodating approximately 30% of animal body and also the most plentiful and ubiquitous animal protein polymer (Khan et al.2009). The unique collagen types are characterized by a huge complexity and diversity in their structure or shape, their splice variants, the presence of extra, non-helical domains, their assembly and function. Collagen has applications in pharmaceutical, nutraceutical field, cosmetics, agriculture because of its excellent biodegradability, biocompatibility and weak antigenicity. Commercially bovine and porcine derived collagen were used but with limited applications and also has ethical and legal issues. Therefore, marine species are the best suitable alternative for collagen. Fish wastes like skin, scales, fins, bone of various fishes are rich source of collagen (Berhie et al.2019). Extraction of collagen from this waste is economically feasible as well as cost effective and mainly helps to reduce environmental pollution burden on eco-system by this waste and there are less ethical issues regarding this.

Gelatin is a fibrous protein having molecular weight 20-200 KDa produced through thermal denaturation of collagen or partial degradation of animal bone and skin. The total or partial dissociation of collagen polypeptide during denaturation is occurred due to weakening and separation of hydrogen bonds which results in loss of original triple helical confirmation. The resulting polymer forms different coiled structure other than original protein designated as gelatin (Milovanovic and Hayes 2018). It is generally used in food, pharmaceutical, medical, cosmetic and photographic industries and has unique physical and chemical properties. Also, it incorporated as stabilizer, gelling, fastener, emulsifier and adhesive agent. It could also be used for diabetics and can reduce body weight. In nutraceutical industry, gelatin is one of the water-soluble polymers that can be used as materials to raise the food elasticity, consistency and stability (Ratnasari et al.2014). Because of gel like properties, it is an effective food packaging material and useful food additive in different food products like gelatinous desserts, gummy candies and many yogurts. It has various properties like thickening, emulsifying, binding and adhesive properties and also able to attract impurities in fruit juices, wine and vinegar. It

can be used in skin creams and lotions face masks, shampoos, hair conditioners, hair sprays, nail polishes, lipsticks also used for craft and decorative purposes.

Fish oil is the good source of polyunsaturated fatty acids (PUFA), especially omega-3 long chain ecosapentanoic acid (EPA) and docosahexanoic acid (DHA). Omega-3 fatty acids are polyunsaturated fatty acids characterized by presence of a double bond three atoms away from the terminal methyl group in their chemical structure. Omega-3 long chain fatty acid plays vital role in growth and development throughout the life and also beneficial in prevention and treatment of diseases related to coronary arteries (Suriani and Taulu 2015). Apart from this, it can be used to increase the omega-3 levels in meat and eggs when fed to land animals and is therefore, often added to animal feed. Fish oils are used in the medical and animal feed areas for supply of vitamin A and D; in production of soaps and detergents, paints and varnishes, floor coverings and oil cloths, oiled fabrics and in the processing of insecticides, alkalized resins, cosmetics, metal and processed leather. A growing market for fish oil supplements for human consumption is also imposing competing demands on the availability of sufficient fish oil supplies from wild caught fisheries due to health consciousness (Howieson 2017). Different fishes like silver carp, tuna, mackerel and sardine are good sources of fish oil. The health benefits of omega-3 fatty acids includes reduction in inflammation, fight against anxiety and depression, improves eye health, promote brain functioning, has application in feed and agriculture field and also, has role in skin and hair treatment.

Current research work deals with optimum utilization of fish processed waste (skin, scale, fins, tail and head) to extract commercially important biological macromolecules. Initial study deals with extraction, purification and characterization of collagen, gelatin and omega-3 fatty acids from waste material of Gethar (*Sarda orientalis*). After the biochemical analysis of these components, they are explored for various applications like biomedical, food and agriculture sector. Soil sample from fish waste dumping site was screened for isolation of fish waste degrading bacteria. After the extraction of all components from fish waste, remaining waste was subjected to microbial degradation. The hydrolysate generated after degradation was studied for its plant growth promotion ability. Thus, complete utilization of fish processed waste was carried out. Fruitful results can be obtained if this collagenous waste is treated in an eco-friendly manner using a biotechnological way which may help to minimize environmental pollution caused due to this waste.

1.2. Literature review

1.2.1. Fish preservation and processing

Fish is one of the protein rich foods that need careful handling. Fish spoilage is important aspect in fish processing. Due to high tropical temperature than actual body temperature of fishes, it initiates harmful activities of bacteria, enzymes and chemical oxidation of fat in fish. Therefore, all the processes during preparation, catching, landing, handling, storage and transport requires close attention to deliver a high quality product. The large amount of fish cannot be processed and utilized at same time so; preservation of fish is important aspect. It includes chilling, salting, drying, smoking, roasting and canning. The fish processing industries utilizes following steps for processing: Handling the catch, Removal of the scales and head, Cutting and Filleting of fish. The handling of fish includes catching, sorting, grading, chilling and storing of chilled fishes. Scales and head are inedible parts; it can be removed by mechanical scrapping. The cutting process involves gutting and washing. Gutting means removal of gut of fish and washing with clean water. Gutting and washing of the fish help to prevent bacterial attack before and during processing, preservation and storage. Fillets are nothing but flesh like material of fish was prepared by traditional method or filleting machine. Fillets are dripped in brine to enhance their appearance and to reduce the amount of drip and it also gives a salty flavour. Then put in a freezer at -35°C to- 40°C and stored at -23°C. After all the processing, the processed fish is subjected to formation of different food products like Fish mince, Surimi, Fish sauce and Fish meal (Ugochukwu 2017).

1.2.2. Fish processing waste

Fishery industries generate a large amount of solid waste such as gross fish waste, fish head, viscera, tails, skin, scales, bones, blood, liver, gonads, guts and some muscle tissues while liquid waste consists of wastewater used during fish processing. The average composition was fish was represented in figure no.1.4. Around 75% of the worldwide fish production is used for human utilization and the remaining 25% is treated as fish waste. The disposal of these bulk fish waste is quite complicated and expensive because they are rich source of organic contents such as 58% protein, bioactive peptides, collagen, gelatin, oil, enzymes as well as calcium, 19% fat and trace amounts of minerals mainly copper, phosphorus, magnesium, sodium, potassium, calcium, iron, zinc and manganese. Inappropriate discarding by traditional methods and sea dumping can lead to pollution and other environmental issues. (Ramkumar et al.2016).

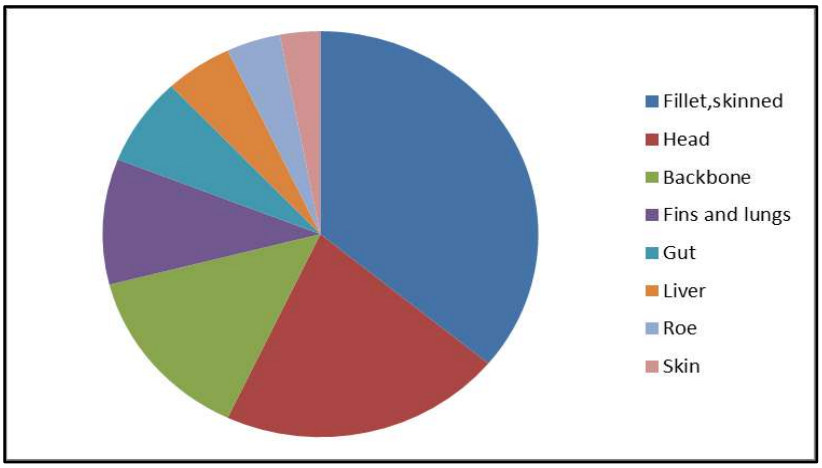

Figure 1.4.: Average composition of fish

1.2.3. Benefits of fish waste

Due to increasing demand of sea food, fish industry wastes are an important environmental contamination source. Fish industry waste and its liquid effluents affect not only surrounding area but also wider coastal zone at distinct ecosystem level. Thus, causes reduction in biomass, density and diversity of the phytoplankton's, zooplanktons and alter normal behaviour of natural food chains and food webs. A remarkable waste reduction approach for the industry is recovery of by-products from fish wastes so as to reduce the problems originated due to fish waste.

1.2.3.1. Animal feed

Recently, use of fish wastes is a substituent for animal feed to reduce the risk of environmental pollution and improve animal health benefits so as to increase currency production from animals. Treatment of fish waste such as head, bones, skin and sometimes whole fish was heated at 60, 80, 105 and 150°C for 12 hours reduces the moisture content to 10-12%. This quality of waste with reduced moisture content is suitable for animal feed as recommended by NRC (National Research Council), 1998. The large amount of minerals, protein (58% dry matter) and fatty acids (19%) like monounsaturated acids, palmitic and oleic acids were present in fish waste also 22% ash content indicates the mineral presence in fish waste (Arvanitoyanni and Kassaveti 2006).

1.2.3.2. Biodiesel/biogas

About 95-96% fuel was produced after filtration, primary and secondary treatments of fish waste. This oil was tested for its density, flash point, heating value, distillation test and sulphur content. The obtained oil was found to have equivalent properties for use in diesel engines. There is no information available about anaerobic

digestion of solid waste from fish industries. The produced biogas may be used to produce thermal and electrical energy (Arvanitoyanni and Kassaveti 2006).

1.2.3.3. Natural pigments

Carotenoids are the important colour pigments which imparts colour to the most of fish and shellfish. The orange-red integument and flesh like structure of more costly seafood like shrimp, lobster, crab, crayfish, salmon, redfish, red snapper and tuna possesses carotenoid pigments. The pigments extracted from fish waste can be effectively used in aquaculture feed formulation and the residue remaining after extraction may be used in preparation of chitin/chitosan (Arvanitoyanni and Kassaveti 2006).

1.2.3.4. Food industry/cosmetics

Some researchers found that, a various useful components can be extracted from fish waste including enzymes, proteins and bioactive compounds have potential antimicrobial as well as antitumor activities. Among different enzymes present in fish waste, protease is the most important industrial enzyme shows useful applications in food industry. Surimi is the new type of fish meat in which fishes are mechanically debones and persevered with the help of cryoprotectants. Fish stomach mucosa is used for the extraction of milk clotting enzyme which is an inexpensive substituent for rennet in cheese manufacturing. This approach may promote the development of new food industry. Some fish hydrolysates were utilized for food processing and preservation. Fish skin, bone and fin were acts as potential source for collagen. It can be used as best alternative to regular collagen in different food, cosmetics and biomedical industries (Arvanitoyanni and Kassaveti 2006).

1.2.4. Fish waste management

Waste management deals with the analysis of solid and liquid pollutants produced by fish processing industries. Environmental issues caused by industrial activity have become one of the major important aspects. The industry which is eco-friendly and socially responsible to nature and will actively help to increase economic strength of nation and also save environment. Waste minimization is one of the interesting approach to meet sustainability of waste management in fish processing and production process (Kurniasih et al.2018). Fishery waste contains useful as well as harmful components which affects adversely on eco-system. The large amount of solid fish waste is discarded near to coastal region; this will lead to change in normal behaviour of food chain. The huge amount of water is required for washing and processing of fish. This will create

liquid waste which is incorporated into water bodies without any treatment. It leads to decrease in oxygen level, change in pH, lowers concentration of ammonia and nitrogen as well as also effects on ecosystem of water bodies.

1.2.5. Composition of fish waste

1.2.5.1. Amino acids

Fish and its derivatives are rich source of proteins with high biological and nutritional values, hence it contains balanced amount of amino acids. Different amino acids are present in fish and its by-products. It includes glutamic acid, arginine, histidine, threonine, tyrosine, valine, methionine, isoleucine, leucine, phenylalanine and lysine. Thus, fish and its waste derivatives are used for fish meal, fish sauce, animal feed and for agricultural purpose (de Souza et al.2017).

1.2.5.2. Enzymes

Fish internal organs such as viscera and gut are great source of enzymes with high catalytic activity at low concentrations. Because of this, fish processing derivatives are used for enzyme extraction. Marine fish viscera have wide variety of proteolytic enzymes like pepsin, trypsin, chymotrypsin and collagenases which are extracted in large scale. Most of enzymes in fish and its derivatives possess high catalytic activity at different environmental conditions like low or high temperature, elevated nutrient concentrations, high pressure and high salt concentrations. Due to these properties, fish proteinases have many applications in food processing industries. The fish derived enzymes also used in isolation of bioactive compounds (Kim and Mendis 2006).

1.2.5.3. Bioactive peptides

There are different bioactive peptides extracted by using enzymatic extraction methods from fish waste or fish waste remaining after processing. Proteolytic hydrolysis of parent proteins using available enzymes and micro-organism releases inactive short amino acid sequences called as bioactive peptides. They have biological and nutritional properties which effects positively on normal functioning of organism’s body and also enhances the food quality (Phadke et al.2017). Bioactive peptides have large demand in pharmaceutical fields due to their beneficial effects on human physiological system.

1.2.5.4. Collagen

Collagen is a naturally occurring protein. About 30% of the total protein present in animal body is largely composed of this structural fibrous protein. It is one of the important component of extracellular matrix of various connective tissues such as the skin, cartilage, bone, tendons, ligament of vertebrates, while in invertebrates body walls,

cuticles showed presence of collagen. It helps in tissue remodelling, adhesion and also plays vital role in maintaining structure of different tissues. It is left handed triple helical structure of protein stabilized by hydrogen bond. It has a diameter of about 1.6 nm with length of about 300 nm. The molecule has specific characteristic repetitive sequence of triplet Gly-X-Y. The structural levels of collagen were given in figure no.1.5.

Different types of collagen are exhibited based on X and Y, that is amino acids linked to glycine. Generally, X and Y are the imino acids proline and hydroxyproline respectively. There are 29 different types of collagen are available. About 90% of the collagen present in our body is the fibril forming collagen. It includes collagen type I, II, III, V and XXVII. Type I is most abundant in all connective tissues except hyaline cartilage (Silvipriya et al.2016). Thus, this fibrous protein plays vital role in various connective tissues and helps to maintain structure of bones, relieve joint pain, improve skin health and also has some other health benefits.

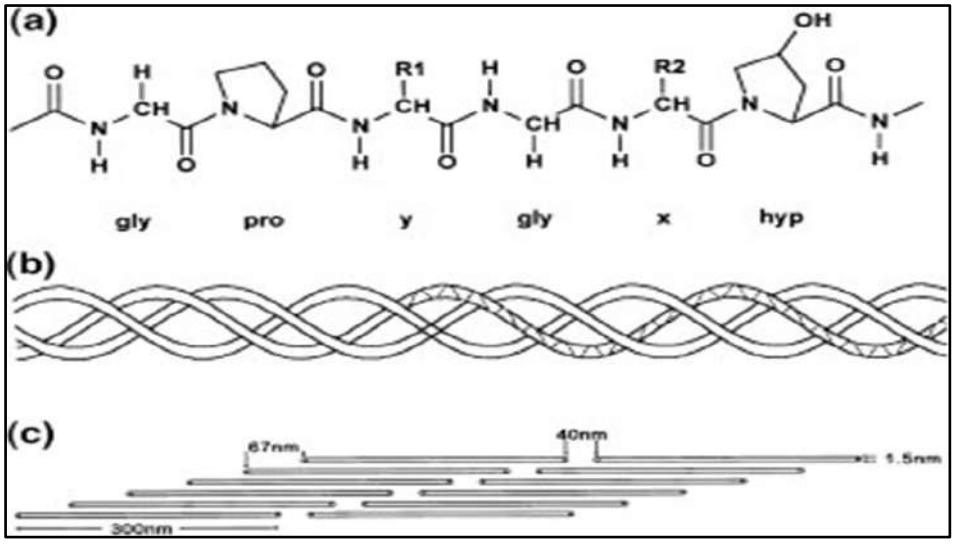

Figure 1.5.: Structural levels of collagen type I (a) amino acid sequence indicating primary structure (b) secondary and tertiary structure with left and right handed triple-helical and (c) collagen quaternary structure (Friess 1997)

1.2.5.5. Gelatin

Partial hydrolysis and heat dissolution at alkaline or acidic pH of skin collagen, bone and tendons of animals forms the proteinaceous hydrocolloid polymer gelatin. It is heterogeneous mixture of 300 to 4000 amino acids, single or multi stranded polypeptide with left handed proline helix confirmation (Kommareddy et al.2007). Chemical configuration of gelatin was showed in figure no. 1.6. It has high protein value having water soluble property and has ability to form transparent gel under

specific conditions. Molecular weight of gelatin ranges from 80 to 250 kD which contains 88% protein, 10% moisture and 1-2% salts. During the gel formation, it retains 50% moisture. Thus, Gelatin is an excellent biopolymer widely utilized in food and pharmaceutical industries (Wasswa et al.2007).

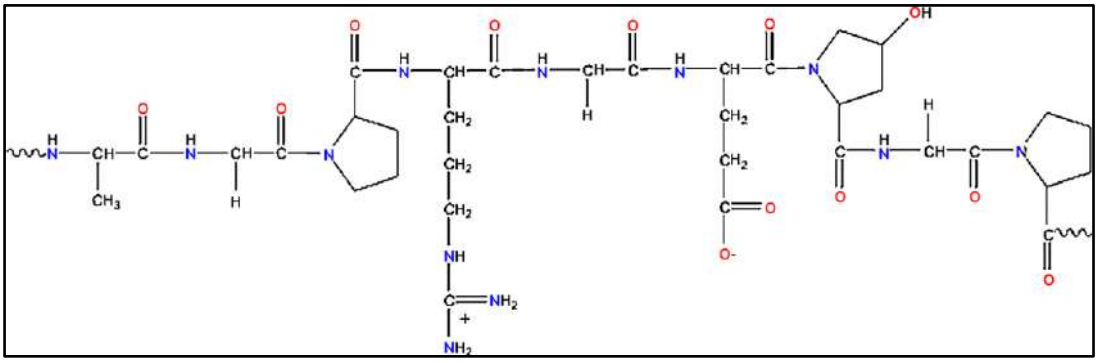

Figure 1.6.: Chemical configuration of gelatin (Chaplin 2012)

1.2.5.6. Oil

Fish oil is a rich source of polyunsaturated fatty acids (PUFA), especially omega-3 long-chain EPA and DHA. There are three types: eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and alpha-linolenic acid (ALA). These fatty acids also called as omega-3 oils or n-3 fatty acids. They are characterized by the presence of a double bond, three atoms away from the terminal methyl group in their chemical structure. Structure of fish oil containing EPA and DHA was depicted in figure no.1.7. Omega-3 long chain fatty acid plays crucial role in growth and development throughout the life. It helps in prevention and treatment of different coronary arteries diseases like arteriosclerosis, hypertension, arthritis, and impaired immune response. Also helps in development of central nervous system and prevention of heart disease (Suriani and Taulu 2015).

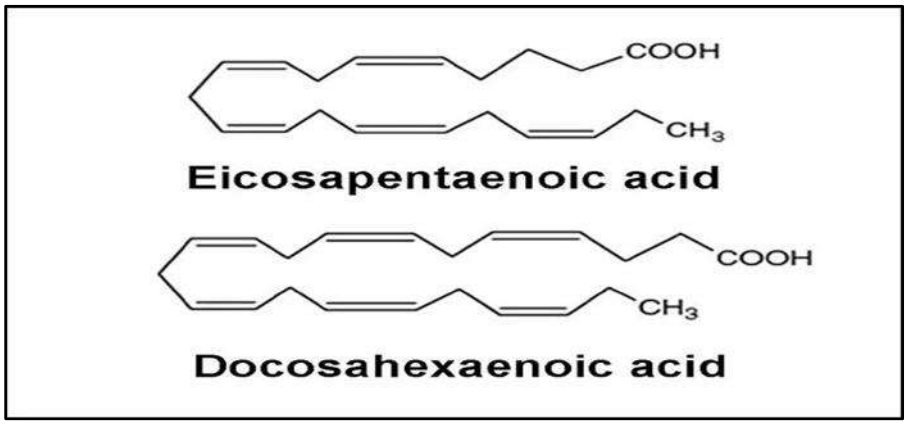

Figure 1.7.: Structure of Omega 3 fatty acids

1.2.6. Sources of collagen

The collagen obtained from bovine and porcine source was mostly used for industrial purpose. Skin and bones of cow and pigs were used for effective extraction. These two sources have different ethical and legal issues in different religions. Marine source is used as efficient alternative source for the extraction of collagen. It has some advantages, like high content of collagen, less immunogenic response, eco-friendly and showed negligible amount of contaminants. The waste materials derived from marine invertebrates and vertebrates were extensively used for this purpose. This will also help to reduce environmental threats caused due to fish waste. Besides all the traditional sources, some other sources like chicken, kangaroo tail, rat tail tendon, duck feet, sheep skin and frog skin also used. But, these sources may show some allergic and immunological response. Therefore, recombinant human collagen was under investigation which has lower immunogenicity as compared to other sources (Silvipriya et al.2015).

1.2.7. Types of collagen

About 25 to 30% of total protein of animal body is composed of most abundant fibrous collagen protein. It plays major role in maintaining biological and structural characteristics of extracellular matrix (ECM) and helps in physiological functions. Almost type I to type XXVIII different collagen forms has been studied. Out of this, type I, II, III and V are important in bone, cartilage, tendon, skin and muscle (Das et al.2017).

Family	Type	Distribution	Application
Fibril-forming	I	Skin, bone, tendon (non-cartilage), dermis, cornea, ligament	Membranes for guided tissue regeneration
	II	Cartilage, vitreous humor, nucleus pulposus, lung, cornea, skin, bone	Cartilage repair, arthritis treatment
	III	Extensible connective tissue (skin, lung, vascular system viz. artery), skin, vessel wall, reticular fibers of most tissues (lungs, liver, spleen)	Hemostats and tissue sealants
	V	Co-distributed with Type-I,	Corneal treatment

		especially in cornea	
	XI	Along with type II, vitreous body and cartilage	mAbs development for osteoarthritis
Basement membrane	IV	Basement membrane	Attachment enhancer of cell culture (mouse neuroblastoma) and diabetic nephropathy indicator
Microfibrillar	VI	Muscle, dermis, placenta, lungs, intervertebral disk, cartilage	Hemostat
Anchoring	VII	Dermal epidermal junction, skin, cervix, oral mucous	Treatment of dystrophic epidermolysis bullosa (DEB)
FACIT	IX	Along with type II in cornea, cartilage and vitreous body	
	XII	Tendon, perichondrium and ligaments	Regulator in early stages fibrillogenesis
	XIV	Along with type I in vessel walls, placenta, liver, dermis and lungs	
	XIX	Many tissues, human rhabdomyosarcoma	Antiangiogenic and antitumoral properties
	XX	Corneal epithelium, sternal cartilage, embryonic skin, tendon	
	XXI	Many tissues, blood vessel wall	Contribute to matrix assembly of vascular network during blood vessel formation
Transmembrane	XIII	NM junction, skin, hair follicle, intestine, lungs	Involved in inflammation and vasculogenesis, regulate bone mass
	XVII	Epithelia, skin hemidesmosomes	Teeth formation
Multiplexins	XV	Associated with collagens close to basement membranes, kidney, smooth muscle cells,	

		pancreas	
	XVI	Many tissues including keratinocytes and fibroblasts	Drug target and biomarker
	XVIII	Close structural homologue of XV, liver, lungs	Retinal structure, closure of neural tube
Miscellaneous	VIII	Endothelium	
	X	Hypertrophic cartilage	
	XXII	Tissue junctions	
	XXIII	Limited in tissues, mainly trans-membrane and shed forms	
	XXIV	Developing cornea, bone	
	XXV	Brain	
	XXVI	Testis, Ovary	
	XVII	Embryonic cartilage	
	XXVIII	BM around Schwann cells	

Table 1.3: Different types of collagen and their distribution in human body (Raman and Gopakumar 2018)

1.2.8. Collagen extraction methods

For extraction of collagen from fish waste includes two steps. First step deals with pre-treatment and second was effective collagen extraction.

1.2.8.1. Pre-treatment process

Pre-treatment of raw material (fish waste) is the crucial step during extraction. Different pre-treatment processes were applied on waste material to remove other biological constituents and to improve the efficiency of collagen extraction. Biological constitutes includes proteins, pigments, lipids and enzymes while fish bone and scale consist of calcium and some inorganic components which may interfere in collagen extraction. Alkaline pre-treatment by 0.1 M NaOH was used to remove non-collagenous proteins and pigments (Hamdan and Sarbon 2019). It also excludes the effect of endogenous protease. Fish bone and scales contains little calcium and some phosphate like components. It can be decalcified by using ethylenediamine tetraacetic acid (EDTA)

(Pati et al.2010). It releases phosphate into solution resulting in demineralization of waste. Lastly, the pre-treated sample is subjected to removal of fat and it was done by 10% butyl alcohol (Aminudin et al. 2015).

1.2.8.2. Extraction methods

There are various techniques employed for collagen extraction and based on this, collagen divided into discrete types. The types include alkali soluble, acid soluble (ASC), salt soluble (SSC), pepsin soluble (PSC) and ultrasound assisted collagen (UAC). Among these methods, acid and enzyme extraction methods are most common.

1.2.8.2.1. Alkali mediated collagen extraction

Many acidic and basic moieties are present in collagen. Extraction of collagen by alkali is not effective and produces very less yield. The alkali like CaO, $Ca(OH)_2$, Na_2CO_3 etc. were used for extraction. Acid-base neutralization under alkaline condition causes the cleavage of collagen into peptides. Yield depends upon concentration of alkali and time required for extraction. Alkaline method may be used to extract bone collagen but it requires more time, denaturation of protein structure and produce lesser yield. The collagen disintegrates and deteriorates in alkali solution, alters its structure, hydrolyses the peptide chains and reduction in isoelectric point. Thus, due to these disadvantages alkaline extraction of collagen is not suggested (Lian et al.2017).

1.2.8.2.2. Acid mediated collagen extraction

Acidic extraction is broadly applied method for productive extraction of collagen from marine waste products and it is designated as acid soluble collagen (ASC). Various concentrations of acetic acid are beneficially used as a solvent for extraction of collagen, since it generate increased yield and thermally stable collagen. It was reported that, collagen was effectively extracted by using 0.5 M acetic acid and gives more yield under controlled biological conditions (Chinh et al.2019). Principal monomeric subunits in collagen could be effectively solubilized in acetic acid. It has more extracting capacity and produces more yield than any other organic and inorganic acids (Veeruraj et al.2013). The concentration behind 0.7 M generates minimum yield and less active collagen. Inorganic acids also utilized for extraction but it gives rise to lower efficiency and low yield of collagen than organic acids (Hadfi and Sarbon 2019).

In addition to acetic acid some organic and inorganic acids such as citric acid, lactic acid, tartaric acid, formic acid, hydrochloric acid and sulphuric acid were employed for the extraction of collagen from fish waste (Bhuimbar et al.2019; Kothai and Premlatha 2018). Fish wastes including skin, scale, bone and fins are important by-

products from fishery industry which has more collagen content. Studies suggested that, acetic acid is applied to extract collagen form various fish processing waste including skin, scale, fins and swim bladder. The high collagen content about 50-70% was reported from skin waste (Nagai et al.2000; Pang et al.2013; Kumar et al.2016). Some studies suggested that, yield of collagen was dependent on fish species, raw materials, habit and habitat of fishes, biological conditions, type and time of extraction, concentration and type of acid used for pre-treatment processes. More concentration of acid may hydrolyse the collagen leading to lesser yield (Das et al.2017; Pamungkas et al.2019). Thus, acid extraction method was widely employed for collagen extraction.

1.2.8.2.3. Enzyme mediated collagen extraction

Commercially, collagen is retrieved from marine animals by using an acetic acid without inclusion of enzyme. Collagen extracted from sea food processing waste was less soluble in acidic condition. Acidic condition may give minimum production of extracted collagen. Therefore, enzyme mediated collagen extraction has been discovered to increase the yield which called as enzyme soluble collagen. Enzymes like pepsin, papain, collagenase isolated from microbial origin were implemented to increase the production of collagen (Junianto et al.2018; Rochima et al.2017; Min Li et al.2015). The acidic and enzymatic method of collagen extraction was depicted in figure no.1.8.

1.2.8.2.4. Salt mediated collagen extraction

Extraction of collagen from fish waste by using NaCl is carried out. But, it is rarely used due to its minimum yield (3.02%). The salt extracted collagen is referred as salt soluble collagen (SSC). Some researcher used 0.45 M sodium chloride for extraction of collagen from fish waste. Sodium chloride extracts free molecules that were not bounded by covalent interaction, thus it has least ability to solubilize collagen and produce smaller yield (Wang et al.2014).

1.2.8.2.5. Ultrasound mediated collagen extraction

Ultrasound is extensively employed method to enhance the shifting of mass by wet processes which are important for extraction and drying. Ultrasound generates excessive frequency sound waves (more than 16 kHz). Ultrasound mediated collagen extraction gives more yield of product in minimum processing period. Ultrasonic frequency of 20 kHz in presence of 0.5 M acetic acid exhibited larger yield in less extraction time. This wave does not alter the structural specificity of extracted collagen. Ultrasonic waves in combination with pepsin gives better yield than conventional

technique, reduces extraction time, greater efficiency and higher thermal stability without disturbing structural and functional quality of extracted collagen (Schmidt et al.2016).

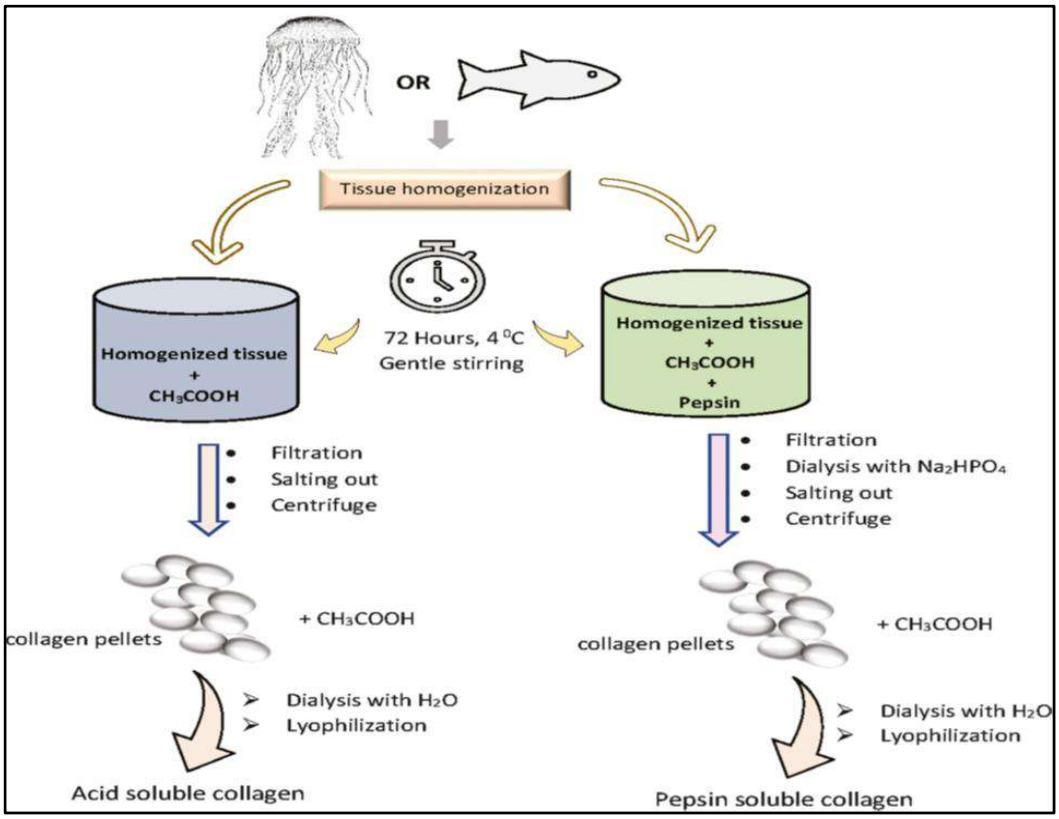

Figure 1.8.: Collagen extraction by acidic and enzymatic method (Felician et al.2018)

1.2.9. Applications of collagen

Collagen extracted from different marine sources has potential applications in biomedical and pharmaceutical industries, food additives and supplements, cosmetology and in healing materials.

1.2.9.1. Biomedical applications

The biodegradable, easily available, biocompatible and highly versatile biopolymer collagen has various applications in biomedical field such as tissue engineering, wound healing, ophthalmic surgery and delivery vehicle. This biomaterial used extensively to increase repair of bone, tendon, cartilage, skin, ligament and connective tissues. It has structural similarity with native collagen in human body so it also functions in cell support system. Collagen sponges were used for wound healing which contains keratinocyte and fibroblast. They are capable of proliferating cytokines and growth factors. Thus, provides skin regeneration and wound healing action in burn patients. Gel structure of collagen has special properties like flowability, injectability and

biocompatibility. Therefore, it effectively acts as delivery vehicle for drug, gene and proteins. Collagen fused with synthetic polymer and collagen based diffusion membranes were applied for controlled drug delivery and prolonged drug release therapy. Collagen-glycosaminoglycan fusion with insulin like growth factor-1 provides an efficient non-viral tool for gene delivery (eg. cartilage regeneration). Calcium phosphate coated (apatite) collagen acts as delivery vehicle for therapeutic proteins such as bone morphogenetic protein-2 (BMP-2) and carried out its safe release. Collagen-microsphere based delivery system was the safe way for delivery of protein based products (Muthukumar et al.2018).

1.2.9.2. Cosmetic potential

Collagen is major constituent of various cosmetic formulations because; it has potential benefits as a humectant and moisturizer. The type I collagen was effectively possess cosmetic potential, good activity to retain water therefore can be used as moisturizer. Collagen in its peptide form utilized as an anti-aging and anti-wrinkling product in cosmetic formulations for personal care. The analysis of molecular markers of collagen indicates no irritation and inflammation of human skin. Due to high molecular weight protein, it cannot binds to stratum corneum of the skin and carried out hydration of skin. Hence, it protects the wounded tissue from microbial infections by moisturization effect (Alves et al.2017).

1.2.9.3. Collagen supplements

Collagen is linked with different health benefits which lead to the development of collagen supplements industry. It has moisture absorption activity and its fractions have ample valuable nutritive fibres so it is used as protein source in human diets. Collagen synthesis will decrease as human gets older and tissues become thinner and weaker. Supplements of it are important to maintain collagen levels in skin, hair, nails and body tissues. Nutricosmetics are usually offered collagen in the form of liquids, pills or functional foods. Due to water absorption ability of collagen, it hydrates skin dermis and epidermis which helps to increase the smoothness and reduces wrinkling. Collagen and hydrolysed collagen contains natural creatine and arginine. These two components boost up muscle gain, decrease recovery time, reconstruct damaged joint structure and improve cardiovascular performances. Type II collagen is effectively used in rheumatoid arthritis. Collagen is effectively supplemented in sports nutrition (Hashim et al.2015).

1.2.9.4. Collagen as carrier

The lipids present in meat were oxidized due to various processes and leads to formation of other compounds which have unfavourable effect on quality and nutritive value of meat products and also decreases the shelf life of processed meat. Collagen is less allergic to humans and other animals, so it is potential carrier for biologically active substances like vitamins, drugs, minerals in medicine, pharmacology and nutraceutical. Collagen and its fractions are extracted from readily available, cheap, raw materials. Due to minimum production cost and different beneficial characteristics, it is a valuable agent in food industry including meat processing plants. Thus, collagen preparations are introduced into processed meat which improves its quality. Collagen fibres have better carrier capacity than collagen hydrolysates. Therefore, collagen fibres acts as carriers of rosemary extract in production of processed meat and its products which improve the antioxidant activity of meat (Waszkowiak and Dolata 2007).

1.2.9.5. Food applications

In current years, collagen has become key additive toward the evolution of healthy energy rich nutrients. Nutritive fibres were important for normal digestion and adsorption in the gastrointestinal tract. Improper diet as well as age is responsible for depletion in collagen production in body. Peoples refused to use collagen capsules, powder and injections to increase the amount of collagen. So, there is need to obtain collagen from nutritious food. Therefore, collagen integrates with numerous food and beverages to link up the required concentration of collagen in body. It is essential nutritive fibre which efficiently performs diverse biological functions. Some studies reported that, about 2-2.5% collagen decreases the time required for mincing and mixing components. Specially treated collagen may be employed for sausage casings. Due to cheap degree of alteration, ecological clarity and easy production method collagen fractions were mostly used for manufacturing of emulsion-type sausages in worldwide (Neklyudov 2002). Collagen or its fractions improves technological and rheological properties of many crude materials. So, it was approved that collagen fibre (heat treated) is native substitute to synthetic emulsifier and can be applied in acidic food products (Santana et al.2011). Collagen behaves as barrier that manages the relocation of oxygen, supplies permeability to water vapour as well as increase the shelf life of food. Thus, the collagen films or edible coatings can be utilized for wrapping, dipping, brushing or spraying the food (Rojo et al.2018).

1.2.9.6. Feed applications

For continuing different metabolic processes, animals need a stable nutrition that offers appropriate energy. The improvement in growth rate was detected in some birds supplemented with collagen in their regular diet. It has major role in broiler chicken feed which helps to increase the muscle growth and nutritional value. Poultry diet mainly consists of methionine and lysine as a source of energy and minerals. Collagen composed of methionine (6%) and lysine (19%) acts as a good protein supplement. Glycine in collagen has anti-inflammatory potential and provides protection against diverse diseases. Thus, due to presence of important amino acids, collagen provides valuable protein supplement for broiler chicken feed with minimum time period by increasing muscle content and health of poultry birds. The poultry feed along with collagen or collagen containing food enhances the growth rate, body weight and strength of muscle. Anti-inflammation and immunity improving capacity of glycine decreases the mortality. Antioxidant capability of collagen hamper the oxidative mechanisms of free radicals, thus remarkably decline the mortality rate. Collagen extracted from fish provides improved nutrient quality than commercial feed. Therefore, fish collagen can be supplied as substituent and productive feed in broiler chicken industry (Nurubhasha et al.2019).

1.2.9.7. Agricultural applications of collagen

The bio-composites derived from collagen and other proteins used in agricultural applications. It is used to enhance germination, nutrition, managed liberation of nitrogen to maintain the nitrogen balance and plant protection for different stages of plant development (Niculescu et al.2019). Collagen and its hydrolysates used in fertilization of horticultural crops and soil remediation. It is used to enhance the seed germination, plant growth stimulant, maintain nitrogen balance, minimize the use of insecticides and fungicides, increases fertilization rate and lowers the effect of iron deficiency caused due to calcareous soil. Collagen films have better elasticity, so that it can degrade easily and releases the tiny peptides. The water vapour permeability of film cause easy respiration of plants and seedlings, thus maintain the moisture content in soil (Niculescu et al.2017).

1.2.9.8. Hydrolysed collagen

Hydrolysed collagen (HC) or collagen peptides are tiny, 0.3-8 kD molecular weight structure synthesized from original collagen present in bones, skin and connective tissues of animals. Because of low molecular weight, HC has several benefits over native collagen like they are digested easily, absorbed easily and dispersed in the various tissues of human body. Different methods of HC extraction, effect on quality and molecular size.

The figure no.1.9 showed the structure of hydrolysed collagen. Hydrolysed collagen is rich in distinct amino acids like glycine, proline and hydroxyproline. During the assimilation of hydrolysed collagen, some proteases (e.g. pancreatic proteases; small intestinal brush-border proteases; peptidase) breakdown it into di and tri peptides or free amino acids (Sibilla et al.2015). The thermal analysis of collagen more than 40°C results in hydrolysis of collagen by proteolytic enzymes (alcalase, papain, pepsin, and others) and forms hydrolysed collagen. Solubility and functional properties (antioxidant, antimicrobial) of HC depends on the type and degree of hydrolysis and enzymes used for extraction. Acidic and alkaline methods are extremely corrosive and generate elevated salt concentration in the finishing product after neutralization. Thermal processing (100°C to 374°C) and pressure (22 MPa) treatment also used for extraction (Lopez et al.2019).

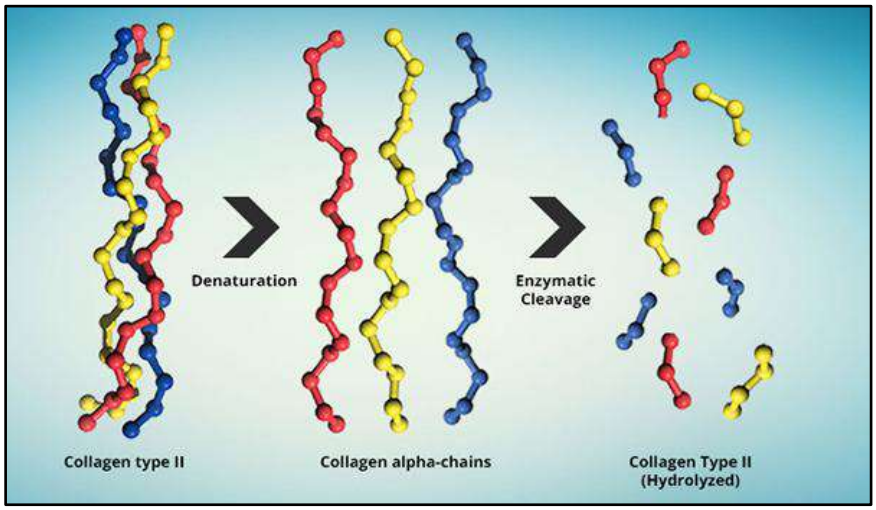

Figure 1.9.: Structure of hydrolyzed collagen

1.2.9.8.1. Applications of hydrolysed collagen

Hydrolysed collagen or collagen peptides maintain metabolic function of body by supplementing low molecular weight collagen which absorbed easily in the gastrointestinal track (GIT). Free amino acids and peptide fragments of HC speedily absorbed through the walls of intestine into blood stream. Collagen peptides were assist nutritional development of skin, hair and nail. Lysine and arginine also present in HC, lysine helps to maintain muscle growth and metabolism while arginine acts as precursor for the energy storage molecule creatine. Thus, hydrolysed collagen is beneficial for athletes and body builders for maintaining muscle growth, expansion of metabolism and supply the structural material for curing of injuries (cartilage, ligaments, tendons, bones

and discs). Some amino acids involved in the formation and repair of collagen of body which supplied by hydrolysed collagen. Due to presence of more amount of glycine, HC provides protection against the toxic substances like chemicals, pollutants, alcohol or tobacco.

HC also helpful in curation of mood related problems by increasing alertness, enriched energy, enhanced concentration, steady mood and improved sense of well-being. Collagen peptides include 18% nitrogen which is helpful to regulate positive nitrogen balance (Haltiwanger 2014). It was observed that, collagen peptides extracted from fish skin exhibit anticancer potential and provides inhibitory action against Angiotensin I converting enzyme. ACE inhibitory activity is initiated by aromatic acids present at C-terminal end. It gives rise to apoptosis of tumor cells by inducing caspases activity. Thus, hampers the cancer cell proliferation (Baehaki et al.2016).

1.2.10. Types of gelatin

The hydrolyzed form of collagen is gelatin synthesized from common sources of collagen such as cattle bones, pig skin and fish. The collagen derived from connective tissues of vertebrates is a major component of mammalian gelatin. It includes the type A gelatin from porcine and type B from bovine sources with molecular weight between 10 to 400 kDa. Fish waste and the waste derived from fish processing industries contains ample amount of collagen which is a major source for gelatin. In some countries, edible insect gelatin can be used as an alternative source of gelatin (Mariod and Adam 2013).

1.2.11. Gelatin extraction methods

1.2.11.1 High pressure processing (HPP)

It is a non-thermal preservation technique employed for inactivation of micro-organisms in food without changing its freshness and nutritional value. This is also designated as Ultra High Pressure (UHP) or High Hydrostatic Pressure (HHP) and considered as one of the best method for gelatin extraction. At certain pressure up to 500 Mpa, it does not cause significant change in collagen structure and extend the protein structure. Because of high pressure it brings protein denaturation and alters the balance of non-covalent bond association which stabilize the native arrangement of numerous proteins. Thus, results in easy extraction of gelatin from fish waste (Jaswir et al.2017).

1.2.11.2 Alkaline extraction method

Gelatin can be extracted from skin and fin of sliver carp fish using alkali such as calcium hydroxide and is common method for extraction from fatty fishes. Raw sample was soaked in solution for 4 weeks. Longer pre-treatment or high concentration of alkali

may reduce the quality of gelatin. The pre-treated gelatin solution was filtered and mixed with 5% (w/w) chloridric acid. The extracted gelatin solution concentrated in rotary vacuum evaporator and dehydrated at 50-60°C for the formation of dry and thin layers of gelatin (Tavakolipour 2011).

1.2.11.3 Acidic extraction method

Acidic method has more yield and good quality of gelatin than alkaline method. Skin of some marine fishes was soaked in 0.1 M sodium hydroxide to remove non-collagenous proteins (Hue et al.2017). Various organic acids like acetic, citric, lactic, malic or tartaric of 0.05 M were added in 1:3 (w/v) ratio. All the extraction processes were carried out under constant stirring for 4 hours (Khiari et al.2011). The extracted gelatin was dehydrated and dried.

1.2.11.4 Enzymatic extraction

Food and pharmaceutical industries has wide applications of microbial protease. Among these alkaline protease produced by *Bacillus licheniformis* was mostly studied. Chemical method has some negative effects on quality and purity of gelatin. Thus, this microbial protease is an effective agent for gelatin extraction from marine waste (Kouhdasht et al.2018).

1.2.12. Applications of gelatin

1.2.12.1. Biodegradable food packaging film

Biodegradable food packaging films has increasing demand than traditional, commonly used packaging polymers due to its large source, sustainability, eco-friendly nature, effective applications and more compatible to various foodstuffs. Gelatin is one of the interesting special materials produced by partial hydrolysis of collagen and increasing demand in various food industries for packaging and coating purpose. Among the distinct biopolymers having film forming ability, it is the best suitable material with advisable film forming properties. Gelatin films were effectively resisting the solvents with significant extent of flexibility and antioxidant activity. The gelatin biopolymer is hygroscopic in nature, thus it has huge moisture contents which expand or dissolve when exposed to the food surface. The different polymers in combination with gelatin were used to synthesize composite films for food packaging. Gelatin films were used to increase shelf life of food and agricultural products. Thus, gelatin play a crucial role in packaging and considered as excellent packaging agent having efficient oxygen barrier capacity which required for packaging resolution than other biopolymers (Hanani et al.2014).

1.2.12.2. Health benefits of gelatin and gelatin-derived peptides

Gelatin and its derived peptides have substantial biological advantages, like antioxidant, antihypertensive, anticancer, antiphotoaging and cholesterol lowering capacity. Due to presence of specific linkages of amino acids, several gelatin peptides derived from fish skin exhibit the ability to scavenge free radicals and reactive oxygen species and also chelate the metal ions. The peptides like His-Gly-Pro-Leu-Gly-Pro-Leu, Pro-Ala-Gly-Tyr and peptides containing hydrophobic amino acids (Gly, Pro, Ala, Val, Leu) at N-terminus and amino acids like Tyr, Met, Ile, Glu, Trp at C-terminus correlated with the high radical scavenging activity.

The gelatin hydrolysates acquired from Atlantic salmon skin using flavourzyme contains peptide Gly-Pro-Ala-Glu and Gly-Pro-Gly-Ala. It inhibits the activity of dipeptidyl-peptidase IV and therefore, gelatin derived peptide can be effectively employed in functional foods and pharmaceutical products against type 2 diabetes. The enzymes including protamex, trypsin, neutrase, savinase and alcalase were implemented for the extraction of gelatin hydrolysates from squid revealed greater cytotoxic effect against cancer cell lines MCF-7 (human breast carcinoma) and U87 (glioma); especially alcalase extracted gelatin were employed for this reason. The free radical scavenging and metal chelating ability of gelatin hydrolysates derived from blacktip shark skin using papaya latex crude enzymes minimizes the oxidation of human low density lipoprotein (LDL) cholesterol by 8.3–39.2%. The peptide Leu-Ser-Gly-Tyr-Gly-Pro from tilapia skin gelatin has potential to protect antioxidative system of mice, thus causes less damage to lipids and collagen (Liu et al.2015).

1.2.12.3. Feed application

Fish derived gelatin acts as protein source and natural stabilizer in diets for the several fish species instead of commercial binders like starch, agar, molasses and wheat meal. Gelatin extracted from dried fish skin has greater amount of crude protein. Regular fish feed in addition of 2% gelatin improves the quality, chemical and physical properties of feed also influence positively on growth parameters of fish. It helps to increase protein amount, flexibility, durability, stability and functional properties of fish feed also decrease the water solubility of normal diet. Thus, fish derived gelatin can be applied in normal fish feed to improve its characteristics (Dubakel et al.2015).

1.2.12.4. Gelatin in plant growth promotion

Greenhouse studies reported that, animal derived gelatin can be acts as bio-stimulant during seed treatment, thus it helps to improve plant growth and performance.

Gelatin capsule treatment significantly increases the crop growth, improves nitrogen uptake, expansion of total leaf area and dry weight. It also helps to induce expression of amino acid and nitrogen transporter genes that may be responsible for root nitrogen uptake improvement (Wilson et al.2018).

1.2.13. Types of omega-3 fatty acids

Omega-3 fatty acids are generally considered as long chain polyunsaturated fatty acids (PUFA). They have more than one carbon-carbon double bond. The first double bond is between third and fourth carbon from tail end so considered as omega-3-polyunsaturated fatty acids. The main types are alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). ALA is found is vegetables source like flaxseeds, canola (rapeseed) oil, soybeans, pumpkin seeds, walnuts and their derivative oils while EPA and DHA is mainly found in marine animals like fatty fishes includes sardine, salmon, mackerel, tuna (Gammone et al.2018). Also, some amount of EPA and DHA are present in waste material of marine animals such as skin, scales, head and tail. EPA is 20 carbons long and DHA is 22 carbons long. The daily requirement of both fatty acids ranges between 250-500 mg per day. These two fatty acids perform various important roles in normal functioning of human body.

1.2.14. Omega-3 fatty acid extraction methods

1.2.14.1. Supercritical fluid extraction method

In last few years, supercritical fluid extraction (SFE) has been used extensively to extract fish oil from fish by-products. It uses medium temperature and oxygen free media which reduces the oxidation of omega-3 fatty acid during extraction. SFE avoids the co-extraction of other polar lipids with inorganic impurities and only allows extraction of lower polar lipids. Because of carbon dioxide, it can be used as alternative method for conventional physical and chemical extraction method. Maximum extraction by this method requires moisture content less than 20% and raw material must be finally cut. The major limitation of this method is high production cost, use of high pressure equipment and the freeze dried machine which maintain moisture content below 20% (Rodriguez et al.2012).

1.2.14.2. Microwave assisted technique

This technique requires minimum time for solvent extraction (soxhlet) and therefore considered as efficient method. Microwave power of 100 W and extraction time of 10 minute using hexane as solvent effectively extracts omega-3 fatty acids. This

technique extracts good quality of oil but may effect on colour and odour of it. Due to high heat generation, it may leads to destruction of omega-3 fatty acids (Li et al. 2018).

1.2.14.3. Enzymatic hydrolysis technique

Among the various methods, enzymatic hydrolysis is suitable method of extraction which includes enzyme and water. Generally, protease, lipase and alcalase are used for extraction. Different temperature range, enzyme concentration and incubation time are important parameters in this technique. Enzymes catalyse hydrolysis reaction which produces more amount of omega-3 fatty acid than any other method (Iberahim et al.2018).

1.2.14.4. Wet rendering process

Wet rendering has been widely used extraction method having some advantages over other. By excluding harmful chemicals, less extraction cost, does not disturb the environmental balance and maintain the natural state of fish waste components. This process involves lower temperature for shorter time with centrifugation and higher temperature for longer time without centrifugation. It uses water as a solvent instead of harsh chemicals which results in cost effective, non-toxic and easily available. Thus, this technique is the green method for extraction of omega-3 fatty acids from fish waste material (Rahman et al.2018).

1.2.15. Applications of omega-3 fatty acids

1.2.15.1. Anti-inflammatory role

Omega-3 polyunsaturated fatty acids (PUFA) mainly EPA and DHA inhibit the synthesis of pro-inflammatory eicosanoids. By using different substrates, it uses same cyclooxygenase (COX) and increase the production of anti-inflammatory eicosanoids without blocking the COX. Due to slow action of omega-3-PUFA, takes more time for effect. It is the best alternative anti-inflammatory agent for long term chronic inflammatory stage. EPA and DHA minimize the pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6. It also initiates the cascade of adhesion molecules followed by injury. This causes increasing movement of immune cells, such as neutrophils, monocytes, B cells and T cells. In both chronic and acute inflammation omega-3 PUFA carried out inflammatory response by inhibiting the inflammatory hallmarks. It also inhibit section of lipopolysaccharides (LPS) in cardiac disease by limiting the nuclear factor kβ (NF-kβ) and toll-like receptor 4 (TLR-4) inflammatory pathways. Thus, omega-3 PUFA can be effective agent in cardiac inflammatory response (Ye and Ghosh 2018).

1.2.15.2. Omega-3 fatty acids and fetal development

Proper supplementation of EPA and DHA during pregnancy provides various benefits for infant. DHA with other nutrients were transferred through placenta from mother to foetus. The long chain omega-3 fatty acids, EPA and DHA play major role in fetal brain and retina development, cell membrane functions, eye and hand co-ordination. It also prevents the premature birth of baby by increasing gestation period. It is caused due to decreasing production of prostaglandin E2 and prostaglandin F2a thus; reduce the inflammation of uterus. It was also suggested that, EPA and DHA supplementation may protect children against allergies (Swanson et al.2012).

1.2.15.3. Anticancer effect

The two main omega-3 polyunsaturated fatty acids that are eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA) were studied extensively for its anticancer potential. Combination of these two fatty acids showed their anticancer activity by effecting multiple targets in distinct stages of cancer development consist of cell proliferation, cell survival, angiogenesis, inflammation, metastasis and epigenetic abnormalities that are important for development and progression of cancer. Daily uptake of 250-500 mg of omega-3 fatty acids decreases the risk of common cancers including skin, colorectal, prostate and breast. EPA and DHA have varying effects on cancer cells indicating their importance in daily routine. DHA initiates the cell death by suppressing cell proliferation while EPA reduces the loss of adipose tissue and protect skeletal muscle. The EPA and DHA efficiently prevent the cancer initiation and development, inhibit cell cycle, increases cell death, reduce inflammation, angiogenesis and metastasis by both epigenetic and genetic changes. Thus, provides supportive agent in anticancer treatment (Jing et al.2013).

1.2.15.4. Neuroprotective agent

There are some evidences that omega-3 PUFA have therapeutic potential in neurology and psychiatry. Omega-3 PUFA provides effective protection in spinal cord injury (SCI) and traumatic brain injury (TBI). Docosahexaenoic acid (DHA) is efficiently acts as a neuroprotective agent against injury. It provides protection to central nervous system (CNS) from damage by kainic acid. It reduces neuronal cell death, prevents cell loss, decrease oxidative stress by mild TBI, reduces trauma induces in cellular homeostasis and inflammatory process. Thus, improve the neurological function. In neurotrauma, it was observed that combination of proper amount of omega-3 PUFA immediately after injury provides a significant therapeutic potential because it helps in various mechanisms of body and provides effective benefits (Titus 2017).

1.2.15.5. Feed applications

Omega-3 polyunsaturated fatty acids is the vital nutritional component may utilized in poultry feed lead to better production, enhanced quality of chicken and poultry products which is also beneficial for human consumption. Poultry feed supplemented with omega-3 fatty acids has remarkable role in lowering the cholesterol and lipid content in blood and egg yolk. The increased content of fatty acids in poultry feed showed positive effect on chicken development. It helps in nervous system development, increase immunity, good quality of meat and eggs also maintain the normal functioning of chicken body. Thus, fatty acids will ultimately enter into human body which provides protection against cardiovascular diseases, having anti-tumor and anti-inflammatory effects. Direct incorporation of omega-3 PUFA in poultry feed increases the amount of important fatty acids in broiler chicken meat. Utilization of fatty acids leads to neutralization of oxidants and causes increase in antioxidant level which minimizes effect of oxidative stress. Thus, use of omega-3 fatty acids in poultry feed supplements increases the nutraceutical value of poultry products and also provides health benefits (Alagawany et al.2019).

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CHAPTER II

MATERIALS AND METHOD

2.1 Chemicals

All chemicals used in this study were highly purified, analytical grade and purchased from local suppliers. Bovine achilles tendon collagen, type I calf skin collagen, p- dimethylaminobenzaldehyde, Bovine serum albumin, standard docosahexanoic acid (DHA), coomassie brilliant blue R-250 gallic acid, quercetin and DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) were purchased from Sigma-Aldrich, USA. Acrylamide, bisacrylamide, agarose, ammonium persulphate, Bio-Rad precision plus protein standard (10-250 kD), leucine, hydroxyproline, gelatin, NaCl (sodium chloride), NaOH (sodium hydroxide), $CuSO_4$ (copper sulphate), Na_2CO_3 (sodium carbonate), $FeCl_3$ (ferric chloride), folin ciocalteu reagent, EDTA (ethelenediamine tetra-acetic acid), TEMED (N,N,N,N'- tetra-methyl ethylenediamide), IMVIC test reagents, ascorbic acid, chloroform, methanol, butanol, acetic acid, lactic acid, formic acid, phosphoric acid, oxalic acid, sodium dodecyl sulphate and all other chemicals required for current study were brought from Hi-media and Sisco Research Laboratories (SRL) chemicals, India. The ingredients used for value added food products preparation were of food grade.

2.2. Optimization of extraction method

The yield of interested end product is influenced by the different extraction conditions. Acidic extraction is commonly employed method for collagen and gelatin recovery from fish waste. Type of solvent, its concentration, time of extraction and temperature as well as recovery conditions has effects on final yield of product. Hence, optimization of these conditions is necessary for maximum recovery. The one factor at a time method is employed for extraction of acid soluble collagen, gelatin and omega-3 fatty acid from fish waste.

2.2.1. One factor at a time method

Many scientists and engineers make use of one factor at a time (OFAT) method for designing experiments, in which only single factor is varied at a time while other factors remains unchanged and response is recorded. This technique requires more experiments to obtain results hence its very lengthy and time consuming. The changes of one constituent at a time in increasing way only describe about their influence on the production (Czitrom 1999).

2.3. Extraction methods

2.3.1. Acid soluble collagen extraction

The ideal procedure for extraction of collagen from marine sources is acid solubilisation. In this protocol weak acids are effectively utilized for collagen recovery. Acetic acid is referred as most significant solvent for extraction with 0.5 M concentration and it was called as acid soluble collagen (ASC). It was proposed that acidic environment resembles positive charge primarily on collagen molecules. Solubilisation of collagen in the extracting acidic solution mainly depends upon the concentration of acid utilized and its swelling properties (Kiew and Don 2013). Thus, the higher repulsive forces between precursor tropocollagen lead to better solubilisation of collagen in extracting solvent.

2.3.2. Acid mediated gelatin extraction

Gelatin is by-product of collagen formed after its partial hydrolysis by heat treatment. Gelatin derived from acid treated precursor referred as type A while type B gelatin was derived from alkali treated precursor. Acid pre-treatment enable to remove impurities from waste so 0.1 M sodium hydroxide (alkali) effectively utilized to remove non-collagenous protein impurities. After this, sample was subjected to acid extraction followed by final extraction with water. The various organic acids were employed for acid mediated gelatin extraction from waste. Concentration of acid and time of heating influences on gelatin yield. Heat treatment during extraction causes disruption of hydrogen bonds in collagen molecule and results in irreversible solubilisation of three dimensional structure of collagen to form gelatin (Kim et al.2020).

2.3.3. Solvent extraction of omega-3 fatty acid

Among all commercial techniques, solvent extraction followed by enzymatic concentration is the only method carried out on laboratory scale. Interaction among the solvents and hydrophobic or hydrophilic regions of molecule causes pure lipids soluble in multiple solvents. The main principle of solvent extraction includes addition of solvent to the reaction mixture which helps to remove one or more components from it. Chloroform: methanol is preferred as binary solvent system, generally employed for efficient extraction of fish oil from waste. Chloroform-methanol may be the best lipid extractant and gives more yield than other solvents, but it is absolutely not safest from health and environmental aspects, thus the research is carried out to find out best replacement for it (Mohanarangan 2012).

2.4. Purification techniques

After the production of sufficient amount of product, next important step is its purification. Steps involved in purification of collagen, gelatin and omega-3 fatty acid can be summarized as below.

2.4.1. Precipitation

The basic step used for protein purification and concentration is precipitation by using salts. It is carried out by changing solvent conditions in order to alter the solubility of interested protein than other proteins in reaction sample. Generally, ammonium sulphate is mostly preferred salt for precipitation and it's more advantageous than any other precipitants because of its good ability to stabilize protein structure, highly soluble, comparatively inexpensive, easy availability of pure material and lesser density of a saturated suspension than any other salting out components (Burgess 2009).

Increase in salt concentration results in saturation of solution which causes decrease in solubility of protein and it get precipitated in the reaction solution called as "salting out". Increase in salt concentration at low ionic strength results in increasing solubility of proteins referred as "salting in". Some examples of precipitants other than ammonium sulphate include NaCl, TCA, ethanol and acetone etc. After precipitation it was subjected to dialysis to remove unwanted substances that may be interfere with the further experimental procedures.

2.4.2. Centrifugation

Centrifugation is a mechanical process that utilizes an applied centrifugal force to separate the components of a mixture according to density and/or particle size. Random Brownian motion results in net movement of solute or suspended particles from regions of higher concentration to regions of lower concentration, a process called diffusion. Thus, diffusion works in opposition to centrifugal sedimentation, which tends to concentrate particles (Zheng et al.2013). The centrifugal force is proportional to the rotation rate of rotor. The centrifugal force (speed of centrifugation) and time has effects on separation. The more centrifugal force causes effective separation between small and large proteins but may cause structural changes in protein structure.

2.4.3. Dialysis

Process of dialysis separates molecules from solution based on their diffusion rate through a semi permeable membrane. Figure no.2.1 depicted the process of dialysis. Technique efficiently eliminates unwanted particles and prevents their possible interference in further purification process. Unwanted particles and small molecules

diffuse through semi-permeable membrane i.e. dialysis bag, into the adjoining medium (buffer or water) leaving behind the protein into dialysis bag. This technique is also useful for concentrating solution.

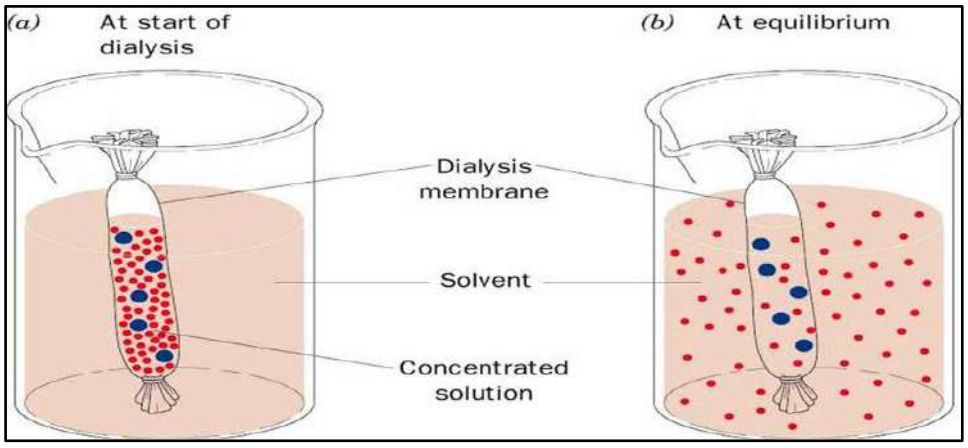

Figure 2.1.: Process of dialysis (Source: Wikipedia)

2.4.4. Filtration

In filtration process solid particles in a liquid or gaseous fluid are removed by use of a different filter medium (Whatmann's filter paper) which permits fluid to pass from it and retains solid particles and separation of mixture achieved. These filter papers useful for qualitative or quantitative analysis of biomolecules or fine separation mixtures. It has different paper grades o which it can be used for various applications. Whatmann filter paper composed of cellulose fibres of high quality cotton containing more alpha cellulose which gives high wet strength and not allows any impurities to enter into the filtrate or sample. Hence, filtration is a basic purification technique for any biological samples or mixtures.

2.5. Characterization techniques

Various characterization techniques were employed for efficient characterization of macromolecules extracted from fish waste. The biochemical and biophysical analysis determines structural and functional properties of respected molecules.

2.5.1. Ultra violet-visible spectroscopy (UV-Vis)

UV-vis spectrophotometer is a scientific tool useful in structural elucidation of bio-organic molecules. It is a powerful technique for superior characterization and quantification used in scientific research. This technique has advanced properties like accuracy, efficacy, quick analysis (speed), easy handling and cost effective. UV-vis

spectroscopy is an absorption spectroscopy involved ultraviolet and visible spectral region employed for quantitative and qualitative investigation (determination) of different metal ions, organic constituents and biological macromolecules. The principle behind it is, Beer-Lambert law based on relation between concentration of sample concentration and intensity of light. This law stated that absorptive capacity of a dissolved substance is directly proportional to its concentration in a solution. Whereas, Lambert law states that path length and concentration of sample is directly proportional to the absorbance of the light.

At fixed path length, concentration of sample in solvent can be determined by measuring absorbance of the respective solvent. Absorbance was measured when electrons in solution get excited at lower transition to upper transition state. Molar extinction coefficients or calibration curve can be resolved for quantification. Therefore, analyte concentration of solution is calculated at fixed path length by computing its absorption and also implemented for qualitative as well as quantitative determination of protein and nucleic acid at absorption wavelength 280 nm and 260 nm respectively. The mechanism of UV-visible spectroscopy was showed in figure no.2.2.

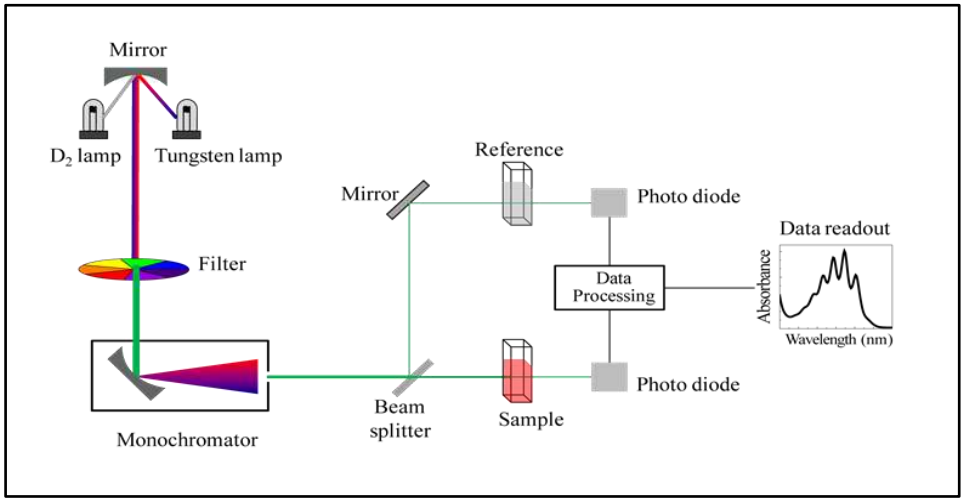

Figure 2.2.: Mechanism of UV-visible spectroscopy (Source: Wikipedia commons)

2.5.2. SDS-PAGE electrophoresis

Gel electrophoresis is most frequently executed for electrophoretic separation of biological molecules. Technique gives good results with better resolution and is comparatively easy to carry out than any other separation procedures. Thus, it is generally employed for qualitative as well as quantitative determination of the protein

molecules (Garfin 2003). An analytical approach of SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) is normally applied for molecular weight determination based on electric mobility. Mainly in this technique influence of properties of biomolecules are important like nature (amphipathic), charge of molecule and pH of medium. Figure no.2.3. illustrated the process of SDS-PAGE gel electrophoresis. By this qualities and properties it acts as an effective reducing agent employed to degrade native polypeptide into unfolded structure and gives negative charge to protein. Polymerization of acrylamide-bis-acrylamide forms gel like network for appropriate separation of proteins of precise size. Consequently, due to electric mobility charged protein fragments migrate from one site to another and separation was achieved. Separated proteins in gels are examined by staining with anionic dye CBB R-250 or by silver nitrate followed by destaining to visualize protein bands (Meyer et al.1965; Garfin 2003).

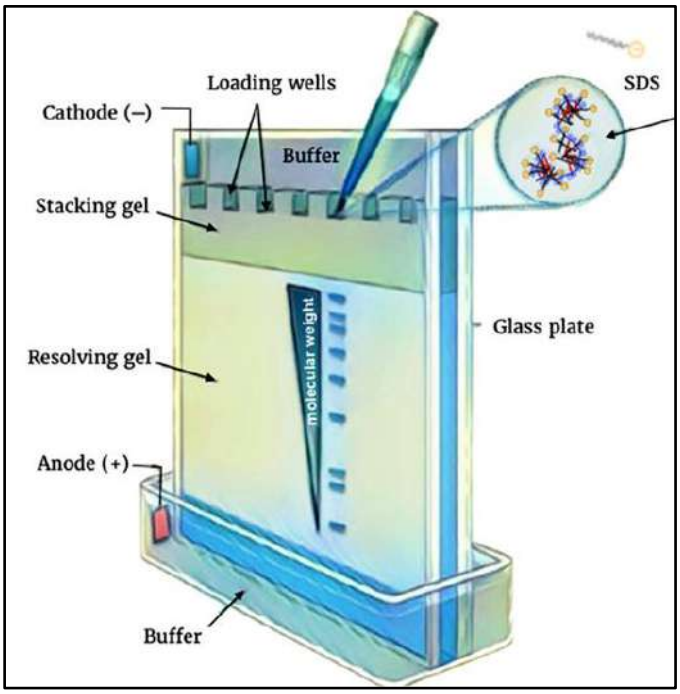

Figure 2.3.: Process of SDS-PAGE gel electrophoresis (Source: Researchgate)

2.5.3. Hydroxyproline content determination

Hydroxyproline evaluation is easy, colorimetric process employed to determine concentration of collagen as well as its partially hydrolysed product gelatin. An oxidizing agent p- dimethylaminobenzaldehyde brings out oxidation of hydroxyproline which results in formation of yellow colored end product. HyP is a prime imino acid helps in equilibration of triple helical structure of collagen. It also plays vital role in maintenance

of thermal stability of protein by establishing hydrogen bonds between polypeptides present in protein. Higher degree of denaturation temperature of collagen and gelatin is related to the more amount of hydroxyproline in it (Rosenbloom et al.1973). HyP is the main component of collagen and it is present in very less concentration or totally absent in any other proteins, thus HyP determination is used to calculated collagen from waste material.

2.5.4. Fourier transform infrared spectroscopy (FTIR)

Fourier transform infra-red spectroscopy is the analytical method applied for the qualitative and quantitative exploration of various composites using infrared absorption of molecules and also gives details about secondary arrangement of proteins (Pelton and McLean 2000). Mechanism of FTIR spectroscopy was given in figure no.2.4. Due to the slow speed of scanning, conventional IR instrument is not suitable for fast data processing thus, FTIR spectroscopy is invented. Basically, FTIR is mostly related to the vibrations and stretching of molecules which have distinctive vibrational frequencies in the IR range and therefore, it is suitable for identification of entire organic molecular assemblies and constituents. The FTIR frequency range is between 4000-400 cm^{-1}. Infrared radiations were absorbed by the bonds existing in the atoms in the molecule and its bending as well as stretching is occurred to generate FTIR spectrum.

Infrared radiations fall on sample and part of it was absorbed while remaining part of radiations is transmitted through the sample. As a result of this, molecular fingerprint of analyte was generated by combination of absorption and transmission peaks. Upon bombardment with the IR radiations, molecules get excited and shifted to the higher energy level from lower energy level. During this transition energy transfer is occurred with the help of molecular bond vibrations, rotations, electron ring shifts and translations. Each specific component has its characteristic vibrational frequency in the infrared region. The FTIR is a quick method utilized for structural analysis of that give complete information about collagen at its molecular level like nature of functional groups, forms of connection and conformation between bonds.

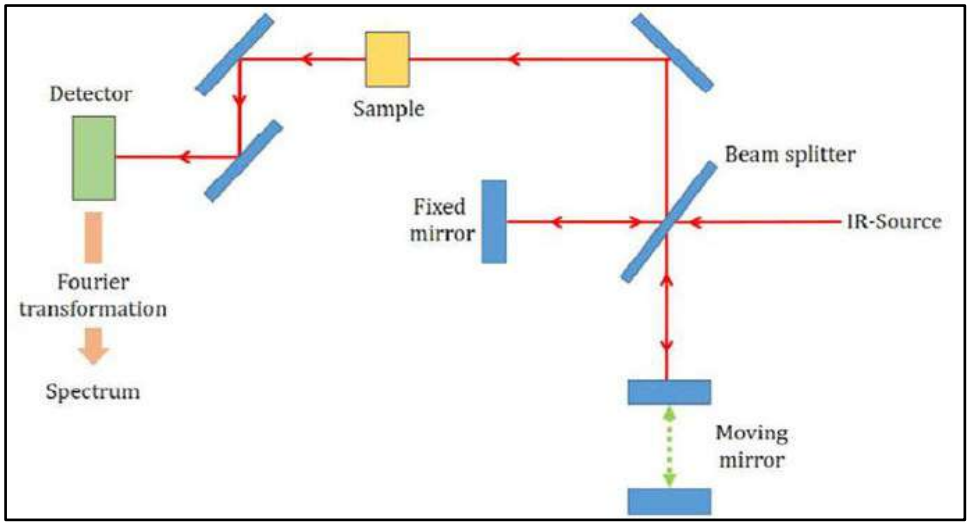

Figure 2.4.: Mechanism of fourier transform infrared spectroscopy (Source: Researchgate)

2.5.5. X-ray diffraction technique (XRD)

X-ray diffraction (XRD) is more convenient analytical technique to acquire information about arrangement of crystalline substances like crystal size and lattice spacing. The diffraction pattern of compounds is used for its identification and characterization. In XRD, monochromatic radiation (X-ray) was fall on atom and photons in it was scattered in every lattice phase. The constructive interference is created by scattered rays that construct peaks for the particular sample. The mechanism of XRD was given in figure no.2.5. XRD is used to analyse diffraction patterns of different biological components, thin films or powder, solid etc. Collagen contains more amount of imino acid and repeating (Gly-X-Y) motif in sequence and therefore it displays triple-helical conformation with semi crystalline organization as well as also interpret distribution and alignment of collagen fibril (Bigi et al.2001).

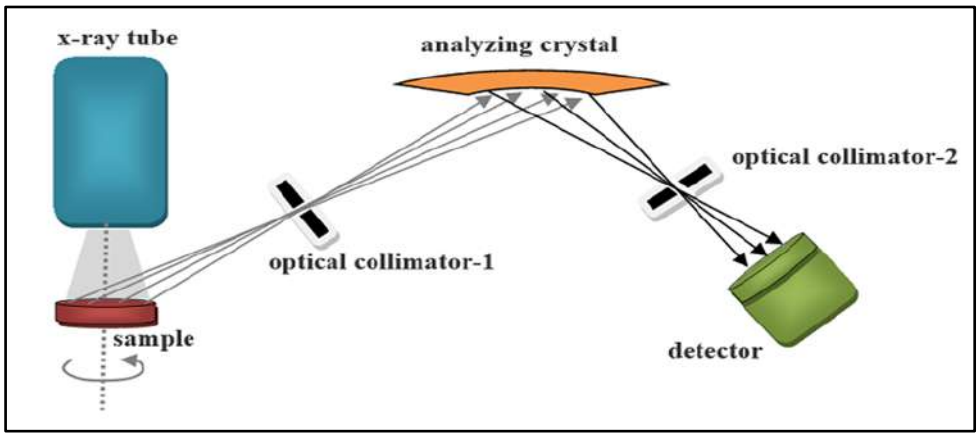

Figure 2.5.: Mechanism of X-ray diffraction (Source: Nuclear ecology)

2.5.6. Scanning electron microscopy (SEM)

Scanning and transmission electron microscopy (SEM, TEM) is the modern analytical techniques of microscopy. SEM studies surface of various materials, chemicals as well as biological samples to compute and assesse fine particulars of it through image analysis. The narrow electron beam produces images of better resolution and magnification with more depth of field thus, helps to evaluate surface topography of the analyte. In SEM, electron beam is focused on the sample surface and upon interaction it will generate different signals associated with surface topography and composition of sample. For SEM characterization of biological material, it should be totally dry and secured with chemical fixative for firmness and structural integrity. After that, the material was covered with gold on stab and placed on sample analyser stage of electron microscope to generate good resolution image of it. Figure no.2.6 represented the diagram of SEM. Thus, this microscopy analyse orientation of collagen fibrils and provides information about its structural properties (Raspanti et al.1996; Starborg et al. 2013).

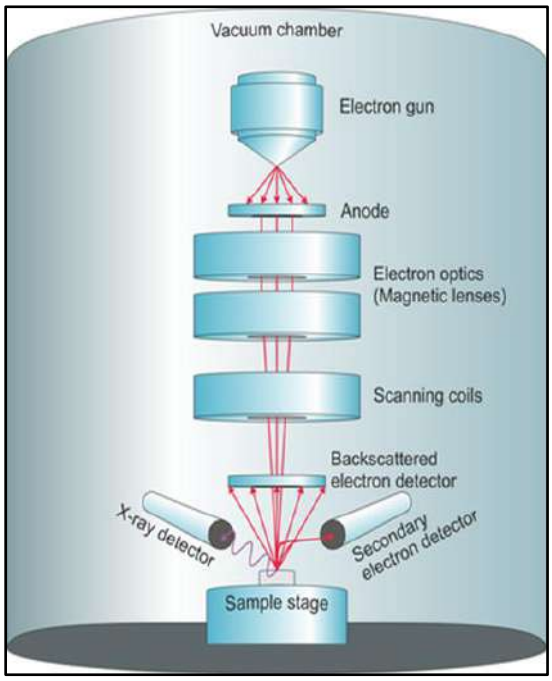

Figure 2.6.: Diagram of scanning electron microscopy

(Source: Applied micro spectroscopy)

2.5.7. Differential scanning calorimetry (DSC)

DSC is an important thermo analytical method employed for characterization of the temperature dependent conformational changes in structure of proteins as well as other biomolecules. The capacity of a material to store energy is referred as heat capacity

(Cp) which is determined by DSC as function of the temperature. Block diagram of DSC was shown in figure no.2.7. Basically, it evaluates the heat capacity difference between two cells under the continuous and concurrent heating of two cells (Ibarra-Molero and Sanchez-Ruiz 2006). Several industries like printing, polymers, pharmaceuticals, paper, semiconductors, food, manufacturing and electronics broadly utilizes DSC. This technique helps to measure the different parameters comprising induction period of oxidation, reaction energy and temperature, heat of melting and fusion, glass and crystalline region conversion temperature, specific heat and energy and also determines melting as well as denaturation temperatures.

The stable and continuous increase in the temperature, results in start of unfolding of protein while the alteration in heat flow raises significantly and attains an extreme point (Tm) and after complete unfolding it will start to decline, generating a peak in the heat flow *vs.* temperature graph. The purified protein exhibited only single peak which describes the heat absorption related to the denaturation of the protein (Ibarra-Molero and Sanchez-Ruiz 2006). Hydroxyproline content present in collagen helps in the thermostability of collagen which can be assessed by differential scanning calorimetry (Komsa-Penkova et al.1996). This method records the change of calorimetric energy due to heating procedure that effects on collagen denaturation. The continuous heat results in unfolding of protein by absorbing heat and DSC was recorded (Bruylants et al.2005).

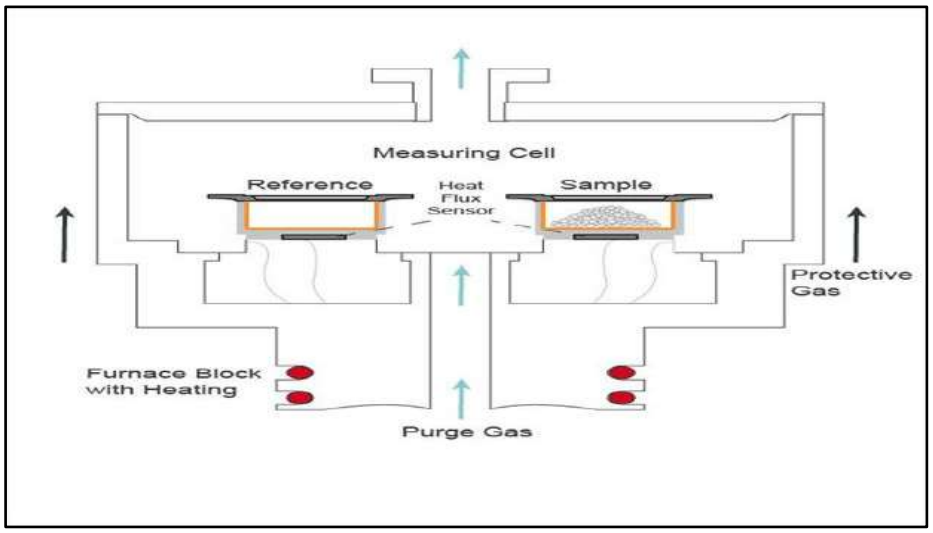

Figure 2.7.: Block diagram of DSC (Source: Particle technology labs)

2.5.8. Particle size analysis

Particle size analyser with zeta potential is used to measure particle and molecular size from less than a nanometre to several microns using dynamic light scattering and

zeta potential by using electrophoretic light scattering. Particle diameter has effect on zeta potential. Because particles of small diameter are easily affected by the random movement of fluid flow and other particles, then the absolute value of effective zeta potential of small particles is greater than that of large particles. Particle size analysis is employed to analyse the size distribution of particles in a given sample. It can be applied to study particle size of solid materials, suspensions and emulsions. The most common techniques to determine particle size distribution are dynamic image analysis (DIA), static laser light scattering (SLS, also called laser diffraction), sieve analysis and dynamic light scattering (DLS). The particle size analyser instrument is based on different technologies such as high definition image processing, analysis of Brownian motion, gravitational settling and light scattering (Rayleigh and Mie scattering) of the particles. In biological field, it is used to measure size of protein aggregation.

2.5.9. Zeta potential

Electrostatic repulsion helps in the stabilization of maximum aqueous colloidal structures. Interaction between the charged colloidal particles causes interactive repulsion at prolonged distances. Some colloidal particles exhibited particular ionisable assemblies that are covalently attached to their surfaces. The pH of the particles varies according to the pH of the surrounding medium, thus it may have positive, negative or net zero charge. The pH of the medium has intense effect on the net charge of the suspended particles and also provides stabilization during its aggregation. Zeta potential is to evaluate total charge of the colloidal particles. Zeta potential is the potential difference existing between the surface of a solid particle immersed in a conducting liquid (e.g.: water) and the bulk of the liquid. Figure no.2.8 depicted the mechanism of zeta potential.

Micro electrophoresis method is utilized to define the zeta potential of colloidal suspensions. The voltage is applied through a couple of electrodes at both end of the cell comprising the particle diffusion; velocity of the charged particles is recorded and determined as unit field strength as their mobility. Particles having high positive or negative zeta potential repelled from each other and causes very less flocculation of particles in suspension. The particles with less zeta potential unable to inhibit the accumulation of particles. Particles having zeta potential value between -10 mV and +10 mV may acts as neutral while particles having value much negative than -30 mV or more positive than +30 mV are generally behaves as steady particles (Clogston and Patri 2011).

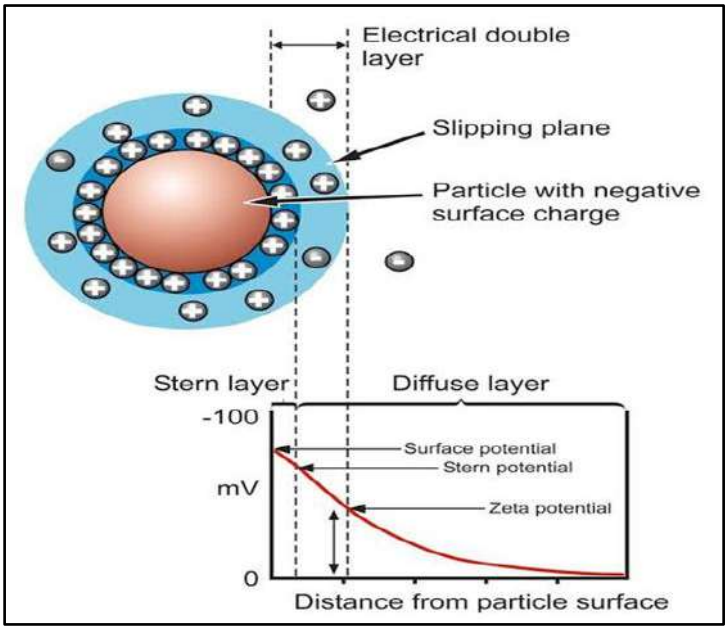

Figure 2.8.: Mechanism of zeta potential

(Source: Encyclopaedia of membranes)

2.5.10. Gas chromatography mass spectroscopy analysis (GCMS)

Chromatography is commonly utilized practise for the evaluation of saturated, mono and polyunsaturated fatty acids in foods as well as biological samples. It is beneficial technique because of rapid separation of mixtures comprising several types of acids, along with more sensitivity given by various types of detectors, such as mass spectrometers (Brotas et al.2020). GC-MS is an instrument that associates the features of gas-chromatography and mass spectrometry to detect different organic compounds presents in the organic material, which contains alkanes, fatty acids, alkenones, sterols etc. The Gas Chromatography/Mass Spectrometry (GC/MS) distinguishes chemical mixtures (the GC component) and recognizes the constituents at molecular level (the MS component). GC can separate various volatile and semi-volatile compounds but not always specifically distinguish them whereas MS can selectively distinguish many compounds but not always separate them. It has very low detection limit that is about sub-ng (nanogram) is measured.

Currently, in GC-MS ion source is directly inserted into capillary column. At 300ºC the effluent comes out from the GC (temperature may goes up to 400ºC). When effluent (single component) release from the GC, it gets enter into electron ionization detector which is commonly used MS detector. Ions can be generated by ion trap or time-of flight. Upon production, these ions are bombarded by a stream of electrons results into its fragments. The mass to charge (M/Z) ratio is calculated as mass of the fragment

divided by the charge. The charge is always +1 while M/Z ratio signifies the molecular weight of the fragment. The quadrupoles of the computer scan (continuous cycle) these fragments one at a time until the range of M/Z is retrieved. This will create the mass spectrum which is a graph of signal intensity (comparative abundance) versus M/Z ratios (particularly molecular weight). Each component has a specific fingerprint and software is easily available to offer a library of spectra for unknown components (Sneddon et al.2007). Figure no.2.9 showed the block diagram of GCMS.

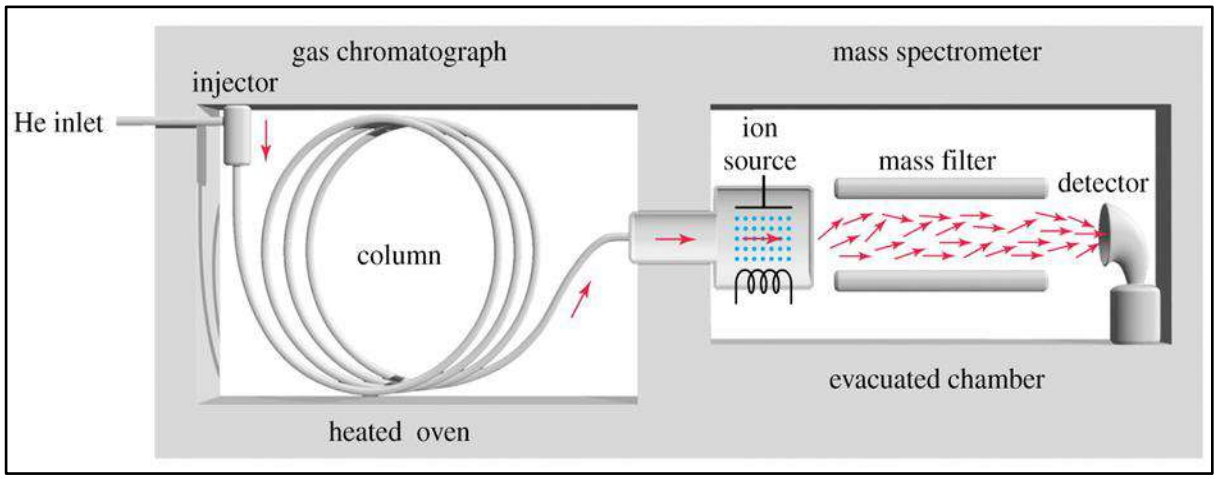

Figure 2.9.: Block diagram of GCMS (Source: Organic spectroscopy)

2.6. Molecular identification of microorganism

16S rDNA sequencing procedure is commonly employed for molecular identification of microorganisms particularly bacteria. This method offers sequence of isolated bacteria and after that it was compared with previous bacterial sequences present in database for identification. BLAST (Basic Local Alignment Search Tool) can be utilized for query sequence comparison with known sequences in several databases (Altschul et al.1997).

2.6.1. Phylogenetic analysis

Phylogenetic analysis is the study of the evolutionary development of a species or a group of organisms or a particular characteristic of an organism. The query sequence was compared with already known 16S rDNA sequences present in GenBank database. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura 1980). The 16S rDNA gene sequence was employed to carry out BLAST with the database of NCBI GenBank database. Based on maximum identity score first ten sequences were selected and aligned using multiple

alignment software program Clustal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 7.

2.7. References

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Objectives of thesis:-

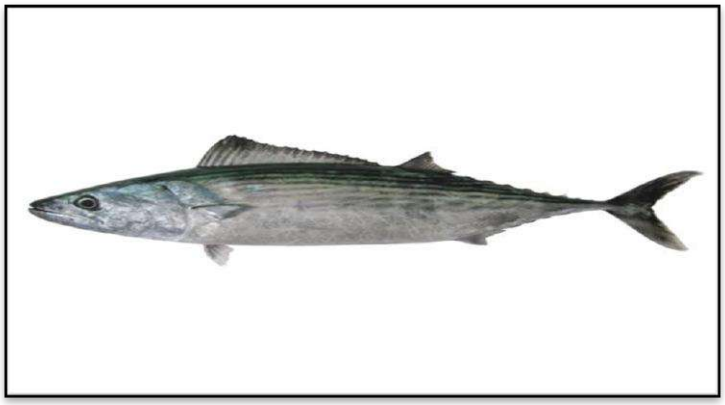
- Collection of fish waste from fish processing industries.

- Extraction, purification and characterization of collagen, gelatin and omega-3 fatty acids extracted from fish waste.

- Application of residual fish waste.

Classification of fish

Kingdom	:- Animalia
Phylum	:- Chordata
Class	:- Actinopterygii
Order	:-Scombriformes
Family	:- Scombridae
Genus	:- *Sarda*
Species	:- *orientalis*
Common name	:- Gethar



CHAPTER III

EXTRACTION, CHARACTERIZATION AND PURIFICATION OF COLLAGEN FROM WASTE MATERIAL OF GETHAR (*Sarda orientalis*)

3.1. Introduction

Fishing industry is one of the conventional food sector which offered adequate quantity of food to consciously increasing population. Fish and its foodstuffs are one of the most-traded food commodities worldwide. The fishery industry in India rises speedily day by day. India stands 2nd and 7th place worldwide in case of fish captures from inland and marine sources respectively. Between these two, fish production through inland sources increases tremendously indicating requirement of fish as a food source in non-coastal areas of India (FAO 2014). The fish processing is the agro based occupation that make use of fishery products as a raw material to generate new products. During fish processing, waste material also produced which increases environmental difficulties. For example, fishing industry utilizes more amount of water (80%) for cleaning purpose, thus generates 20 m^3/tonnes of waste water (Kurniasih et al.2018).

An ample quantity of waste (50% to 70%) generated from original fish raw materials during processing of fish products. This waste contains scales, skin, bone, viscera and head which composed of high nutritive components which can be supplemented as feedstuff or fertilizer along with conventional products. The improper disposal of waste into water bodies or dumping site may disturb ecological balance. Sometimes, poisonous gases may release from it which creates offensive odour. Therefore, optimal exploitation of these wastes for manufacturing of value added by-products is a promising approach to upsurge the income of producers and to minimize the disposal management cost of these wastes (Nagai and Suzuki 2000 and Wang et al.2007).

Collagen is a most important structural protein in extracellular matrix of numerous connective tissues in the body (i.e. skin, bones, ligaments, tendons, and cartilage). It is endogenously synthesized protein by organisms made up of three helical chains of amino acids. The polypeptide chains composed of repeated sequence of (Gly-X-Y)n, in which X and Y positions are usually occupied by proline and 4-hydroxyproline.The Gly-Pro-Hyp is the most common tripetide (10.5%) found in collagen (Shoulders and Raines 2009; Davison et al.2019; Silva et al.2014).

In current scenario, the commercial collagen is easily obtained from skin and bone of bovine and porcine. Due to the outbreak of bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and foot-and-mouth disease (FMD), there are some limitations on consumption of collagen and supplemented products produced from these animals. Also there are some ethical issues behind the use of collagen extracted from bovine and porcine sources. Thus, there is need to find out

alternative source of collagen especially from aquatic/marine origin to overcome these problems (Gaurav and Suresh 2015).

Therefore, the more research is carried out for the utilization of marine by-products as it is a potential source of collagen. These marine derivatives offer some advantages such as simple extraction, more collagen content, easy absorption by human body due to its small molecular weight, biocompatibility, minimum risk of animal and pathogenic diseases, eco-friendly, low amount of biological contaminants and toxins, fewer religious and ethical limitations as well as less regulatory and quality control complications (Jafari et al.2020).

Collagen extracted from marine origin has good biocompatibility, biodegradability, better cell attachment properties and weak antigenicity. Thus it has many applications in food, cosmetic, pharmaceutical, tissue engineering and biomedical sectors (Gomez-Guillen et al.2011). Collagen and collagen containing hydrolysate possesses superior gelling ability, texturizing, solidifying, and water holding capacities. Also it has swelling and solubility capabilities, emulsifying and foaming abilities, adhesion-cohesion properties, protective colloid function and film developing capacities. Due to this wide range of different properties, it generally utilized in food, cosmetic and pharmaceutical fields (Gaurav and Suresh 2015). The surrounding environment and body temperature of fish species has direct effect on thermal stability and applicability of collagen from several sources. In pharmaceutical sector, the collagen can be employed for generation of wound bandages, transparent implants and as transporters for drug delivery. It acts as a healing agent to treat burn patients, for reconstruction of bone as well as for surgical purposes related to dental, orthopaedic and cosmetic region. Also utilized for formation of edible coverings in meat processing industries (Mahboob et al.2014).

Due to good antioxidant potential, collagen is added into the skin moisturizing cosmetic products and can be analysed by numerous techniques, comprising DPPH (1,1-Diphenyl-2-Picrylhydrazyl), FRAP (Ferric Reducing Antioxidant Power), and CUPRAC (Cupric Reducing Antioxidant Capacity). Antioxidants are composites that attach to free radicals and very reactive molecules to prevent its oxidation which results in prevention of cell destruction. Collagen and some other peptides act as good antioxidant agent. The use of collagen containing products in cosmetics is the best treatment to decrease oxidative stress on the skin (Ardhani et al.2019). This supplementation can protect normal skin cell function by inhibiting free radical accumulation. Biomolecules and their

hydrolysate having strong antioxidant potential is widely applicable in anti-aging research as it reduces the oxidative stress which is the key reason of aging (Nurilmala et al.2019).

Gethar (*Sarda orientalis*) also called as striped bonito. It is tuna like fish of length 102 cm. It belongs to species of marine perciform fish and family *Scombridae*. It is distributed through the Indo-Pacific and East Pacific region and occurs at depths from 1 to 167 meters from sea surface. The present work was designed for; (i) collection of fish waste; (ii) optimization of extraction method; (iii) extraction of acid soluble collagen and (iv) purification and characterization of collagen for further potential applications.

3.2. Materials and methods

3.2.1. Collection of waste

The skin, fin, tail waste of *Sarda orientalis* (Gethar) were collected from fish market Ratnagiri (MS, India) and Gadre fish processing industry, Ratnagiri (MS, India) by maintaining suitable conditions. The same type of fish waste was collected from local fish market Kolhapur for further studies.

3.2.2. Proximate analysis of fish waste

The standard methods approved by AOAC (1980) were carried out to determine proximate composition of fish waste. The content of moisture, fat, ash and protein was evaluated on wet (WWB) and dry weight basis (DWB).

3.2.3. Pre-treatment of waste

The collected waste was separated into skin, fin, tail and other waste parts. These are washed twice with cold tap water and scrapped with knife to remove flesh and other contaminants. After that, waste was washed with cold distilled water and cut into small pieces with the help of scissor for collagen extraction. All process of pre-treatment and extraction were carried out at 4^{o}C.

The cleaned waste material was subjected to pre-treatment by NaOH and butyl alcohol according to method of Kumar and Nazeer (2013) with slight modifications. The non-collagenous proteins were removed by 0.1 M NaOH. The waste pieces (10 gm) were soaked into 0.1 M NaOH with 1:30 (w/v) ratio for 24 hr. The solvent was changed after every 4 hr. After incubation period, the soaked material was washed with cold demineralized water till neutral pH to remove traces of alkaline solution. Then, deproteinized waste material was subjected to defatting by 10% butyl alcohol with 1:30 (w/v) for 48 hr. The defatted residue was washed with cold demineralized water till pH 7.0 and used for further extraction.

3.2.4. Effect of NaOH

Pre-treatment with NaOH was useful for the removal of non-collagenous proteins on fish waste. To study the effect of NaOH, the method of Widowati (2017) was slightly altered. The waste pieces of fish (5 gm) were soaked in 0.1 M NaOH solution (1:30 w/v) for 24 hr. The NaOH solution was changed after 4 hr interval to know the effective time for removal of non-collagenous impurities. The protein concentration was measured by Lowery method using BSA as a standard.

3.2.5. Extraction of ASC from waste and effect of extraction conditions

ASC (acid soluble collagen) was extracted according to method of Bhuimbar et al. (2019) with minor modifications. Entire process of extraction was carried out at 4ºC. The optimization of method was carried out with respect to acid type, concentration of acid and extraction time. The pre-treated fish waste was suspended in 0.5 M concentration of acetic, lactic, formic, phosphoric and oxalic acid and kept for incubation at 72 hr to carry out extraction. Also inorganic acids such as hydrochloric acid (HCl) and sulphuric acid (H_2SO_4) were tested for extraction. After swelling, the skin was squashed and viscous solution was filtered through muslin cloth. The clear solution was obtained by centrifugation at 5000× g for 20 min. The 2 M NaCl was used for the precipitation of protein. The precipitate was collected by centrifugation at 5000 × g for 20 min. The sticky gel like nature of collagen was dissolved in minimum volume of respective acid (1:5 w/v). The final solution was dialyzed against cold demineralized distilled water for 48 hr using dialysis bag MW cut-off 12 kD, lyophilized and used for characterization and applicatory studies. The acid which gives maximum collagen yield was utilized for further optimization studies. The molar concentration of acid was optimized from 0.1 M to 1 M while extraction time ranges from 4 to 72 hr. Also waste: acid ratio (w/v) was optimized. The optimization of process was carried out to determine suitable condition of extraction which gives maximum yield. The acid which gives maximum yield in less extraction time was further used for mass production of collagen from waste.

3.2.6. ASC yield and hydroxyproline content

Yield of ASC on wet weight basis was computed using formula:

$$\text{Yield (\%)} = \frac{\text{weight of freeze dried ASC (g)}}{\text{weight of initial skin (g)}} \times 100$$

Hydroxyproline analysis of collagen samples was accomplished by the method of Neuman and Logan (1949) using L-hydroxyproline as standard. Percentage of hydroxyproline was calculated as:

$$\text{Hyp (\%)} = \frac{\text{conc.of Hyp (µg ml}-1)}{\text{conc.of protein (µg ml}-1)} \times 100$$

3.3. Characterization of extracted ASC

3.3.1. UV Visible spectrum

The UV-Visible spectrum of acid extracted collagen was determined according to method of Zhang et al. (2011) using a Shimadzu spectrophotometer UV-1800, Japan in the range of 200-400 nm. 1 mg of ASC was dissolved in respective acid to determine UV-visible spectra.

3.3.2. Electrophoretic pattern of ASC

The separation of collagen fragment based on their molecular weight was carried out by using SDS-PAGE according to the method of Laemmli (1970). Polyacrylaminde gel was prepared using 8% resolving gel and 5% stacking gel. About 100 µl protein samples with gel loading dye was loaded into the gel wells and electrophoresis was carried out. After electrophoresis, staining was performed by coomassie brilliant blue R-250 followed by distaining. The bands were observed on gel and then it was compared with standard collagen to determine molecular weight.

3.3.3. Fourier transform infrared spectroscopy (FTIR)

The different functional groups attached to collagen during extraction were analysed by Fourier transform infrared spectroscopy. FTIR analysis of gethar extracted collagen was carried out using Nicolet iS10 Mid FT-IR spectrometer (Thermo electron scientific, Madison, USA) in the range of 500-4000 cm^{-1}.

3.3.4. Scanning electron microscopy (SEM)

The scanning electron microscopy was used to study structural morphology of acid extracted collagen (TESCAN, Czech Republic).

3.3.5. Differential scanning colorimetry (DSC)

The differential scanning colorimetry (DSC) analysis of Gethar extracted ASC was studied according to method of Liu et.al.2012. The DSC was performed on DSC Q20 V24.11 calorimeter (Netzsch-Geratebau GmbH, Germany). The instrument was standardized for enthalpy and temperature by using indium as the standard and the measurements were carried out when the samples were continuously eradicated with ultrahigh-purity nitrogen at 50 cm^3/min. The lyophilized protein sample was precisely weighed into aluminium pan, wrapped and scanned from 25ºC to 400ºC at a heating rate of 5ºC /min. For reference a vacant wrapped aluminium pan was used. The highest

temperature was noted by the software referred as maximum denaturation temperature (Tmax).

3.3.6. Particle size analysis of collagen with zeta potential

The zeta potential of ASC along with particle size analysis was evaluated according to method of Chen et al. (2016) with slight modifications. The particle size was helps to analyse size of collagen based on intensity.3 ml of collagen dispersion was prepared in distilled water and homogenized for its hydrolysis. The hydrolysed sample was subjected to particle size analysis. For zeta potential, collagen sample was dispersed in acetate solution (0.05gm/100 ml) and kept for stirring for 6 hr. The zeta potential and particle size analysis of sample was studied by using Malvern Zetasizer Ver. 7.11.

3.3.7. Amino acid analysis

The amino acid composition of extracted collagen was checked on a Waters-PICOTAG amino acid auto analyser high performance liquid chromatography (Model: Waters 501) connected to the automatic amino acid estimating software. The Waters-Pico Tag column (size:- 3.9×150 mm) was employed for this purpose.

3.4. Functional properties of ASC

The ASC with different functional properties is suitable for various applications in food, pharmaceutical and agricultural field. So there is need to analyse Functional properties of ASC which includes emulsifying and foaming properties, water holding capacity, turbidity and oil absorption capacity.

3.4.1. Turbidity of collagen

Turbidity of collagen was determined as per method of Shon et al. (2011) with little modifications. About 10% (w/v) collagen was dissolved in double distilled water (DDW) and kept undisturbed for 1 hr. After incubation absorbance was measured at 600 nm (Shimadzu spectrophotometer UV-1800, Japan) and it was expressed as turbidity of collagen sample.

3.4.2. Solubility of collagen

The method of Shon et al. (2007) was slightly modified to determine solubility of gethar extracted collagen. At neutral pH, 0.7 gm of collagen powder was dissolved in a centrifuge tube containing 7 ml of 10 mM imidazole buffer. Pre-wrapped (black paper) tubes were vortexes for 15 sec and kept in undisturbed condition for 5 min. After incubation tubes were centrifuged at 7000 × g for 25 min. The supernatant was removed and tubes were dried and weight was taken. The solubility was expressed as;

$$\text{Insolubility (\%)} = 100\ \frac{\text{insoluble sample weight}}{\text{sample weight}}$$

$$\text{Solubility (\%)} = 100 - \text{insolubility (\%)}$$

3.4.3. Viscosity of collagen powder

The methodology of Gomez-Guillen (2000) was little bit modified to determine viscosity of collagen sample. 5% (w/v) collagen was dispersed in distilled water (D/W) and heated at 70ºC with constant increase in temperature. The viscosity was recorded by Brookfield digital viscometer (Model DV-II) in centipoises (cP) units.

3.4.4. Determination of water-holding capacity

The water holding capacity of acid extracted collagen was determined according to method of Pan et al. (2018). 10 mg of collagen was dispersed in 1 ml D/W and allowed to incubate at 20ºC for 1 hr. The tube was vortexes after every 15 min. The resultant sample was centrifuged at 5000 × g for 15 min. The supernatant was removed carefully without disturbing pellet remaining at the base and this tube was weighed. Water-holding capacity is the ratio of the weight of the absorbed water per milligram of collagen sample.

3.4.5. Oil absorption capacity

Oil absorption capacity (OAC) of collagen was studied according to method of Wani et al. (2015) with slight changes. About 0.5 gm of collagen was mixed with 10 ml soyabean oil and kept for incubation at room temperature (RT) for 30 min. After incubation, centrifugation was carried out at 4000 × g for 25 min at RT The volume of supernatant (free oil) was measured and OAC was calculated as gm of oil/gm of collagen.

$$\text{OAC (gm)} = \frac{V0 - V1}{W}$$

3.4.6. Emulsifying properties of ASC

The method of Pearce and Kinsella (1978) was executed for determination of Emulsion activity index (EAI) and Emulsion stability index (ESI) of collagen with minor modifications. In this experiment, 6 ml of collagen (1%, 3% and 5% w/v) and soyabean oil (2 ml) was mixed and homogenized for 10 min. After emulsion formation 100 µl sample was pipetted at 0 and 10 min then mixed with 5 ml of 0.1% SDS solution. The absorbance of solution recorded instantly (A_0) and after 10 min (A_{10}) at 500 nm using a spectrophotometer (Shimadzu UV-1800 Japan). The following formulae were used to calculate EAI and ESI;

$$\text{EAI } (m^2g^{-1}) = \frac{2 \times 2.303 \times A0}{0.25 \times \text{protein weight in g}}$$

$$\text{ESI (min)} = \frac{A0}{\Delta AXt} \quad \text{where } (\Delta A = A0 - A10,\ t = 10 \text{ min})$$

3.4.7. Foaming properties of ASC

The process utilized by Celik et al. (2019) was used to determine foaming capacity (FC) and stability (FS) of collagen. 1%, 3% and 5% w/v collagen solution was homogenised for 5 min at RT. After foam formation, the whipped solution was transferred to 25 ml measuring cylinder. Total volume of beaten solution was recorded immediately at 0 min and after 30 min by incubating it at 20ºC. The FC and FS of collagen was determined by using following formulae;

$$\text{Foaming capacity (\%)} = \frac{VT - V0}{V0} \times 100$$

$$\text{Foaming stability (\%)} = \frac{Vt - V0}{V0} \times 100$$

3.4.8. Effect of pH on collagen solubility

The effect of pH on collagen solubility was evaluated by method of Rodriguez et al. (2014) with minor modifications. 1 ml of collagen dispersion was taken into an Eppendorf centrifuge tube and pH was adjusted from 2.0 to 14 with either 6 N HCl or 6 N NaOH. The final volume of each pH tube were adjusted to 1.5 ml with distilled water and adjusted to same pH. The reaction mixtures were centrifuged at 5000 × g for 25 min at 25ºC and protein content was determined by Lowery method. Relative solubility was calculated with respect to pH having highest solubility.

3.4.9. Effect of NaCl on collagen solubility

Effect of NaCl on collagen solubility was studied to determine effective concentration of NaCl at which precipitation of collagen in acidic solvent was occurred. For this purpose, the methodology of Jongjareonrak et al. (2005) was used with some changes. About 10 ml of acidic solvent was precipitated by using NaCl ranging from 0.5 M to 3 M concentration. After precipitation all reaction mixtures were centrifuged at 5000 × g for 25 min at 25ºC and protein content was determined by Lowery protocol. Relative solubility of each sample was calculated with respect to NaCl concentration having highest solubility.

3.4.10. Sensory assessment

The sensory analysis of fish waste extracted collagen was carried out as per protocol of Aichayawanich and Parametthanuwat (2018) along with calf skin collagen as control. The sensory characteristics like appearance, color, fishy odour and overall

acceptability were tested by non-trained 7 member panel from Food Science and Technology department, Shivaji University, Kolhapur using 5 point hedonic scale (from 1:- dislike very much; 2:- dislike slightly; 3:- neither like nor dislike; 4:- like slightly and 5:- like very much).

3.5. Result and discussion

3.5.1. Proximate composition of fish waste

The Ward and Courts (1977) stated that, pre-treatment process during extraction eliminate several cross linked constituents present on waste as well as used to remove impurities and unwanted ingredients. More values of protein and ash were observed when raw material taken on dry weight basis for analysis. However, proximate composition may be influenced by seasonal variation and habitat of fish. Proximate composition like moisture, protein, lipid and ash content of fish waste on wet and dry weight basis were tabulated in table no.3.1. The result showed that, gethar fish waste contains 65.11±0.27% moisture, 34.69±0.38% protein, 6.48±0.24% lipid and 1.20±0.05% ash on wet weight basis while 15.79±0.27 moisture, 72.07±0.56 protein, 8.39±0.18 lipid and 2.52±0.28 ash was calculated on dry weight basis. The obtained value is slightly similar to the proximate composition (%) of small spotted catshark (Blanco et al.2019), shark, rohu and tuna (Hema et al.2013) as well as deep and shallow sea orbicular batfish (Pan et al.2018).

Samples	Moisture (%)	Protein (%)	Lipid (%)	Ash (%)
WWB	65.11±0.27	34.69±0.38	6.48±0.24	1.20±0.05
DWB	15.79±0.27	72.07±0.56	8.39±0.18	2.52±0.28

Values were means ± SD from triplicate determinations

Table 3.1.: Proximate analysis of fish waste

3.5.2. Effect of NaOH on non-collagenous protein removal

The non-collagenous proteins were removed by pre-treatment process. High temperature may degrade or denature collagen so, all experiments was carried out at cold condition (4ºC). For pre-treatment, alkaline solutions were preferred instead of acidic. The alkaline solutions were more effective to remove non-collagenous proteins and have lower level of protein loss than using acidic (Suptijah et al.2018). During pre-treatment, reaction between fish waste and NaOH take place which results in chemical changes in solution. The de-proteinase action was occurred on fish waste hence, excess NaOH turn to turbid during soaking condition. By the action of NaOH, the disruption of telopeptide

region on collagen molecule was happened which causes enlargement of fish tissues and successive removal of protein. High NaOH concentration with prolonged soaking time will increases the amount of dissolved proteins. Thus, it may dissolve collagen protein along with non-collagenous proteins (Zaelani et al.2019). The figure no.3.1 depicted the decrease in non-collagenous protein concentration within 24 hours. The results were confirmed that, pre-treatment with 0.1 M NaOH for 24 hr effectively eliminate non-collagenous impurities without damaging collagen structure.

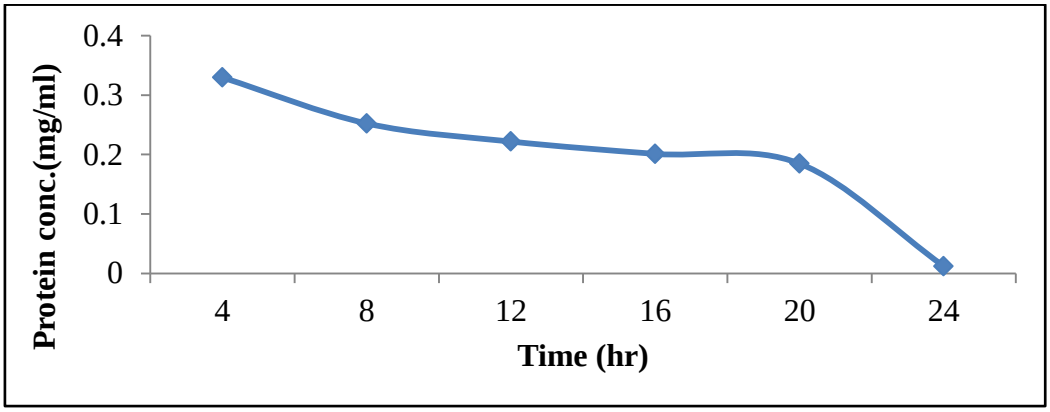


Figure 3.1.: Effect of NaOH on non-collagenous protein removal

3.5.3. Influence of extraction parameters on collagen yield

There are numerous parameters such as acid type, molar concentration of solvent, extraction time and waste to acid ratio affect the yield of collagen from fish sources. Effect of each of these parameters should be considered and optimized to determine suitable conditions to extract collagen.

3.5.3.1. Effect of acid

The different fish waste like skin, tail and fin were subjected to acidic extraction by using various organic and inorganic acids. The figure no.3.2 gives the general extraction procedure of collagen. The swelling of collagen was occurred in acidic solvent and loses its fibrillar appearance, thus generates viscous solution. Figure no.3.3 showed, the effect of acid on collagen yield. Among organic acids, lactic acid exhibited maximum collagen yield (34%) followed by acetic acid (26%), formic (6%) and phosphoric acid (2%). Cheng et al. (2009) reported that, utilization of inorganic acid (hydrochloric and sulphuric acid) for the extraction of collagen from animal tissues results in lesser efficacy and yield than the organic acids. It produces shorter amino acid chains of results in weak stability of collagen structure.

Liu et al. (2015) stated that, organic acid causes break down of inter strand crosslinks in collagen and solubilisation of chain. The exposure of protein to acid induces its hydration. The solubility of protein totally depends on type of acid, ionic strength and pH which effects on swelling characteristics. The H+ ion of acid causes entry of water in collagen fibres and enriches its recovery from the raw material (Gomez-Guillen 2001; Skierka and Sadowska 2007). Oxalic acid was also an organic acid but is not preferred for extraction because it disrupts the collagen structure during precipitation. The yield was less than collagen from skin of medusa fish extracted by lactic acid which was found to be 45% (Bhuimbar et al.2019) but more than 13.6% of collagen from black pomfret extracted by acetic acid (Maboud et al.2014).

The yield variation of collagen is due to difference in fish species, protein content of fish, its habitat conditions as well as pre-treatment and extraction method (Junianto et al.2018). Sometimes, combination of acid with enzyme (pepsin) may increase the extraction yield (Munasinghe et al.2014). Alkali was not suitable for extraction of collagen because it lost the ability of collagen to produce fibrils at neutral pH under physiological conditions (Hattori et al.1999).

Figure 3.2.: Collagen extraction procedure from fish waste

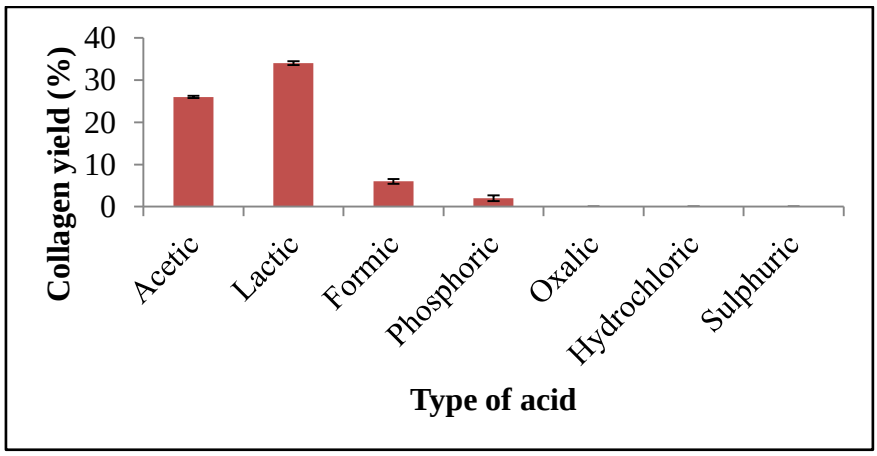


Figure 3.3.: Effect of acid on collagen yield

3.5.3.2. Effect of solvent concentration

Among various organic and inorganic acids employed for extraction, lactic acid gives more yield. Hence, optimized to find out its effective molar concentration ranging from 0.1 to 1 M while other component remains constant. Mostly collagen extraction was carried out using 0.5 M acetic acid because of its high productivity (Wang et al.2008; Veeruraj et al.2012; Tamilmozhi et al.2013), indicating 0.5 M is the effective concentration for extraction. In current study, 0.2 M lactic acid (11.46±0.23%) extracts more collagen than other acids (figure no.3.4) but slightly reduced at 0.3 M and 0.4 M that is 11.21±0.46% and 11.04±0.26% respectively. Comparatively better yield was obtained till 0.7 M (9.43±0.33%) beyond this it was start to decrease and very low yield was recorded at 1 M (1.28±0.41). It was found that, the increasing acid concentration beyond 0.7 M decline collagen yield due to degradation by excess acid (Jafari et al.2020). The similar study was carried out using 0.1 M to 1 M acetic acid on the collagen yield of sole fish skin (Arumugam et al.2018) and hybrid catfish skin (Kiew and Don 2013).

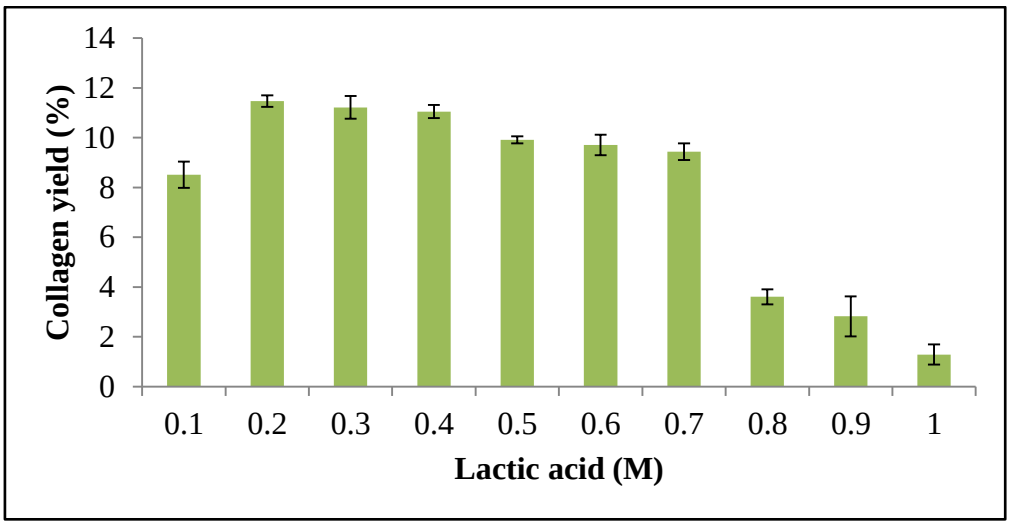


Figure 3.4.: Effect of solvent concentration on collagen yield

3.5.3.3. Effect of extraction time

The high yield of collagen was obtained for 0.2 M lactic acid, thus it was selected to optimize extraction time ranging from 4 to 72 hr. Extraction is strongly influenced by diffusion mechanism, which is time-dependent. The mass transfer rate explains the impact of extraction time on collagen yield. Therefore, increased extraction time is related to the more recovery of collagen. However, prolonged extraction period may cause disintegration of the peptides. In this condition, collagen polypeptide chains were

broken by the acid solution resulting decomposition of it and reduce the final extraction yield.

Additionally, lengthy extraction time would make the extraction procedure inappropriate for industrial scale-up (Jafari et al.2020). The effect of extraction time on collagen yield (%) was shown in figure no.3.5. It was found that, the yield was increased up to 11.44±0.53% within 40 hr. Later on, slight decrease was observed and further, it was constantly improved up to 72 hr (9.91%±0.44). Thus, optimum extraction time for gethar waste collagen was recorded as 40 hr with maximum yield. The obtained result was closely related to extraction time of sole fish skin collagen in which 36 hr gives more yield and after that it reduced (Arumugam et al.2018). But, reported extraction time in present study was more than 15 hr for collagen from wami tilapia (Alfaro et al.2014).

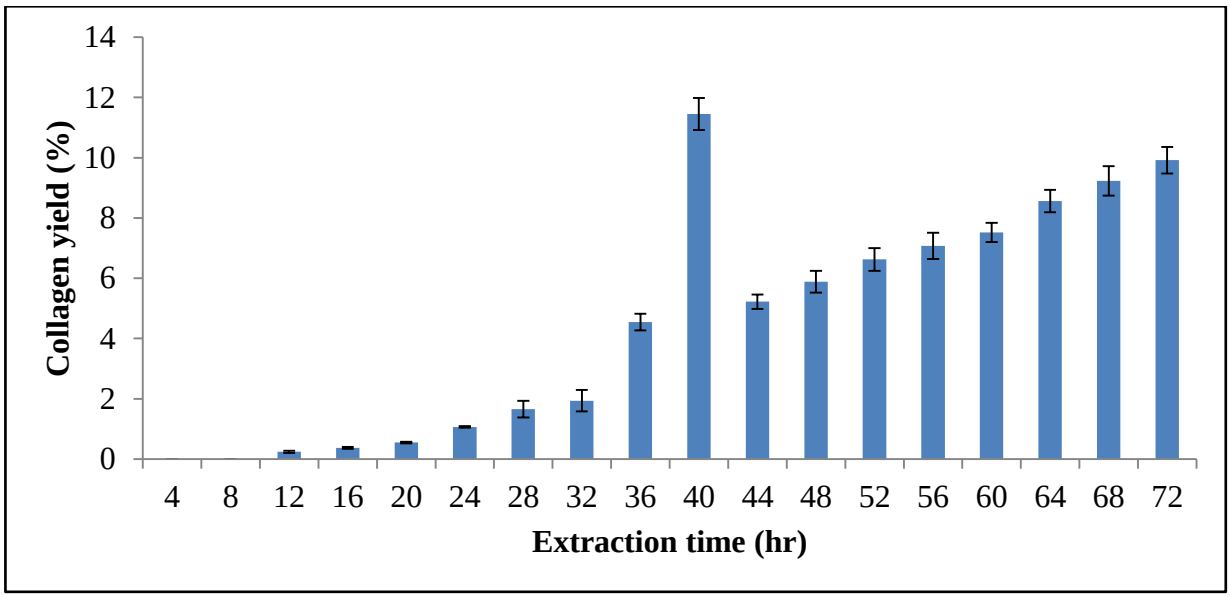


Figure 3.5.: Effect of extraction time on collagen yield

3.5.3.4. Effect of solid to liquid (S/L) ratio

The solid to liquid ratio is the quotient of quantity of solid collagen source divided by liquid solvent utilized for extraction. Commonly, increasing solvent amount results in improved interaction between free protons and amino acids of collagen chain thus stimulates separation of crosslinks present in the collagen helix. The reduced S/L ratio rises depolymerisation rate of peptides, since a more amount of acid causes disintegration of collagen chains, thus generates high quantity of lower molecular weight peptides (Arumugam et al.,2018). The figure no.3.6 depicted the effect of S/L on collagen yield. The 1:30 ratio gives maximum yield of collagen (12.59±0.42) and further it was decreased. The obtained ratio was less than 1/10 of collagen from skin of tilapia

and cod (Salamone et al.2016; Carvalho et al.2018) but higher than 1/60 of collagen from grass carp skin and giant croaker (Liu et al.2015; Coelho et al.2017). As the amount of solvent is improved, the fish tissue will be exposed to a more quantity of new solution, thus enhancing the degree of solubilisation of collagen peptides (Jafari et al.2020).

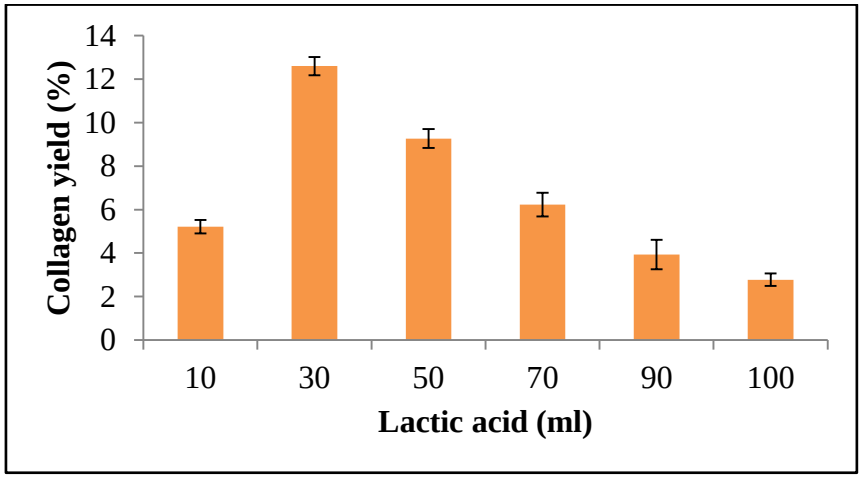


Figure 3.6.: Effect of solid to liquid ratio on collagen yield

Additionally, collagen extraction temperature was kept constant at 4ºC and it was depends on type of substrates used. The denaturation temperature of collagen was between 30-40ºC and therefore, it was more beneficial to carry out overall extraction procedure at 4ºC. The constant extraction temperature avoids degradation of protein and maintains its structural integrity (Coelho et al.2017; Jafari et al.2020).

3.5.4. Hydroxyproline content

The hydroxyproline (HyP) content is directly proportional to protein amount and is the major amino acid present in collagen (Lopez et al.2018). Hydroxyproline is a marker imino acid that stabilizes triple helical collagen structure. Additionally, hydroxyproline is exclusively present in collagen and showed very insignificant concentration in other proteins and can be used to estimate collagen quantitatively. Hydroxyproline content of collagen obtained after different acid treatments was tabulated in table no.3.2. Lactic, acetic, formic and phosphoric acid extraction exhibited 5.83±0.36 mg g^{-1}, 3.38±0.14 mg g^{-1}, 2.77±0.46 mg g^{-1} and 1.95±0.30 mg g^{-1} of hydroxyproline respectively. The lower amount was observed in phosphoric acid treated sample due to loss of collagen during extraction. HyP was detected in oxalic acid as well as HCl and H_2SO_4 extracted sample as they were ineffective for collagen extraction. The HyP amount in all acid samples was much less than swim bladder of catfish (Lopez et al.2018).

Sample	Hydroxyproline mg g^{-1} sample
Lactic acid	5.83 ± 0.36
Acetic acid	3.38 ± 0.14
Formic acid	2.77 ± 0.46
Phosphoric acid	1.95 ± 0.30
Oxalic acid	00
Hydrochloric acid	00
Sulphuric acid	00

Table 3.2.: Hydroxyproline content of ASC

3.5.5. Structural properties

3.5.5.1. UV-Visible absorption spectra

The basic and simple way to characterize collagen is to scan the sample by using UV-visible spectroscopy with range 200-800 nm. The UV visible spectra of lactic acid extracted ASC was given in figure no.3.7 with absorption maxima at 269 nm. The protein has maximum absorption at 280 nm but tryptophan did not present in collagen while very less amount of tyrosine was detected. The spectrum was nearly similar to collagen from skin of tuna, dog shark and rohu (Hema et al.2013; Kumar and Nazeer 2012).

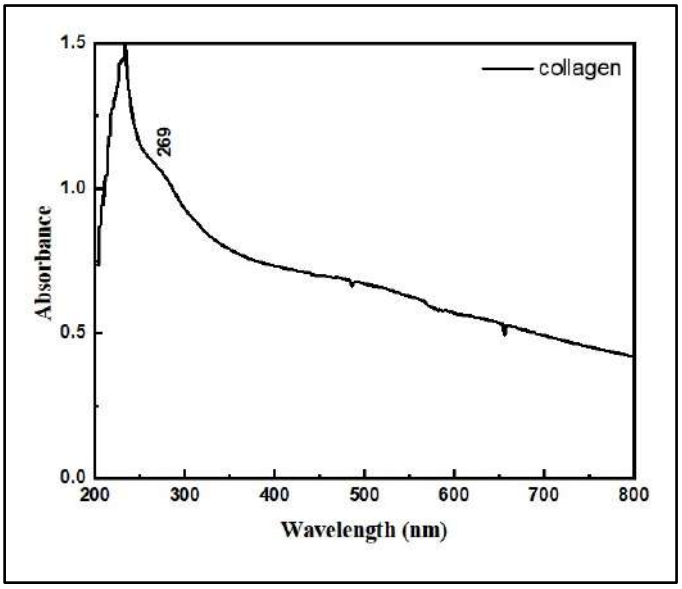


Figure 3.7.: UV–Vis absorption spectra of ASC

3.5.5.2. SDS-PAGE analysis

The figure no.3.8 depicted the SDS-PAGE analysis of acid extracted collagen along with standard calf skin collagen. Depending on electrophoretic mobility and subunit arrangement, it was concluded that collagen from gethar fish waste was type I collagens and were composed of monomer of β chain and dimer of α chain (α1 and α2). The bright β band was observed due to association of 2 α subunits while light band was because of α subunit (Seixas et al.2020). The presence of two different subunits confirmed that extracted collagen is the type-I (Ogawa et al.2003). Similarly study was observed in collagen from skin of black pomfret, nile tilapia and cuttlefish (Maboud et al.2014; Potaros et al.2009; Krishnamoorthi et al.2017) exhibited two α chains (α1 and α2) and one β component in the structure. The band pattern of collagens extracted using various acids are similar with that of standard.

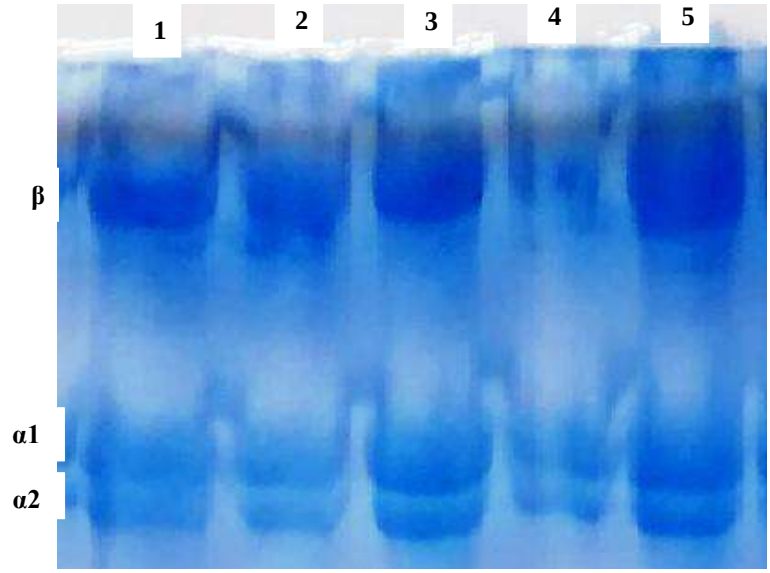


Figure 3.8.: SDS-PAGE of collagen: lane 1-standard calf skin collagen and lane 2-5 showed collagen extracted using lactic acid, acetic acid, phosphoric acid and formic acid respectively

3.5.5.3. FTIR spectroscopy analysis

The Fourier transform infrared spectroscopy is used to analyse various functional groups attached to protein. The figure no.3.9 depicted FTIR spectra of gethar waste extracted collagen. The weak stretching present at 1216 cm-1 corresponds to the vibrational modes of amide III functional groups (Payne and Veis 1988). The stretching at 1612 cm-1 represents the amide I bond. The broadening of –OH group was analysed between 3000-4000 cm-1 wave number range. The deep widening at 3370 cm-1 was occurred due to amide A (N-H stretching). The –OH stretching band was disintegrated into fragments whose frequencies were correlated to various O-H bond lengths, i.e. water molecules forms array in the different vibrational energies (Bridelli et al.2017). The conformation of the protein backbone may have positive influence on FTIR spectra of protein molecules. The theoretical studies of the vibrational force fields of the polypeptide chain revealed that α-helix, β-sheet and β-turn structures generates distinct positions for the amide I, II, and III bands (Payne and Veis 1988). Similar type of spectra was observed for collagen from elasmobranch by-products (Seixas et al.2020) and bone of torpedo scad and tigertooth croaker marine fishes (Kumar et al.2012).

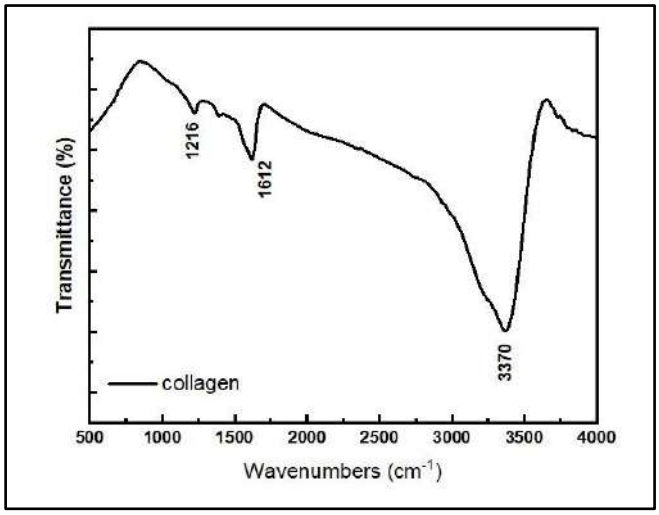


Figure 3.9.: FTIR spectra of ASC

3.5.5.4. SEM analysis

The SEM analysis of extracted collagen was shown in figure no.3.10. Under low magnification it exhibited highly porous nature with random windings of coil-like structures which specifies the fibrous nature of collagen with smooth and regular surface. At more magnification, coil-like fibrils were observed as a sheet interrelated to each other. The space between inter coiled sheets provides porosity to collagen, which will promote easy incorporation of any important drug into collagen structure (Shanmugam et al.2012; Arumugam et al.2018). The similar SEM structure was reported for collagen from scallop mantle, skin of cuttlefish and sole fish (Choi et al.2013; Shanmugam et al.2012; Arumugam et al.2018).

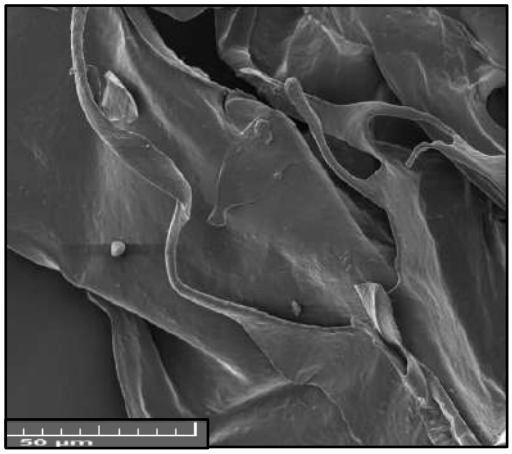


Figure 3.10.:SEM image of extracted ASC

3.5.5.5. DSC analysis

DSC remains as an incomparable method to evaluate the thermodynamic steadiness of proteins in a particular buffer condition. DSC determines heat capacity as a function of temperature. The DSC analysis of gethar extracted ASC was depicted in figure no.3.11 which exhibited single broad endothermic peak at 88.64ºC and three smaller peaks at 61.56 ºC, 129.82 ºC and 244.46 ºC. These peaks describe the thermal denaturation (Td) of collagen due to heating. The variation in thermal stability of collagen is occurred due to physicochemical changes during extraction. The 61.56ºC was observed because of alteration in covalent cross linkages which was more than 54.18°C and 59.17°C of chicken collagen (Akram and Zhang 2020) but less than bovine skin collagen (63-65ºC; Zhao and Chi 2009). The other peaks were associated to the constant conformational modifications of super helix and accordingly with the destruction of materials (Huang et al.2016).

The thermal stability of collagen was related to body and environmental temperature of fish habitat. The Td values determine the unfolding of helix assembly of protein to the random coil that alters the physicochemical properties of protein (Akram and Zhang 2020). It was studied that, thermal stability of collagen of various species depends on imino acid concentration (proline and hydroxyproline). The more number of imino acids imparts higher stability to collagen to maintain its helical structure (Abedin et al.2013). The Td values were accompanying with the amino acid profile and were justified with amount of hydroxyproline and proline (Okazaki and Osako 2014). The collagen with high thermal stability, good heat resistance and superior structural strength might be useful as probable substitutes for mammalian collagen (Huang et al.2016). Thus, extracted ASC possess good thermal stability so it can be utilized in nutraceutical, cosmetics and biomaterials product.

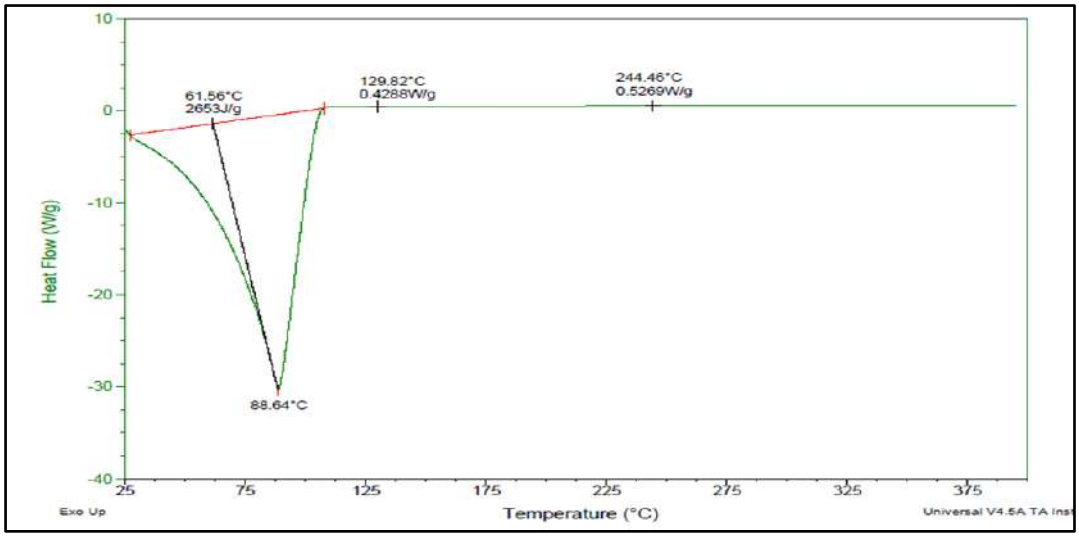


Figure 3.11.: DSC analysis of ASC

3.5.5.6. Zeta Potential

Generally, zeta potential technique was employed to study the surface net charge variation of the proteins along with collagen. At pH more than sample compound isoelectric point (pI); there is negative charge on surface of it. Usually, at the pH above and below of the protein pI, it possess net negative and positive charge respectively. This was occurred due to the de-protonation and protonation of the amino acids, respectively (Benjakul et al.2010). The protein has least solubility when the pH of mixture is same as to its pI. In aqueous sample, the protein has zero net charge while positive and negative charges counterbalances each other at their pI. At the pI, electrostatic repulsion will be reduced and precipitation as well as an aggregation of collagen is occurred. The zeta potential of gethar fish extracted ASC was illustrated in figure no.3.12 and it was recorded as -0.345 mV. The similar results were observed for collagen from skin of seabass (Sinthusamran et al.2013) and bamboo shark (Kittiphattanabawon et al.2005). Constant increase in pH results in decrease of charge on collagen. The pI of collagen was in the acidic range as it was related to the greater amount of acidic amino acids such as aspartic and glutamic than the basic including lysine, histidine and arginine. Also alteration in amino acids composition and distribution on the surface domains of collagen molecules may effect on pI (Muhammed et al.2018; Upasen et al.2019).

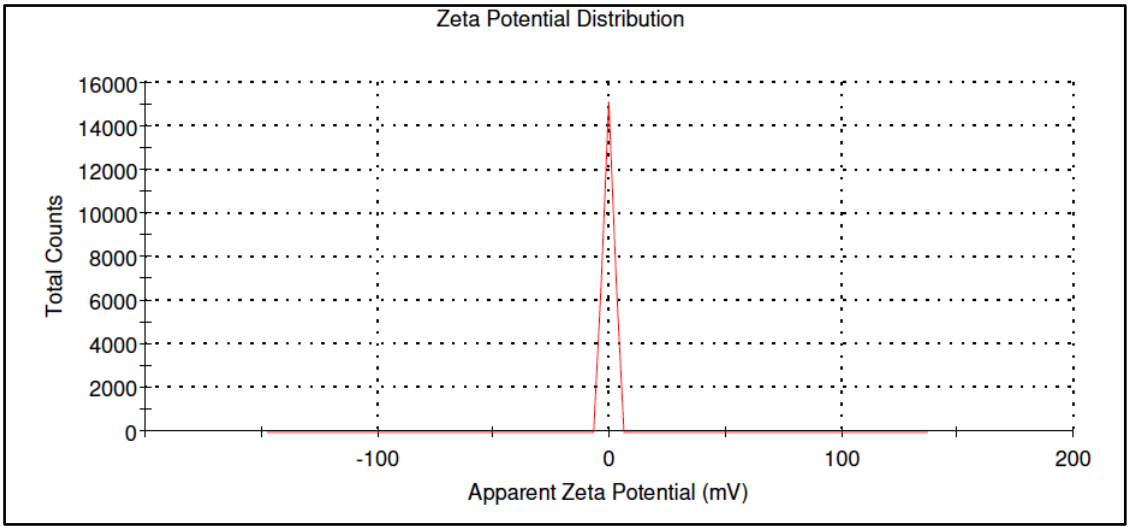


Figure 3.12.: Zeta potential of ASC

3.5.5.7. Particle size analysis

Particle size analysis was carried out to characterize size distribution of particles in a specific sample. The size distribution of gethar extracted ASC based on its intensity was represented in figure no.3.13. The mean particle size of ASC ranges from 3.12-14.51 d.nm (diameter values in nanometres).

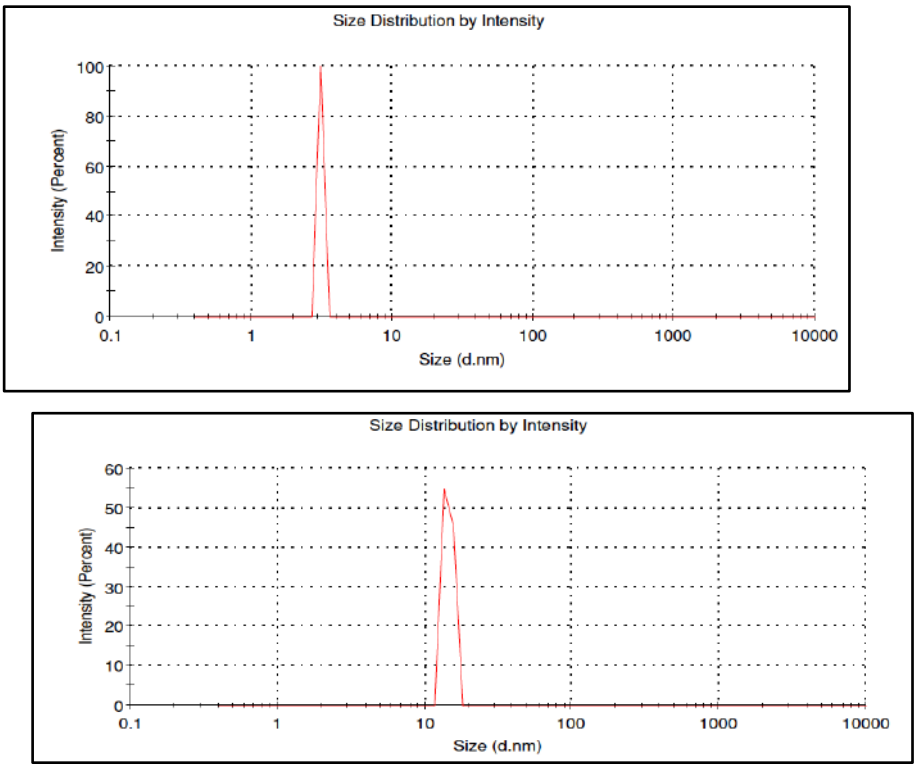


Figure 3.13.: Particle size distribution of ASC

3.5.5.8. Amino acid composition

The amino acid composition of gethar extracted ASC was tabulated in table no.3.3. The collagen contains more amount of proline, glycine (329 residues) and alanine (132 residues) while comparatively low amount of hydroxylysine (6 residues) which is general characteristic of collagen (Lee et al.2009). The stability of helices depends on content of imino acid. Therefore, the quantity of proline (111 residues) and hydroxyproline (82 residues) was found to be significantly higher and may have probable effect on thermal stability and structural integrity of collagen (Akram and Zhang 2020). The higher degree of proline hydroxylation effects on the triple helical structure of collagen (Xu et al.2017). About 6/1000 residues of hydroxylysine were reported in extracted ASC which was similar to collagen from shark skin (Kittiphattanabawon et al.2010). The analogous amino acid composition was observed in red snapper, black drum and sheepshead seabream skin collagen as well as *Axinella cannabina* and *Suberites carnosus* sponge collagen (Zaelani et al.2018; Ogawa et al.2003; Tziveleka et al.2017).

Amino acid	No.of amino acid per 1000 residues	% of amino acid
Alanine	132.33±1.24	13.23
Arginine	54.33±1.27	5.43
Aspartic acid	42±0.86	4.2
Cysteine	0	0
Glutamic acid	72.66±1.21	7.26
Glycine	329.33±1.24	32.93
Histidine	5.66±0.47	0.56
Isoleucine	14±0.89	1.4
Leucine	22.66±0.94	2.26

Lysine	26±0.86	2.6
Hydroxylysine	5.66±0.42	0.56
Methionine	15.66±0.89	1.56
Hydroxyproline	82±0.97	8.2
Proline	111.33±2.05	11.13
Serine	26.33±1.71	2.63
Phenyl alanine	5.33±0.14	0.53
Threonine	26.33±0.47	2.63
Tyrosine	5.33±0.54	0.53
Valine	23±0.81	2.3

Table 3.3.:Amino acid analysis of extracted ASC

3.5.6. Functional properties

3.5.6.1. Turbidity of ASC

Turbidity value reveals concentration of residual lipid and other colloidal material that were appear in collagen. As concentration of collagen increases, the turbidity value also increases and values are largely relying on performance of the clarification (filtration) process. The protein-protein interaction leads to the aggregation of particles having size larger than wavelength of light which results in more turbidity value. Protein aggregation in collagen dispersion occurred by heating at 60ºC for 30 min and increases the turbidity of solution (Kim et al.1994; Gomez-Guillen 2000). The turbidity of gethar waste extracted collagen was found to be 0.229±2. It was least than turbidity of skate skin collagen 0.28±0.04 (Shon et al.2011).

3.5.6.2. Solubility of collagen

The solubility of collagen was more at acidic pH (2.0-5.0) with relative solubility higher than 80% and decline suddenly at the neutral pH. Some collagens exhibited variation in solubility between pH 6.0 to 10.0. The difference in solubility of proteins with respect to pH was occurred due to variation in pI (Foegeding et al.1996). At acidic pH of 1.0, the some amount of collagen undergoes degradation results in solubility

reduction. At pH near to pI, there is reduction of molecular charges on collagen molecule occurred leads to declined solubility (Montero et al.1991). The solubility of extracted ASC was 57.23±0.37% while insoluble amount was 42.30±0.41% which was less than skate skin collagen (82.7±1.87%) (Shon et al.2011). The alterations in pH maxima for solubility among collagens from skin and bone might be happened due to various molecular characteristics and conformations between collagen while skin collagen exhibited a lesser degree of molecular cross-linkages and weaker connections than bone collagen (Kittiphattanabawon et al.2005).

3.5.6.3. Viscosity of collagen

The stronger electrostatic repulsion between molecular chains of collagen results in more viscosity which is important physicochemical characteristic of it. Type of acid used has impact on relative viscosity of collagen. The hydrogen bond balances triple helical alignment of collagen is irreversibly damaged and transformed into random coil arrangement. The destruction of hydrogen bonds was occurred due to heat treatment which minimizes viscosity of collagen. At high temperature molecules transfer quickly and enhance the molecular exchange, as thermal energy increases, molecules become extra moveable, thus reduces the viscosity (Lower 2013; Hadfi and Sarbon 2019). The large proportion of more molecular weight β and γ-chain in the solution at specific temperature causes increase in viscosity. But, the weak configuration may be formed due to reorganisation of α, β and γ-chain which could lead to the reduction in viscosity (Shahiri et al.2012; Pan et al.2018). The temperature more than 40°C disorganizes hydrogen bonds and random coil alignment of collagen molecule results in dissociation of it (Normah and Nur-Hani Suryati 2015). The viscosity of ASC was found to be 2.18±0.04 cP which was more than orbicular batfish (1.27±0.12 cP) but less than chilean mussels collagen (2.35±0.27 cP) (Pan et al.2018; Rodriguez et al.)

3.5.6.4. Water holding capacity

The solubility, particle size, micromorphology and physicochemical atmosphere of protein are responsible for variation in water holding capacity (Wu et al.2011). Proteins with more water absorption and retention capacities are estimated to have comparatively more polar residues with the property to produce hydrogen linkages with water (Hou et al.2012). Water holding capacity (WHC) is the function of proteins to inhibit water from being removed or excluded from their three-dimensional structure. Water binding and holding capacity is the very significant feature in development of confectionary food products. The sample having these two characteristics contains

amount of water in the form of hydrodynamic water, bound water and physically entrapped water. The polar and non-polar species attached to water showed effect on water binding properties. The increase in water holding capacity is observed due to the higher hydrophilic properties of lower molecular weight peptides (Barzideh et al.2014). The water holding capacity of gethar extracted ASC was recorded as 19.7µl/mg which was more than ASC from orbicular batfish (25.22 µl/mg) and tilapia scale (9.4-15%) (Pan et al.2018; Huang et al.2016). Most important functional characteristics of proteins are associated to their interconnection with water, thus the choice of proteins with a suitable WHC is prime in food development. The dominant protein-protein interactions and the zero net charge on protein results in lower value of WHC (Akram et al.2020).

3.5.6.5. Oil absorption capacity (OAC)

Oil absorption capacity is related to texture contributed by interaction between oil and other compounds. The OAC of a protein is correlated to its non-polar amino acid residues. Hydrophobic association among the non-polar amino acids of protein component and hydrocarbon residues on oil regulate OAC of protein. The OAC value determines the number of non-polar amino acids present on ASC. The result was in accordance with the contact angle investigation, where ASC possesses more hydrophobic groups than PSC (Chen et al.2019). OAC of gethar extracted ASC was found to be 12.2±0.21 gm/gm of collagen which is higher than OAC of scallop gonad protein (5.2 mL/g) (Han et al.2019) but much less than OAC of red stingray ASC (41.41 ±0.47 mL/g) (Chen et al.2019). As stated by Maruyama et al.(1998), proteins with greater value of OAC provide superior shape retention in food, such as meat or candy products ;thus the collagen extracted from gethar waste will be employed in nutraceutical industries.

3.5.6.6. Emulsifying and foaming properties

Emulsification denotes the mixing of two or more components to produce a uniform dispersion of performance in which particles in the form of a liquid, distributed in another liquid (Guo and Ruan 2006). It is an essential characteristic of protein which reflects the ability of protein to associate with water and oil to generate emulsion. It consists of emulsifying activity and stability. Emulsifying activity index (EAI) states that, the property of protein to produce emulsion in presence of water and oil while emulsifying stability index (ESI) is the ability of protein to conserve the oil-water emulsion without its separation and to resist the external stress situations (Zhang et al.2006). The collagen and other higher molecular weight polypeptides act as efficient stabilizer for protein film than lesser peptides in oil-in-water emulsion. Protein

hydrolysate behaves as surface-active materials that stimulate an oil-in-water emulsion due to its hydrophilic and hydrophobic moieties and their charges (Chi et al.2014). The reduction in repulsive intensity increases the probability of oil droplet aggregation. The oil droplet aggregation decrease the association between oil and water required for foaming, thus it decreases the EAI and ESI of collagen.

The foaming capacity (FC) of protein defines the function of protein to produce film at the air-water boundary. The protein with fast adsorption on newly formed air liquid interface throughout bubbling and causes unfolding as well as molecular reorganization at the interface, reveal improved foaming capacity than proteins that adsorb gently and obstruct unfolding at border (Damodaran 1997). The decline in FC and foaming stability (FS) may occur due to reduced solubility and weak electrostatic repulsion between the collagen molecules which was not sufficient to inhibit accumulation of molecules. This accumulation decreases the inter linkage among protein and water essential for foaming results in lesser FC and FS of collagen (Chen et al.2019). The relationship between protein and water has vital role in generation of foam and its stability. Ultrasound treatment can be applied to increase the FC and FS properties of collagen (Akram et al.2020).

As per table no.3.4, 1% ASC has EAI 15.30 m^2g^{-1} and ESI 27.29 min. Further increase in protein concentration from 1% to 5% lowers emulsion forming ability as well as stability. Oppositely, foaming capacity and stability of ASC was enhanced with increased protein concentration. The 5% ASC has 21 ± 0.87% foaming capacity and 15 ± 0.39% foaming stability (table no.3.4). Standard calf skin collagen has greater EAI, ESI and foam properties than extracted ASC; this might be because of source and purity of standard collagen. Remaining lipid in collagen has been recognized as a prime component to influence on emulsifying, foaming and flavour properties. Thus, elimination of residual lipids from collagen results in enhancement of these characteristics (Shon et al.2011). The similar results were obtained for ASC from black ruff skin collagen (Bhuimbar et al.2019). The emulsifying and foaming capacity of gethar waste extracted ASC was mush lesser than ASC from skin of brown bullhead and collagen hydrolysate from skin of spanish mackerel (Chen et al.2013; Chi et al.2014). Therefore, it can be concluded that studied ASC acts as an alternative source for commercial collagen. Due to its good emulsifying and foaming properties it can be employed in baking, beverages, and minor food ingredients (Chen et al.2019).

Sample	Emulsifying Activity Index	Emulsion Stability Index	Foaming capacity	Foam stability
	(m^2g^{-1})	(min)	(%)	(%)
1 % ASC	15.30 ± 0.04	27.29 ± 0.12	6.0 ± 0.02	2 ± 0.09
3 % ASC	8.13 ± 0.03	13.28 ± 0.04	16 ± 1.28	11 ± 2.4
5 % ASC	4.86 ± 0.03	11.32 ± 0.08	21 ± 0.87	15 ± 0.39
Calf skin collagen	56.23 ± 0.14	30.63 ± 0.03	30 ± 0.03	22.5 ± 0.95

Values were means ± standard deviation from triplicate determinations.

Table 3.4.: Emulsifying and foaming properties of ASC

3.5.6.7. Effect of pH on collagen solubility

Isoelectric point (pI) was used to determine influence of pH on protein solubility. When pH of protein aggregation is more or less than pI, then repulsive power between charged residues and chain of protein increases. Thus, it results in enhancement of protein solubility. When the pH is close to pI, then the overall charges on protein molecules are zero and there is rise in hydrophobic interaction. Therefore, it forms precipitation and aggregation of the protein molecule (Jongjareonrak et al.2005; Iswariya et al.2018). Singh et al. (2011) stated that most of collagen exhibited higher solubility in acidic environment and after that reduction was observed. The figure no.3.14 depicted the effect of pH (2-14) on the relative solubility of collagen. The ASC from gethar fish waste has maximum solubility between pH 1-3 (solubility at pH 2.0:- 99.36%) and after that slightly decreased up to pH 8.0 (75.59%). The gradual reduction of solubility was detected up to pH 14 (40.75%). Similar result was obtained for collagen from puffer fish and silver catfish skin collagen (Kirti et al.2015; Hukmi and Sarbon 2018).

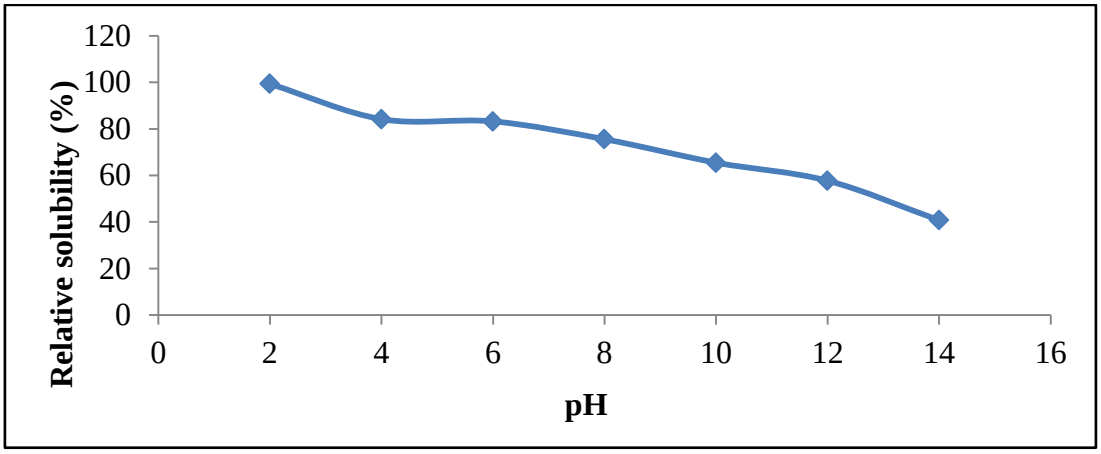


Figure 3.14.: pH dependent relative solubility of ASC

3.5.6.8. Effect of salt concentration on collagen solubility

Effect of NaCl was studied to determine optimum concentration of salt to precipitate collagen from acid solvent. At low NaCl concentration, collagen solution exhibits salting in effect while increase in concentration results in salting out. The figure no.3.15 represents the effect of NaCl concentration on collagen solubility. Solubility was maximum at 2M NaCl (99.96%) and further it was decreases up to 76.50% as NaCl concentration was increased up to 3 M. The improvement in hydrophobic interaction reduces the collagen solubility after 2 M (Abdelaal et al.2020). This result was similar to the solubility of the tilapia skin (Zeng et al., 2009), puffer fish skin (Kirti et al.2015) and brown stripe red snapper fish (Coppola et al.2020). As The more co-operation between ionic salts and water results in precipitation of protein (Kirti et al.2015). The ASC exhibited better solubility at low NaCl concentration (0.5 to 2 M) because the salt ions attaches weakly to charged clusters on the protein molecule (Damodaran 1996).

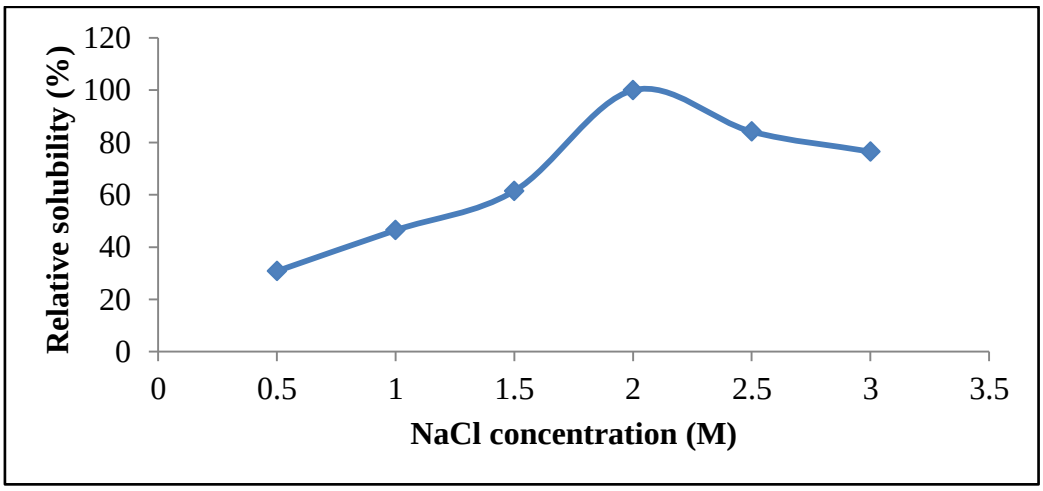


Figure 3.15.: Effect of NaCl on ASC precipitation

3.5.6.9. Sensory evaluation

The quality of food products in market were determined by studying its sensory characteristics. The 5 point hedonic scale was used for this purpose. The consumer dislikes food product, if score is less than 3. The sensory analysis of appearance, colour, fishy odour and overall acceptability was carried out. The result was shown in figure no.3.16. The similar analysis was obtained for red cheek barb scale collagen (Aichayawanich and Parametthanuwat 2018). The sensory analysis score of colour, appearance and overall acceptability of extracted ASC was more than 3 hedonic points. It has slight fishy odour due to raw materials and extracting solvents used. Thus, due to good sensory properties ASC can be exploited for different food applications.

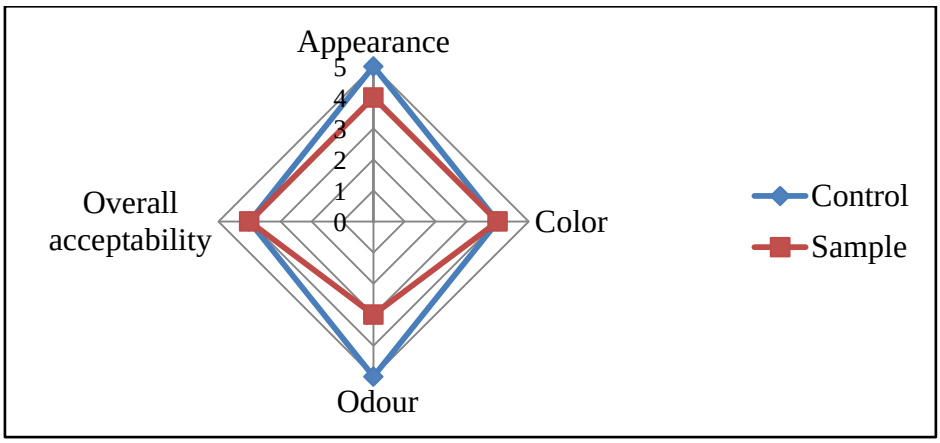


Figure 3.16.: Sensory analysis of extracted ASC

3.6. Conclusion

In this study, fish waste that ultimately responsible for reducing environmental pollution burden was effectively utilized for extraction of medically and industrially important collagen. Lactic acid with 0.2 M concentration was found most efficient among other acids used for collagen extraction from waste material of gethar (*Sarda orientalis*). The 40 hr extraction time and 1:30 (w/v) was sufficient to generate better yield of collagen and also it reduces prolonged extraction time so it can be applicable at small scale level. Electrophoretic pattern, UV visible spectra, FT-IR, SEM, zeta potential and particle size analysis confirmed type I collagen with two α chains (α1 and α2) and one β chain. Extracted collagen exhibited significant emulsifying, foaming properties, water holding and oil absorption capacity as well as possess good solubility, turbidity and viscosity. Thus, it can be useful in formulation of functional foods enriched with collagen. It also has maximum solubility at pH 2.0 and effectively precipitated by 2 M

NaCl. Thus, fish waste was successfully utilized for the extraction of collagen and development of protein rich functional foods as well as in pharmaceutical field due to its antimicrobial, antioxidant, anti-diabetic and anti-tumour properties.

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CHAPTER IV

EXTRACTION, CHARACTERIZATION AND PURIFICATION OF GELATIN AND OMEGA-3 FATTY ACID FROM WASTE MATERIAL OF GETHAR (*Sarda orientalis*)

4.1. Introduction

The seafood industry generates large amount of fish waste through various processes. Different parts of fish have been utilized by fish processing industries to making of fishmeal as well as some by-products for the pharmaceutical and nutraceutical fields as a supplementary component. Each year fishing industries disposes significant quantity of waste after fish processing which include trimmings, fins, tail, frames, head, skin, scales and viscera. Some of these products are used but the majority components are discarded as waste causing various types of environmental pollution. These wastes contains large amount of nutrients like protein with good biological importance, unsaturated fatty acids, vitamins and antioxidants, minerals as well as physiologically valuable amino acids and peptides which acts as substrate for fish meal production (Oladapo and Awojide 2015).

About 75% waste was produced after fish processing and it was major environmental pollutant. Skin and bone part of waste contains higher amount of collagen protein (Guillen et al.2002). Gelatin is one of the largely employed biopolymer (polypeptide) in nutraceutical, pharmaceutical and cosmetic sectors. Due to its distinct physical properties, it utilized both as food additive and functional food in the nutraceutical. However, it plays major role in various food components as a thickener, stabilizer, adhesive and emulsifier in biodegradable films. Also act as a gelling, foaming and microencapsulation agent in several food products like confectionary, jelly, yoghurt, ice cream, cheese, and canned foods (Koli et al.2012). In addition to this, it comprises bioactive properties which includes antimicrobial, antioxidant and also exhibited antihypertensive ability by inhibiting angiotensin converting enzyme (ACE) (Atma and Ramdhani 2017).

Due to diverse functional properties, gelatin has classified into two classes: first one is correlated with gelling process and another related with the surface behaviour of gelatin. The degree of collagen transformation into gelatin is associated with the strength of pre-treatment and extraction method as a function of pH, temperature and extraction period. Depending on the acid or alkaline pre-treatment process, two categories of gelatin were obtained, called as type-A (isoelectric point at pH ~8–9) and type-B (isoelectric point at pH ~4–5) respectively (Sousa et al.2017; Karim et al.2008).

During last few years, the neurological disorders like Bovine Spongiform Encephalopathy (BSE) have been identified in several countries and it may be exported from cow meat and meat products (Baziwane and He 2003). On the other hand,

consumption of pork meat has ethical issues in some religions. Therefore, there is need to find out new source of gelatin, like marine fish wastes which is generated in more amount during fish processing by industries and local markets.

The major distinguishing feature between fish and mammalian gelatin is the quantity of imino acids proline and hydroxyproline, which balances the ordered configuration during gel formation of gelatin. Due to fewer amount of these imino acids gelatin possess low gelling and melting temperature (Tavakolipour 2011). The generation of gelatin from fish waste mainly from fish skin has much importance due to its characteristic properties and qualities. Fish and its products were accepted by almost all religions, so there are negligible ethical issues (Kittiphattanabawon et al.2005). Thus, it imparts a solution for implementation of vast amounts of fish wastes generated.

The total world fisheries accounts about 141.6 million tons (FAO 2006) and it was increases day by day. Thus, there is need to convert this enormous quantity of fish waste into beneficial products such as fish gelatin (Herpandi et al.2011). To get gelatin from unsolvable native collagen, a proper heat was applied which will disrupt the non-covalent bonds. Therefore, it will cause sufficient swelling and break the intra and inter molecular linkages leading to successive solubilisation of collagen (Kim and Cho 1996). The extraction of gelatin from waste material involves acid or alkaline treatment to cleave the collagen crosslinks followed by treatment with hot water. Heat treatment is important to destabilize the triple helix confirmation of collagen to transform its helical configuration into a coiled structure, resulting in a gelatinous nature at cooling temperature (Dincer et al.2015).

There is a necessity to carry out more research to acquire essential fatty acids EPA and DHA for the use of its probable application in food and pharmaceutical trades because these fatty acids has important role in diagnosis and treatment of human disorders. The extraction of these fatty acids from discarded fish waste is economically feasible and also it is a cost effective alternate method to decrease problems of waste disposal and to prevent environmental pollution from it (Rai et al.2013; Nascimento et al.2015).

The increasing demand of highly purified fish oil specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has led researchers to invent nontoxic procedures for the separation of suitable components. Fish oil is present in the form of omega-3 polyunsaturated fatty acids (PUFA) are widely utilized as pharmaceutical supplements, food flavourings and health supplements (Espinosa et al.2002).

Various clinical and epidemiologic studies have revealed positive effect of omega-3 PUFA in new-born improvement, cancer, cardiovascular disorders as well as in mental illnesses such as depression, attention-deficit hyperactivity syndrome and dementia. The De Novo synthesis of PUFA was not carried out in humans and therefore these are essential fatty acids which are acquired from diet. Fish oil, marine animal oil and vegetable oil were naturally available dietary sources of omega-3/6 PUFA. As per the European Food Safety Agency (EFSA) recommended dietary intake of EPA+DHA was 250 mg per day. Thus, PUFA containing fish oil has more importance in nutrition sector and its production has been one of the increasing research area in last few years (Ferdosh et al.2016).

Fish and fish tissues exhibited comparatively high autolytic activities and more amount of polyunsaturated fatty acid and thus they are susceptible to both lipolysis and oxidation. Due to this, there are some difficulties during extraction of oil from fishes. Large quantity of PUFA causes hydrolytic spoilage of fish oil mainly by oxidative deterioration. The oxidation of lipids lead to some drawbacks comprising rancid odours and flavours, decreased nutritional quality and safety which may be harmful to health by creating health issues (Bako et al.2017).

The conventional techniques like wet pressing and solvent extraction as well as modern procedures such as supercritical fluid extraction were employed for fish oil extraction. Also enzymes from fish or other sources (e.g.:Alcalase) were employed for extraction. The habit and habitat of fish has influence on amount of fish oil (Adeoti and Hawboldt 2014).

At industrial level wet pressing is commonly utilized technology for extraction of fish oil and it has four steps: - fish cooking, pressing, decantation and centrifugation (FAO 1986). Another traditional method is, use of solvents for extraction. This method is useful for analytical purposes but not for industrial production because some solvents which are restricted in food industry were utilized for extraction. The basic of this method depends on solubility of lipids in organic solvents and their insolubility in water. The soluble components such as proteins, carbohydrates and minerals can be separated from water. There are numerous methods depending upon type of solvent. The general used methods are Soxhlet and Bligh-Dyer as well as include McGill-Moffatt and Randall-Folch methods were assessed (Mendez and Concha 2018).

In current approach, gelatin was extracted from skin and omega-3 fatty acid were extracted from head waste of marine fish gethar (*Sarda orientalis*), also called as striped

bonito. It is tuna like fish having length 102 cm and belongs to species of marine perciform and family Scombridae along with tuna and mackerel. But it contains fewer amounts of lipids than other two fishes. The enzymatic concentration of fish oil was done to determine amount of omega-3 fatty acid in it. Thus, extraction of gelatin and omega-3 fatty acid were carried out and studied for its structural as well as functional properties and may be useful in different applications in various fields.

4.2. Experimental Methodology

4.2.1. Chemicals

Lactic acid, sodium hydroxide (NaOH), hydrochloric acid (HCl), butyl alcohol, chloroform, Bovine serum albumin (BSA,100% pure), leucine, L- hydroxyproline, sodium dodecyl sulphate (SDS), acrylamide, polyacrylamide, agarose, coomassaie brilliant blue and other chemicals required to perform experiments were purchased from Hi-Media and Sisco Research Laboratory, India. Chloroform, methanol, glacial acetic acid, sodium hydroxide (NaOH), potassium hydroxide (KOH), sodium thiosulphate ($Na_2S_2O_3$), sodium sulphate, iso-octane, *p*-anisidine reagent, carbon tetrachloride (CCl_4), hydrochloric acid (HCl), iodine solution, potassium iodide (KI), phenolphthalein indicator and other chemicals employed for current study were procured from Himedia, India and Sisco Research Laboratory, India. Different chemicals and reagents utilized for this study were of analytical grade.

4.2.2. Collection and cleaning of fish waste

By maintaining suitable conditions skin and head waste of *Sarda orientalis* (gethar) were collected from fish market Ratnagiri (MS, India) and Gadre fish processing industry, Ratnagiri (MS, India) during the period January to April. It was washed with cold tap water and scrubbed with knife to bring out flesh. The cold demineralized water was used for final wash and cut into small pieces for further procedures.

4.2.3. Proximate analysis of fish waste and gelatin

The standard techniques sanctioned by AOAC (1980) were carried out to determine proximate composition of fish waste and extracted gelatin. The content of moisture, fat, ash and protein was evaluated on dry (DWB) and wet weight (WWB) basis.

4.2.4. Pre-treatment of waste for gelatin extraction

Pre-treatment was carried out according to procedure of Kumar and Nazeer (2013) with slight modifications. The whole process was conducted at 4ºC. Non-collagenous proteins were removed by soaking 10 gm skin in 0.1 M NaOH (1:30 w/v) for

24 hr. Treated waste was washed with cold demineralized distilled water (D/W) till pH 7.0 and subjected to defatting by 10% butyl alcohol (1:30 w/v) for 48 hr. After defatting, it was washed repeatedly with cold demineralized water till neutral pH and was subjected to collagen extraction by 0.2 M lactic acid for 40 hr. Also, this pre-treated waste was employed directly to gelatin extraction.

4.2.5. Gelatin extraction

Partial hydrolysis of collagen by acid, alkali or thermal treatment causes formation of gelatin. One method utilizes extracted collagen for gelatin preparation while in another method pre-treated waste material was directly used for gelatin extraction using organic acid. Following two methods were exploited for extraction.

4.2.5.1. By collagen hydrolysis

Method given by Du et al. (2014) was slightly modified for formation of gelatin from collagen. Extracted and purified collagen (10 gm) was mixed with D/W in 1:3 (w/v) ratio. It was kept for thermal treatment at 60ºC-80ºC for 7 hr after that gel like appearance was observed. The water soluble gelatin was recovered by centrifugation at 5,000 x g for 15 min at 20ºC (Remi centrifuge).

4.2.5.2. By acid treatment method

Gelatin was extracted directly from pre-treated waste by the method of Tinrat and Asna (2017) with slight modifications. About 10 gm pre-treated fish waste was mixed with 0.2 M lactic acid in 1:10 (w/v) proportion and placed under continuous stirring for 2 hr at RT. The acid treated waste was washed thoroughly with D/W till neutral pH. Final extraction period was optimized for gelatin extraction. Thus, optimized final extraction was carried out with D/W in a ratio of 1:10 (w/v) and kept for continuous stirring at 80-100ºC for 1, 1.5, 2, 2.5 and 3 hr. Viscous solution was obtained after extraction and was centrifuged at 5000 x g for 10 min to remove impurities then it was filtered through muslin cloth. The resultant filtrate was dried using hot air oven at 60ºC for 48 hr. Powder form of fish gelatin was stored for further characterization and applicatory studies.

4.2.5.3. Yield of gelatin and hydroxyproline analysis

Yield of gelatin extracted by two methods were calculated by following formula (Bordignon et al.2019);

$$\text{Yield (\%)} = \frac{\text{weight of dried gelatin (g)}}{\text{weight of dried collagen (g)}} \times 100$$

$$\text{Yield (\%)} = \frac{\text{weight of dried gelatin (g)}}{\text{weight of dried waste (g)}} \times 100$$

Hydroxyproline analysis of gelatin samples was accomplished by the method of Neuman and Logan (2019) using L-hydroxyproline (HyP) as standard. Percentage of hydroxyproline was calculated as:

$$\text{Hyp (\%)} = \frac{\text{conc.of Hyp (µg ml}-1)}{\text{conc.of protein (µg ml}-1)} \times 100$$

4.2.5.4. Structural characterization of gelatin

Extracted gelatin was characterized by UV visible spectroscopy by using 1 mg/ml sample (Shimadzu UV-1800 Japan) between 200-800 nm and characteristic peak was detected while D/W was used as reference. SDS-PAGE was performed to analyse gelatin on the basis of its molecular weight. For this purpose, 8% resolving gel and 5% stacking gel was used while Coomassie brilliant blue was used as staining agent. 50-200 KD pre-stained protein ladder (Himedia, India) was utilized for comparison of molecular weight. The Fourier transform infrared spectroscopy (FTIR) was carried out to determine different functional groups attached to gelatin. The Nicolet iS10 Mid FT-IR spectrometer (Thermo electron scientific, Madison, USA) in the range of 500-4000 cm^{-1} was utilized. X-ray diffraction pattern gives information about the distribution and orientation of gelatin. The samples were subjected to Cu-Kα radiation at 40 kV voltage and current of 40 mA with scanning range 10°-80° (2θ). X-ray diffraction (XRD) analysis of extracted gelatin was done by Bruker AXS analytical instrument (Germany).

The differential scanning colorimetry (DSC) was performed on DSC Q20 V24.11 calorimeter (Netzsch-Geratebau GmbH, Germany). The gelatin sample was eradicated with ultrahigh-purity nitrogen at 50 cm^3/min and scanned from 25ºC to 400ºC at a heating rate of 5ºC /min. The 1 mg/ml gelatin was dispersed in D/W and subjected to zeta potential and particle size analysis by using Malvern Zetasizer Ver. 7.11 instrument. The electrophoretic mobility of the gelatin dispersion was measured by the instrument and then converted into zeta-potential values. The gelatin sample was hydrolysed with 5 N HCl at 100ºC for 24 hr. The amino acid analysis of extracted gelatin was carried out on a Waters-PICOTAG amino acid auto analyser high performance liquid chromatography (Model: Waters 501) connected to the automatic amino acid evaluating software. The Waters-Pico Tag column (size:- 3.9×150 mm) was used for this purpose. Structural characterization was used to determine physical appearance of gelatin which helps to confirm that, extracted component was gelatin.

4.2.5.5. Functional characterization of gelatin

4.2.5.5.1. Solubility and viscosity of gelatin

The 6.67% (w/v) of gelatin was mixed with D/W and heated up to 40-60ºC for solubilisation. The clearance of water was observed for gelatin solubility.

The viscosity of gelatin (6.67%,w/v) was determined according to procedure of Kuan et al.(2016).The viscosity of sample was carried out by using a Brookfield digital viscometer (Model LV-DV-II, Brookfield Engineering, Middleboro, MA, USA) equipped with a No. 1 spindle (Model LV) at 60 rpm and 40±1ºC.

4.2.5.5.2. Turbidity and clarity of gelatin

Turbidity of gelatin was measured according to method of Khiari et al.(2011) with slight modification. 6.67% (w/v) gelatin was dissolved in D/W by heating up to 70ºC and sample was placed into transparent glass tube. The turbidity of the gelatin samples was measured as formazin turbidity units (FTU) using UV visible spectroscopy (Shimadzu UV-1800 Japan) at 450 nm.

The method of Avena et al. (2006) was used to determine gel clarity of gelatin with minor changes. The 6.67% (w/v) of gelatin was heated at 60ºC for 1 hr to form soluble component. Clarity was determined by measuring transmittance (%) at 620 nm (Shimadzu UV-1800 Japan).

4.2.5.5.3. Melting and gelling temperature

The melting temperature was studied according to the method of Shyni et al. (2014) with slight modifications. Gelatin sample, 6.67% (w/v) was formulated and about 5 ml of aliquot was removed into glass tube in triplicate. The tubes were sealed by using parafilm and heated for 15 min at 70ºC. The tubes were instantly chilled in ice cold water and matured at 10ºC for 20 hr. Five drops of blend of 75% chloroform and 25% red food colour (Asian Food products, Maharashtra, India) was kept on the surface of gel. The formed gel sample was placed in water bath at 10ºC. The temperature at which the dye began to enter into gel was recorded as the melting point.

Gelling temperature was determined according to method of Ratnasari and Firlianty (2016) with minor modifications. About 30 ml of 6.67% gelatin solution was taken in glass tube and it was kept in cool box along with thermometer. The crunched ice cube was added gently until gel nature of gelatin forms and gelling temperature was note down.

4.2.5.5.4. Emulsifying properties

The procedure of Bichukale et al. (2018) was used to study emulsifying stability index (ESI) and emulsion activity index (EAI) with minor modifications. With the help of homogenizer, emulsions were prepared with 1%, 3% and 5% of gelatin in 50 ml of soyabean oil. The emulsion was pipetted at 0 min and 10 min. 0.1% sodium dodecyl sulphate (SDS) was mixed with it and absorbance was measured at 500 nm (Shimadzu UV-1800 Japan).

$$\text{Emulsion activity index } (m^2g^{-1}) = \frac{2^{\times}2.303^{\times}A0min}{0.25^{\times}\text{protein weight in gm}}$$

$$\text{Emulsifying stability index (min)} = \frac{A0min}{A0\,min - A10min} \times \Delta t$$

(where; A_{0min}:- absorbance at 0 min, A_{10min}:- absorbance at 10 min, Δ t:-10 min)

4.2.5.5.5. Foaming properties

To determine foaming properties of gelatin method of Tkaczewska et al. (2019) was slightly modified. 1%, 3% and 5% gelatin sample was prepared in water and heated up to 70ºC to dissolve it. All samples were homogenized to generate foam and the foam capacity (FC %) and stability (FS %) was calculated by comparing the ratio of foam to liquid. The foam stability was recorded by comparing initial volume of foam to the volume of foam after 60 minutes.

$$\text{Foaming capacity (\%)} = \frac{VT}{V0} \times 100$$

$$\text{Foam stability (\%)} = \frac{Vt}{V0} \times 100$$

4.2.5.5.6. Water holding and fat binding capacity

The method of Hue et al. (2017) was slightly modified to determine water holding and fat binding capacities (WHC and FBC) of extracted gelatin. For assessing WHC, 1 gm of gelatin was taken in centrifuge tube and mixed with 50 ml D/W. The tube was held at RT for 1 hr. The solution was vortexes for 5 sec after every 15 min and then centrifuged at 4500 x g for 20 min. The supernatant was removed and tube was tilted on paper to drain out remaining liquid. The WHC was calculated as the weight of the pellet after withdrawing liquid divided by weight of the gelatin and expressed as % weight of dehydrated gelatin.

For FBC determination, 50 ml water was replaced by 10 ml of sunflower oil and same procedure was executed to calculate FBC of extracted gelatin. FBC was expressed as % weight of dehydrated gelatin.

4.2.5.5.7. Effect of pH on gelatin solubility

To study effect of pH on gelatin solubility method of Ratnasari and Firlianty (2016) was slightly revised. The 6.67% (w/v) of gelatin was prepared by mixing it with water and stirred at 60ºC. The gelatin solution was formulated from pH 2.0 to 14 by using HCl and NaOH (6 N). The solution was making up to 10 ml with distilled water and adjusted to previous pH. All samples were centrifuged at 7000 x g for 15 min at ambient temperature. The protein content of supernatant was determined according Lowery method by using bovine serum albumin as standard. Relative solubility at each pH was calculated by relating the solubility value at the pH with highest solubility.

4.2.6. Experimental methodology for omega-3 fatty acid

In first step, solvent extraction of fish oil was carried out while in second enzymatic concentration of omega-3 fatty acid by lipase enzyme was done.

4.2.6.1. Solvent extraction of fish oil

The methodology of Bligh and Dyer (1959) was slightly modified to extract fish oil. 10 gm of cleaned and dry fish head waste was crushed by using mortar and pestle for 5 min. The methanol: chloroform (2:1) was added to it and homogenized for 5 min. In next step 10 ml chloroform was added and homogenized for 90 sec. The final homogenization was carried out for 90 sec by using 10 ml D/W. The resultant mixture was centrifuged at 5000 x g for 15 min at 5ºC and filtered through double layered muslin cloth. The filtrate and solids separated from each other. To the remaining solids methanol: chloroform (1:1) was mixed and centrifuged at 5000 x g for 15 min at 5ºC. The waste material containing pellet was removed and supernatant was filtered out. The concentrated filtrate from both steps was mixed together and kept at stable condition to form biphasic layer of aqueous and organic phase. The lipid containing organic phase was collected and passed through anhydrous sodium sulphate then supernatant was collected through filtration. The solution was evaporated at 50ºC to obtain crude fish oil. Further it was subjected to enzymatic hydrolysis to concentrate omega-3 fatty acids.

4.2.6.2. Enzymatic concentration of fish oil by lipase (*S.sciuri*)

The procedure of Mohammad et al. (2018) was slightly changed to carry out enzymatic concentration of fish oil which helps to determine actual composition of omega-3 fatty acid in fish oil. 30 ml of extracted oil treated with lab isolate *Staphylococcus sciuri* was added together. In this reaction, the lipase activity of enzyme from S.sciuri was used to carry out enzymatic hydrolysis of sample. The reaction was carried out at 37ºC for 24 hr in shaking incubator (Remi incubator) at constant stirring

(95 rpm). The both sample and control with respect to enzyme were analysed by GCMS for determination of omega-3 fatty acid concentration in fish oil.

4.2.6.3. Yield of oil (%)

The yield of extracted oil was calculated by following formula with respect to mass of dry matter.

$$\text{Yield of oil (\%) (w/w)} = \frac{\text{weight of oil obtained (gm)}}{\text{mass of dry matter (gm)}} \times 100$$

4.2.6.4. Fatty acid analysis

The composition of fatty acid present in fish oil were analysed by using gas chromatography-mass spectroscopy (GCMS) technique (TQ 8050 plus with HS 20, Shimadzu, Japan). The amount was computed from the integrated peak area as the percentage of the total area of the entire peak. The fatty acids in sample were identified by comparing retention times with those of known standard.

4.2.6.4.1. GCMS instrumentation

For sample separation, DB-5 MS high resolution capillary column (thickness: 0.25 μm, length: 30 m, diameter: 0.25 mm) was used. For temperature control, the oven was maintained at 80°C for one min and then raised up to 250°C with interval of 10°C per min and kept for 5 min. The 10:1 ratio of split injection was conducted while 0.8 ml/min helium gas was used as carrier with 1 μl sample injection volume. The mass spectrometer was functioned in electron-impact (EI) manner. The other experimental conditions include; pre column pressure: 70 kPa, injection temperature: 250°C, ion source: EI (200°C), interface temperature: 280°C, electron energy: 70 eV and solvent delay: 5.5 min. For qualitative analysis, the full scan mode was carried out with 40–400 m/z scan range.

4.2.6.4.2. Preparation of sample

About 60 mg of enzymatically hydrolysed and un-hydrolysed oil sample was taken in a centrifuge tube. 0.5 M of potassium hydroxide methanol was added to it. The components were rigorously mixed, further tube was filled with argon gas and heated at 60ºC in water bath with intermediate shaking for 20 min to form transparent solution. Boron trifluoride methanol complex solution (3 ml) was mixed with it and resulting mixture was kept for cooling. The argon gas was filled in tube and kept in water bath for 5 min.2 ml of each saturated sodium chloride and n-hexane were added to it and mixed properly. The centrifugation was carried out at 4000 x g for 10 min and 1 μl of

supernatant was used as sample solution for GCMS analysis (Yi et al.2014; Mustafa et al.2015).

4.2.6.5. Analysis of fish oil characteristics

4.2.6.5.1. Free fatty acid (FFA) content

The free fatty acid content (%) of fish oil was determined according to method of Murage et al.(2021). 2 gm of extracted fish oil was taken in 125 ml flask and 10 ml of ethanol were added into it. After that 0.5 ml of phenolphthalein was added into it and titrated against 0.1N NaOH until pink colour was observed. The FFA was calculated using following formula;

$$\text{FFA (\%)} = \frac{\text{NaOH (ml)} \times \text{N} \times 28.2}{\text{mass (g)}}$$

Where;

N = normality of the NaOH

mass (g) = mass of sample used.

4.2.6.5.2. Determination of acid value

The acid value of fish oil was calculated according to method of Sirilun et al.(2016) with some changes. It determines triglyceride amount in oil mixture as well as it's an indicator of rancidity and degradation of oil. 1 gm of oil was taken in Erlenmeyer flask and 25 ml of fat solvent (95% ethanol: ether) was added along with few drops of phenolphthalein. The sample was titrated against 0.1 N KOH till colour of solution forms permanent pink colour. The blank was carried out without addition of oil.

The acid value (mg KOH/g of sample) was given by the following formula:

$$\text{Acid value} = \frac{\text{Y}-\text{X} \times \text{N} \times 56.1}{\text{W}}$$

Where;

X= ml of standard alkali used in the titrating the blank

Y= ml of standard alkali used in the titrating the sample

N= normality of standard alkali

W= gm of sample

56.1= molecular weight of KOH in gm

4.2.6.5.3. Determination of iodine value

The iodine value of oil was determined as per method of Hanus (1966) with minor modifications.1 gm of extracted oil was added along with 10 ml CCl_4 into conical flask. To this, 25 ml of Hane's iodine solution was added and flask was kept in dark for 1 hr with frequent stirring. After incubation 10 ml of 15% KI solution along with 50 ml

D/W was added. The resultant solution was vigorously titrated against 0.1 N $Na_2S_2O_3$. Starch was used as indicator until the blue colour disappears. A blank test was carried out without oil under same conditions. The iodine value was calculated using:

$$\text{Iodine value (mg)} = \frac{(Vb - Vs) \times 12.7 \times 100}{W \times 1000}$$

Where;

V_b = volume of standard $Na_2S_2O_3$ solution used for blank test

V_a = volume of standard $Na_2S_2O_3$ solution used for the test sample

W = weight of oil sample (gm)

4.2.6.5.4. Determination of saponification value

The saponification value of oil was calculated as per procedure of Alam and Uddin (2017) with minor modifications. It gives information about the mean molecular weight of the collective fatty acids. It is the number of mg of KOH required to complete saponify 1 g of fat or oil. 25 ml of 0.5 M alcoholic KOH solution was added into 1 gm of extracted fish oil. An inverted funnel was kept on flask and refluxed for 30-40 min. The resultant mixture was cooled at room temperature and few drops of phenolphthalein indicator were added. The solution was titrated against 0.5 M HCl until a pink endpoint was attained. A blank was performed without addition of oil under similar conditions. Saponification value is calculated as;

$$\text{Saponification value (mg)} = \frac{Vb - Va \times 28.05}{W}$$

Where;

V_b = volume of standardized HCl solution used for the blank

V_a = volume of standardized HCl solution used for the test sample

W = weight of sample (gm)

4.2.6.5.5. Determination of peroxide value

The peroxide value of extracted fish oil was calculated as per method of Bako et al.(2017). 5 gm of fish oil sample was taken into conical flask. About 30 ml of glacial acetic acid and chloroform (3:1) was added into it and mixed thoroughly to dissolve sample completely. 0.5 ml KI was added and the solution was allowed to stand in dark with occasional shaking for precisely 1 min further 30 ml D/W was added. The resulting mixture was titrated against 0.1 N sodium thiosulphate ($Na_2S_2O_3$). Few drops of 1% starch indicator were added and titration was continued with constant shaking till the blue colour vanished. A blank was prepared without addition of oil sample. The peroxide value (milli equivalents peroxide/gm sample) was given by the following formula:

$$\text{Peroxide value} = \frac{(S-B)\times N \times 1000}{W}$$

Where;

B= ml of standard $Na_2S_2O_3$ used for titration of blank

S= ml of standard $Na_2S_2O_3$ used for titration of sample

N= normality of $Na_2S_2O_3$

W= gm of sample

4.2.6.5.6. Determination of p-Anisidine value

The p-Anisidine value is used to assess the secondary oxidation of oil or fat. It is a reagent used for the detection of oxidation products such as aldehydes and ketones in oil or fat. The p-Anisidine value of gethar extracted fish oil was calculated as per method of Hiremath et al., (2018). 0.7 gm of oil was taken in 25 ml volumetric flask and final volume was making up with diluted iso-octane. The 5:1 gethar extracted oil and p-anisidine reagent was taken in test tube and analysed by UV after 10 min. For blank 5:1 ratio of iso-octane and p-anisidine reagent was used. Finally absorbance was measured at 350 nm using spectrophotometer. The p-anisidine value was calculated by using following formula;

$$\text{p-anisidine value} = \frac{25 \times (1.2 \times As - Ab)}{W}$$

Where;

A_S = absorbance of the oil solution: p-anisidine reagent

A_B= absorbance of the oil solution

W= weight of sample (gm)

25= size of volumetric flask used

1.2= correction factor

4.2.7. Sensory evaluation of gelatin and fish oil

The sensory evaluation of gelatin and fish oil from gethar was carried out as per protocol of Oladapo and Awojide (2015) and Sinthusamran et al. (2017) along with market gelatin and mackerel fish oil as control. The sensory characteristics of fish oil like appearance, color, fishy odour and overall acceptability were analysed by non-trained 7 member panel from Food Science and Technology department, Shivaji University, Kolhapur using 5 point hedonic scale (from 1:- dislike very much; 2:- dislike slightly; 3:- neither like nor dislike; 4:- like slightly and 5:- like very much). The sensory properties of gelatin like appearance, odour, colour, texture and overall acceptability were analysed by non-trained 9 member panel from same department of Shivaji University, Kolhapur

using 9 point hedonic scale (from 1:- dislike extremely; 2:- dislike very much; 3:- dislike moderately; 4:- dislike slightly; 5:- neither like nor dislike; 6:- like slightly; 7:- like moderately; 8:- like very much and 9:- like extremely).

4.2.8. Statistical analysis

Each experiment was carried out in triplicates and standard deviation was calculated by using basic statistical programme and graph pad prism software.

4.3. Result and discussion

4.3.1. Proximate composition of fish waste and gelatin

Proximate composition includes moisture, protein, lipid and ash content of fish skin and extracted gelatin. The analysis was tabulated in table no.4.1. According to Ward and Courts (1977), the pre-treatment carried out during extraction abolish some cross linked components present on waste and used to eliminate impurities as well as unwanted materials. Waste material of gethar contains 26.42±1.38% moisture, 42.48±1.70% protein, 7.85±0.05% lipid and 2.35±0.14% ash on DWB while 67.88±2.14% moisture, 50.03±2.72% protein, 8.97±0.13% lipid and 3.03±0.07% ash on WWB. Current waste material contains good amount of protein thus it can be suitable for gelatin extraction. Muyonga et al.(2004) described that, increase in collagen content of material causes maximum production of gelatin from waste. Proximate analysis showed that protein content of gelatin was much higher than waste material (table no.4.1). Other contents like moisture, lipid as well as ash was found to be lower than waste. Extracted gelatin contains 14.19±1.04% moisture, 85.08±2.93% protein, 1.50±0.14% lipid and 1.04±0.04% ash on DWB while 65.45±0.37% moisture, 90.30±1.76% protein, 1.49±0.12% lipid and 1.07±0.02% ash on WWB. Similar results were obtained for gelatin extracted from catfish skin (Ardekani et al.,2013), calf skin, carp scale and pork skin (Dincer et al.,2016).

The proximate composition of gethar fish head based on wet and dry weight were tabulated in table no.4.2. As per wet weight, it contains 82.58±0.20% moisture, 21.43±0.32% protein, 9.85±0.57% lipid and 1.29±0.53% ash. The moisture content was reduced up to 51.37±1.56% upon drying the sample. Based on dry weight, fish head waste comprises 24.48±0.11% protein, 11.73±0.44% lipid and 2.35±0.14% ash. The head part of the fish possesses more amount of lipid than any other body site (Mustfa et al.2015). Thus, more amount of lipid was found in gethar head waste. The results were analogues to the proximate analysis of catfish viscera (Adetuyi et al.2014), catfish and mackerel fish waste (Oladapo and Awojide 2015).

Sample	Moisture (%)	Protein (%)	Lipid (%)	Ash (%)
Fish waste (WWB)	67.88±2.14	50.03±2.72	8.97±0.13	3.03±0.07
Fish waste (DWB)	26.42±1.38	42.48±1.70	7.85±0.05	2.35±0.14
Gelatin (WWB)	65.45±0.37	90.30±1.76	1.49±0.12	1.07±0.02
Gelatin (DWB)	14.19±1.04	85.08±2.93	1.50±0.14	1.04±0.04

Table 4.1.: Proximate analysis of fish skin and gelatin

Sample	Moisture (%)	Protein (%)	Lipid (%)	Ash (%)
WWB	82.58±0.20	21.43±0.32	9.85±0.57	1.29±0.53
DWB	51.37±1.56	24.48±0.11	11.73±0.44	2.35±0.14

Values were means ± SD from triplicate determinations

Table 4.2: Proximate composition of fish head

4.3.2. Yield of gelatin (%) and oil (%)

Time required for final extraction of gelatin with D/W was optimized from 1 hr to 3 hr by using 1 gm of waste for each reaction. Figure no.4.1 showed effect of extraction time on gelatin yield. At 1 hr, very less gelatin yield (0.12±0.02 gm gelatin/gm waste) was obtained while maximum yield was attained at 2 hr (0.63±0.03 gm/gm). The decreased yield was observed after 2 hr extraction time and at 3 hr 0.52±0.04 gm/gm gelatin was obtained. Continual hydrolysis of collagen during its conversion into gelatin may decline the final yield and also changes its functional characteristics (Aberoumand 2011).

The decreased yield of gelatin was attained due to partially unstable cross associations present in collagen (Niveditha et al.2020). Extraction of gelatin from pre-treated waste sample of fish by using acid followed by heat generates economically feasible yield of gelatin with good gel strength (Roy et al.2017). The gelatin recovery from waste sample is mainly depends on type of fish skin, acid concentration, solvent pH, breaking of inter linkages in collagen molecule during washing and swelling procedure (Chavan et al.2018). About 14.33% and 9.7% gelatin was produced from pre-treated waste material of gethar and collagen hydrolysis respectively. Figure no.4.2 depicted nature of gelatin before and after drying. The obtained gelatin yield was less

than gelatin extracted from fish hoof skin (20.85%) and shark (18.65%) (Aberoumand 2011), iridescent shark catfish (17.29%) and black spotted croaker (17.52%) (Chavan et al.2018), pangas catfish (22%), asian red tail catfish 21.28%, striped snakehead 20.25% and nile tilapia 21.93% (Ratnasari et al.2013) but higher than that of tilapia skin (12.24%) (Boulahsen et al.2018) and milkfish (12.93%) (Masirah et al.2017). Gelatin yield from collagen hydrolysis was lower than pre-treated waste method. Thus, mass production of gelatin from pre-treated waste was carried out and utilized for further characterization and applicatory studies.

The chloroform, methanol and water were used as effective solvent for extraction of fish oil from gethar head waste by solvent extraction method (figure no.4.3). The variation in PUFA content was occurred due to changes in environmental conditions around the fish species. Also it is related to seasonal variations, alterations in plankton species in their diet and also in the post-spawning time (Khoddami et al.2009). The yield of oil (w/w) was found to be 27.63±0.24%. It is related to the fish oil from bigeye tuna (27.7-31.5%) (Ahmed et al.2017) and head waste of sardine (26.39%) (Khoddami et al.2009). The yield was more than oil from cobia liver (21.15%) (Santos et al.,2016) and intestine as well as liver of sardine (24.90% and 22.67%) (Khoddami et al.2009).

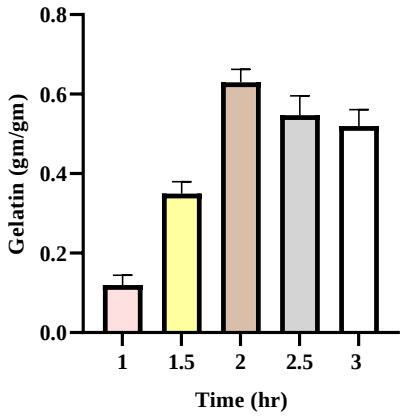

Figure 4.1.:Effect of extraction time on gelatin yield

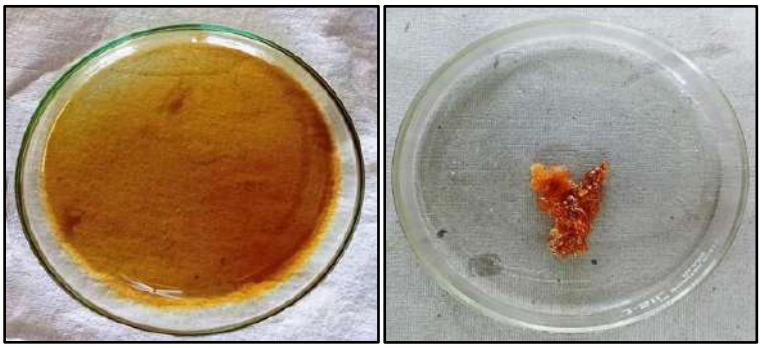

Figure 4.2.:Gelatin a) before drying; b) after drying

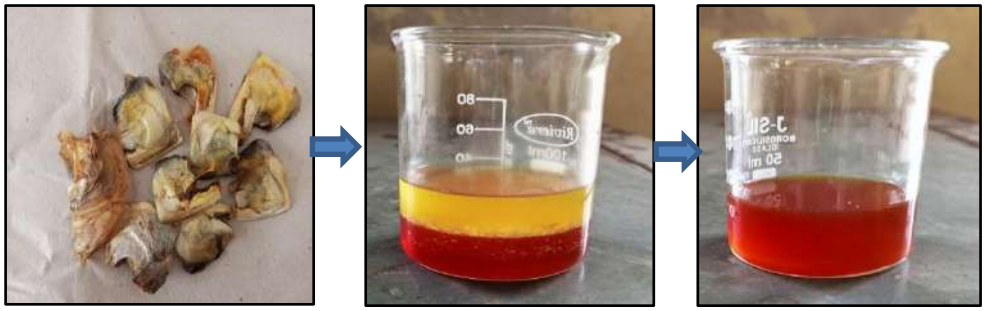

Figure 4.3.: Solvent extraction of fish oil

4.3.3. Yield of Hydroxyproline

Hydroxyproline determination is useful parameter for determination of collagen or gelatin concentration. Hydroxyproline content of gethar skin gelatin was found to be 22.46±0.61%. The yield was less than red tilapia skin gelatin which was 26.84±1.92% (Tinrat and Asna 2017) but higher than 18.5% hydroxyproline in nile tilapia skin gelatin (Zeng et al.2010).

4.3.4. Structural properties of gelatin

4.3.4.1. UV visible spectral analysis

UV visible spectroscopic analysis of extracted gelatin was carried out in the range of 200-800 nm. Chromophore groups present in gelatin exhibited characteristic absorption peak in UV analysis (Hermanto et al.2013). UV absorption spectrum of gelatin was given in figure no.4.4 and it has characteristic spectra at 280 nm. Species of fish, type of raw material used and extraction condition has effect on UV spectra. The n → π* transition of aromatic side chains gives characteristic spectra at 280 nm. The sensitive chromophoric components in the gelatin molecular structure may losses during extraction and therefore it shifts spectra to aromatic region (Xu et al.2017; Maharana and Misra 2018).

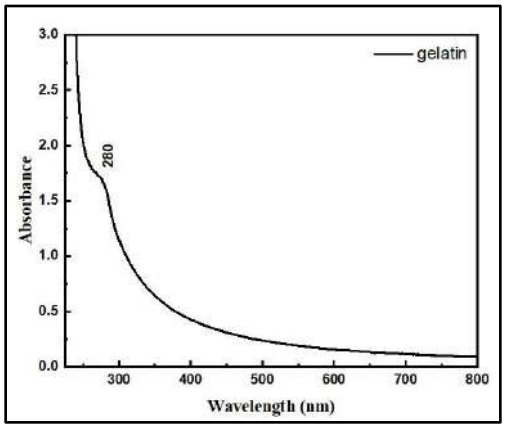

Figure 4.4.:UV spectra of gelatin

4.3.4.2. SDS-PAGE

The SDS-PAGE analysis of extracted gelatin was depicted in figure no.4.5 in which three bands were observed on gel that corresponds to two chains of gelatin. β chain with ~ 200 kD molecular weight and two α-chains (α1 upper and α2 lower) with 116 kD and 97 kD molecular weight respectively. Organic acid used in extraction did not effect on comparative movement as well as molecular weight distribution of α and β chains. The heat treatment during extraction process may cause partial breaking of protein chain into low molecular weight fragments also excess heat causes loss of component of gelatin (Gimenez et al.2005; Shyni et al.2014). It was reported that gelatin with more amount of crosslinking constituents (β- or γ-components) easily forms random coil and generates stable triple helical structure with high gel strength and good viscosity than other gelatins. The generation of degradation fragments is related with decreased viscosity, less melting point, reduced bloom strength and greater foaming properties (Kuan et al.2016). Similar electrophoresis results were obtained for mackerel head, duck feet and bovine gelatin (Khiari et al.2011; Kuan et al.2016).

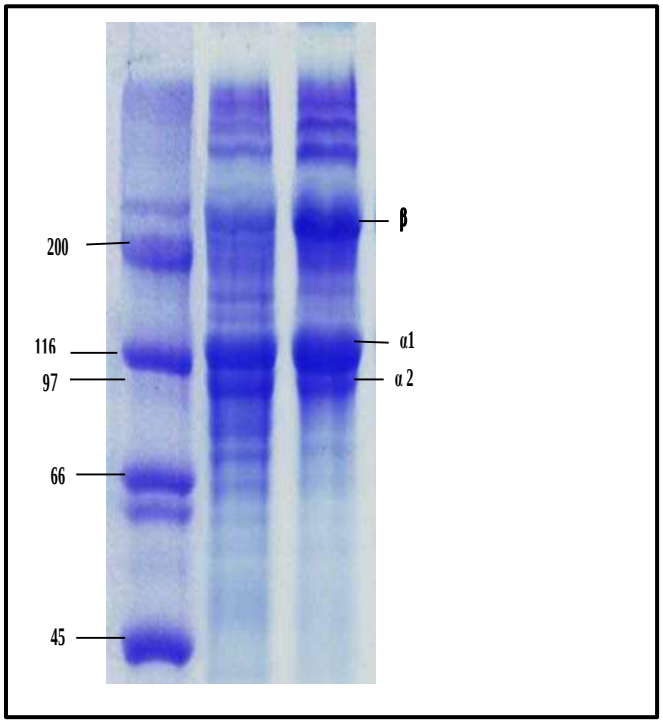

Figure 4.5.: SDS-PAGE analysis of gelatin. Lane 1: protein ladder; lane 2 and 3: extracted gelatin from gethar skin

4.3.4.3. Fourier transform infrared spectroscopy analysis (FTIR)

The various functional groups attached or inserted in gelatin structure during extraction process were analysed by Fourier transform infrared spectroscopy. The FTIR

spectrum of gethar skin gelatin was depicted in figure no.4.6 and it was slightly similar to the FTIR spectrum of catfish skin gelatin (Sai-Ut et al.2012) and black tilapia scale gelatin (Sockalingam and Abdullah 2015). The stretching at 3300 cm^{-1} was due to N-H bond (amide-A band) and it's a characteristic pattern of gelatin (Silva et al.2014). Peaks present at 1384 cm^{-1} and 1448 cm^{-1} were assigned due to methyl group which exhibit symmetric and asymmetric bending vibrations (Das et al.2017).The stretching occurred at 1211 cm^{-1} expresses C-N and N-H in-plane bending while bending at 1625 cm^{-1} occurred due to C=O (Mureithi et al.2017). The peak region present at 1034 cm^{-1} and 1116 cm^{-1} corresponding to Amide I, II and III (Arsyanti et al.2018). The reaction temperature, period of extraction and pH, material used for extraction, cross-linking bonds present in collagen peptide structure have impact on bond formation between gelatin molecule and thus, it exhibited variation in FTIR spectrum (Ahmad et al.2011).

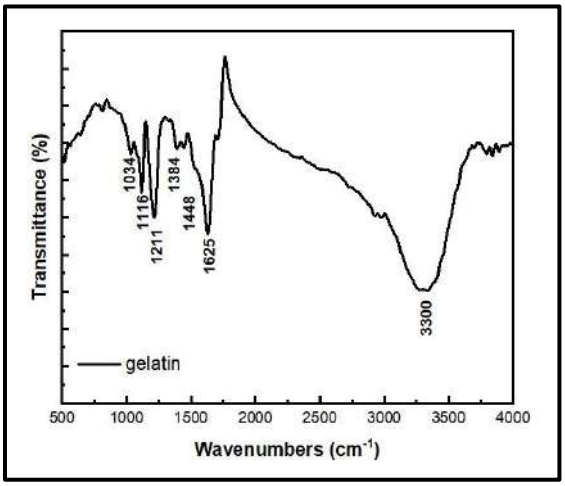

Figure 4.6.: FTIR spectrum of extracted gelatin

4.3.4.4. X-ray diffraction studies (XRD)

The X-ray diffraction analysis was studied to determine crystalline nature of several particles as well as biological structures. The X-ray diffractogram of extracted gelatin were given in figure no.4.7. The graph described partially crystalline nature of gelatin with a broad peak positioned at $2\theta = \sim 19^{o}$. This characteristics peak is occurred due to the triple helical crystalline nature of gelatin (Das et al.2017).The similar diffraction patterns were given by Yakimets et al.(2005) and Pena et al.(2010).

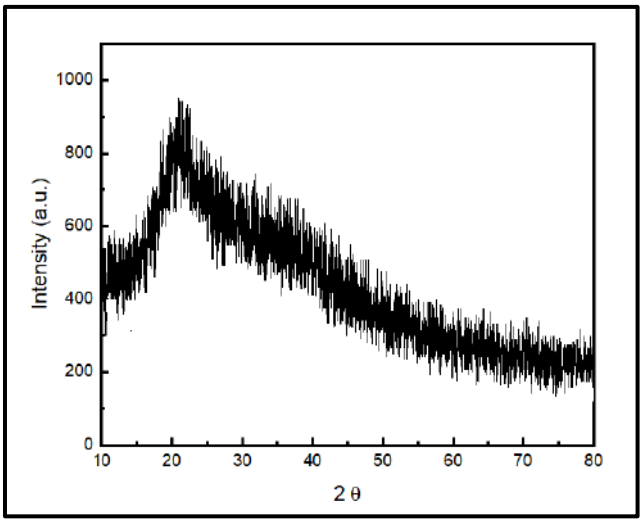

Figure 4.7.: XRD analysis of extracted gelatin

4.3.4.5. Differential Scanning Colorimetry (DSC)

Heat treatment of gelatin during DSC analysis beyond glass transition (Tg) and melting (Tm) temperature results in formation of large endothermic transition peak (Mukherjee and Rosolen 2013). The DSC analysis of gethar extracted gelatin was depicted in figure no.4.8 which exhibited single broad endothermic peak at 119.27ºC and three smaller peaks. Every peak on the heating curve associated to a helix–coil conversion while other number of peaks represents several fractions. The molecular weight distribution data indicates that there are four fractions in gelatin sample. The first small peak at 55.82ºC and broad peak at 119.27ºC is associated with melting temperature of low and high molecular weight fraction respectively (Derkach et al.2019). The breakage of peptide linkages due to polymer decomposition results in weak transition at 226.51ºC (Ghorani et al.2020). The smaller peak at 335.99ºC is occurred due to the isomerization of gelatin sample. The similar results were observed for tailoring cod and calf skin gelatin (Derkach et al.2019; Mukherjee and Rosolen 2013).

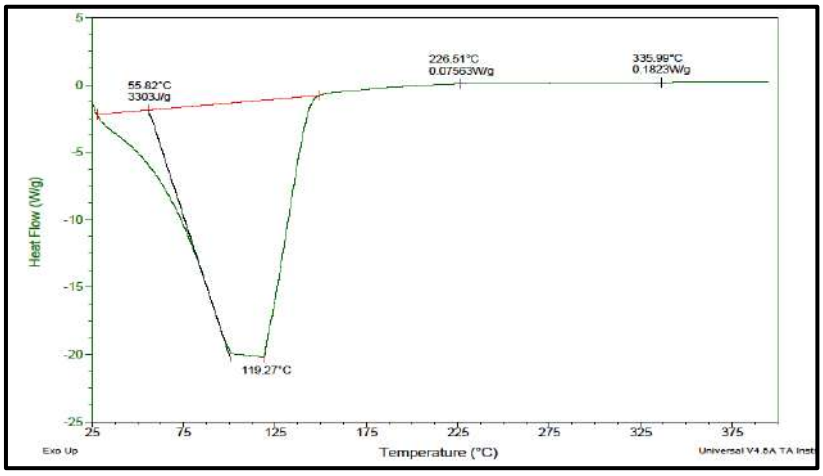

Figure 4.8.: DSC analysis of extracted gelatin

4.3.4.6. Zeta potential analysis

The zeta potential analysis of gethar extracted gelatin was depicted in figure no.4.9 and it was found to be 2.24 mV which is less than 2.56 mV of standard gelatin powder (Ghorani et al.2020). The isoelectric point (pI) of type A gelatin ranges from ~8–9, therefore it results in zwitter ion formation. The solution having pH above pI changes surface charge of the protein into negative and forms more negative carboxylic groups of the amide functional linkages. Nevertheless, acidic environment causes protonation of the amino acid groups thus gives positive charge to the gelatin backbone (Ghorani et al.2020). Usually, gelatin samples exhibited positive charge at acidic pH while negative at alkaline condition. At isoelectric point positive and negative charges were balanced by each other. Therefore, the difference in pI of total gelatin samples is occurred due to difference in their amino acid compositions and distribution. The unequal distribution of amino acid residues may be influenced by size or age of raw material utilized (Sinthusamran et al.2015). The pI of gethar skin gelatin was 8 which is less than pI of young nile perch (8.8) and adult nile perch (9.4) (Muyonga et al.2004).

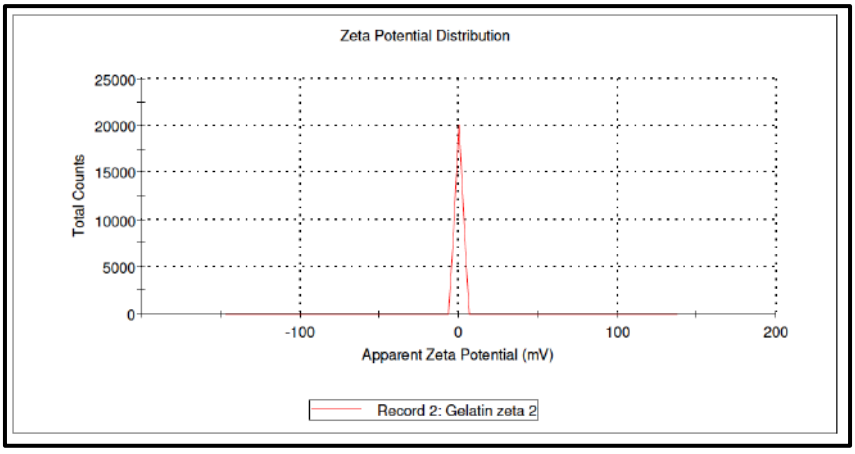

Figure 4.9.: Zeta potential of extracted gelatin

4.3.4.7. Particle size analysis

Particle size analysis is employed to characterize the size distribution of particles in a particular sample. The size distribution of gethar extracted gelatin sample based on its intensity and mass was depicted in figure no.4.10. The mean particle size of gelatin varies from 30-600 r.nm (radius values in nanometres). The yellowish brown colour of gelatin altered to yellowish orange and then to brown when extraction period of was increased (Dinarvand et al.2005).

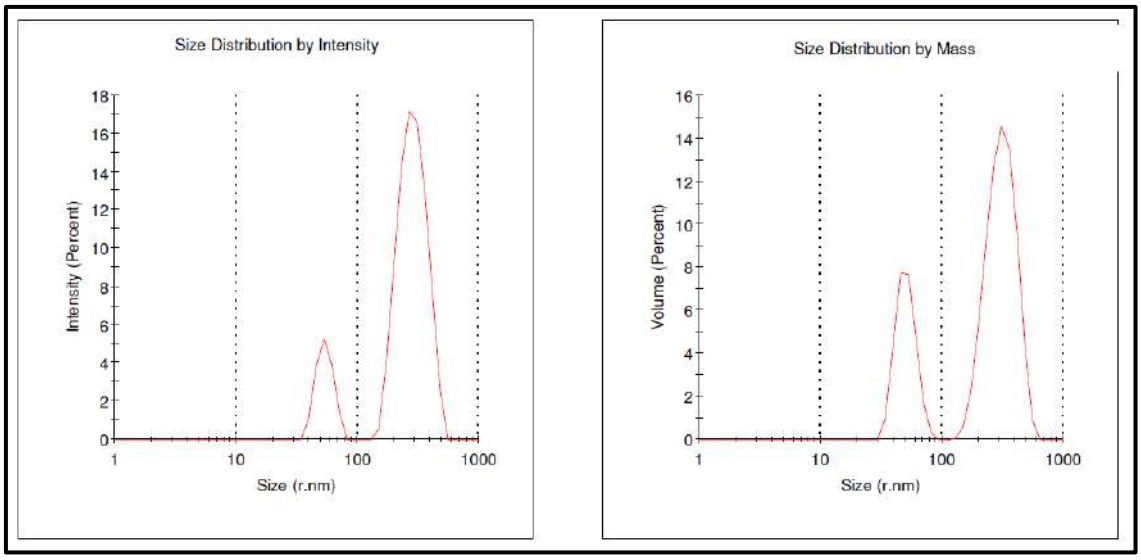

Figure 4.10.: Particle size analysis of extracted gelatin

4.3.4.8. Amino acid analysis

The distinctiveness of the amino acid sequence imparts characteristic coiled coil nature to the collagen molecule. The triple helical structure was steadied by inter chain hydrogen bonds as well as covalent cross linkages (Brodskey and Ramshaw 1997). The arrangement of amino acid determines the physical characteristics of gelatin. These physical characteristics influenced by amino acid arrangement, comparative amount of components as well as aggregation of higher and of lower molecular weight protein fragments. Also, the preservation of raw material is the key factor in determining properties of gelatin (Kala and Mathew 2017). Thermal degradation as well as physical or chemical denaturation causes the breaking of collagen triple helical polypeptide into gelatin (Bigi et al.2004). At temperature of about 40ºC, the aqueous solution of gelatin is in sol state and converts into physical thermo reversible gels upon cooling. During gelling the chains undergoes conformational changes, order alteration and slightly reorganize the triple helical collagen structure (Pezron et al.1991). The less amount of imino acid forms the less sterically hindered helix and may disturb the specific properties of gelatin. The HyP along with proline has important role in the stabilization of the triple helical collagen due to its hydrogen bonding capacity through its –OH group (Gormez-Guillen 2005).

The configuration of amino acids has main importance in determining gelatin gel strength and melting point. The significant amount of imino acid (proline and HyP) and glycine gives better strength to gelatin gel. The composition and alignment of the protein and amino acid differs in every fish species (Hossain et al.2016). The amount of proline and HyP was found to be 115.66±0.94/1000 residues and 92±0.86/1000 residues

respectively. The content of glycine of gethar gelatin (313.66±2.05/1000 residues) was more than bigeye snapper (246.57-259.38 residues/1000 residues; Benjakul et al.2009) but it was less than amur sturgeon skin gelatin (336 residues/1000 residues; Nikoo et al.2011) and shark species (321-322 residues/1000 residues; Kittiphattanabawon et al.,2010).

The acid hydrolysis of gelatin converts some amount of glutamine and asparagine into its acidic forms; i.e. glutamic acid and aspartic acid, respectively, and the associated removal of ammonia (Irwandi et al.2009). The aspartic and glutamic acid was found to be 49.66±1.69/1000 residues and 69.33±1.24/1000 residues respectively. The amino acid analysis of gethar extracted gelatin was tabulated in table no.4.3. The similar results were obtained for gelatin from the skin of farmed amur sturgeon and swim bladder of yellowfin tuna (Nikoo et al.2011; Kaewdang et al.2015).

Amino acid	**No.of amino acid per 1000 residues**	**% of amino acid**
Aspartic acid	49.66±1.69	4.96
Glutamic acid	69.33±1.24	6.93
Serine	44±1.63	4.4
Glycine	313.66±2.05	31.36
Histidine	3.66±0.47	0.36
Arginine	52.66±1.24	5.26
Threonine	27±0.86	2.7
Tyrosine	8.66±0.43	0.86
Alanine	108.33±0.94	10.83
Valine	22±0.81	2.2
Methionine	8.66±0.47	0.86

Cysteine	1	0.1
Isoleucine	17.66±1.24	1.76
Leucine	23.66±0.45	2.36
Phenylalanine	17±0.82	1.7
Lysine	26.66±0.44	2.666
Proline	115.66±0.94	11.56
Hydroxyproline	92±0.86	9.2

Table 4.3.: Amino acid analysis of extracted gelatin

4.3.5. Functional properties of gelatin

4.3.5.1. Gelatin solubility and viscosity

Generally commercial gelatin (eg: Hi-Media gelatin) used for laboratory experiment is hot water soluble while food grade gelatin in market is soluble in warm water. The gethar extracted gelatin was found to be soluble in hot water at 40ºC temperature and this is analogues to solubility of commercial gelatin.

Viscosity is the resistance of a fluid to change in shape or movement of neighbouring portions relative to one another. As per the British standard, gelatin viscosity values range from 1.5 to 7 cP. Figure no.4.11 showed viscous solution of gelatin. The viscosity of gethar skin extracted gelatin was reported as 6.67±0.19 cP. When compared with bovine skin gelatin, the obtained value is much more (1.74±0.03 cP; Khoirunnisa et al.2018) but less than 13.53±0.23 cP of black kingfish gelatin (Koli 2019). The viscosity was influenced by various factors which include temperature, pH, concentration as well as molecular weight and size distribution of proteins. Instrumental value of high gel strength does not give more viscosity value. The more viscosity of gelatin is occurred due to insoluble components present in it and less foam formation capacity (Lin et al.2015).

Figure 4.11.: Viscous solution of gelatin

4.3.5.2. Turbidity and clarity of gelatin

The decreased transparency of sample is generally referred as turbidity. Gel turbidity and clarity are complementary to each other. The effectiveness of purification procedure causes increased or decreased transparency of gelatin gel (Alfaro et al.2013). Gel clarity of sample is depends on temperature during extraction process. The figure no.4.12 illustrated the slightly turbid gelatin solution after clarification. The gelatin turbidity and clarity was influenced by inorganic and muco-substance contaminants which are not removed during purification as well as type of fish and raw material used (Eastoe and Leach 1977).

Clarity of gethar extracted gelatin was found to be 41.1±1.31 while turbidity value was 257.66±6.54 FTU. The gel clarity value of gethar skin gelatin was less than pacu skin and squid skin gelatin which ranges from 49.40±0.06 to 55.62±0.07 (Sahoo et al.2015; Nagarajan et al.,2012). The resulted turbidity value was higher than wami tilapia skin gelatin and mackerel head gelatin which was found to be 67 ± 4.3 NTU (Nephelometric turbidity unit) (Alfaro et al.2013) and 176±3.2 FTU (Khiari et al.2011) respectively. Gimenez et al. (2005) stated that, lactic acid employed for solubilisation of material causes more amounts of aggregation and thus results in greater turbidity value.

Figure 4.12.: Solution of gelatin after clarification

4.3.5.3. Melting and gelling temperature

It was reported that, melting and gelling temperature of fish gelatin was less than mammalian gelatin. Fish gelatin with less melting temperature releases enhanced odour and offered stronger aroma during preparation of food product also, control the consistency and flavour release during chewing of products. Gethar extracted gelatin possesses 10.75±0.65ºC gelling and 29.88±0.41ºC melting temperature. The results were less than gelling (28.69±0.67ºC) and melting (36.40±0.53ºC) temperature of bovine lung gelatin (Roy et al.2016) while more than cod skin extracted gelatin (gelling 4ºC and melting 13ºC; Haug et al.2004). Maturation time of gelatin results in rise of melting temperature (Koli et al.2013). The gelling and melting temperature is influenced by type and composition of raw material used, concentration of amino acid as well as molecular mass distribution of peptides in the gelatin solution. It has been reported that amount of imino acids (hydoxyproline+proline) is important parameter to study gelling and melting temperature (Pavan kumar et al.2017).

4.3.5.4. Emulsifying properties

Oil droplets were surrounded by longer chain so as to form sturdy and firm films and therefore increase the steadiness towards emulsion breakdown (Devi et al.2016). The attraction of protein towards oil droplets depends on surface hydrophobicity of proteins. The fundamental characteristics of protein, methods of formation and solubility have effect on emulsion capacity of protein (Damodaran 1997 and Sikorski 2001). Emulsifying characteristics of food proteins describe its capacity to stabilize newly generated emulsion and also provide strength to emulsion against stress conditions. Gelatin is a good surface active agent and has better emulsifying activity in oil-water emulsion (Zakaria and Bakar 2015). Table no.4.4 gives information about emulsifying activity index (EAI) and emulsion stability index (ESI) of 1%, 3% and 5% gelatin. EAI of gethar skin extracted gelatin ranges from 21.78±0.06 m^2g^{-1} to 29.24±0.06 m^2g^{-1} and it was highest for 3% gelatin with 37.17±0.12 m^2g^{-1}. ESI of gelatin varies from 12.63±0.29 min to 28.95±0.09 min and it was more for 3% gelatin with 28.95±0.09 min. The ESI and EAI of control gelatin were much higher than extracted gelatin. EAI was more than EAI of gelatin from skin of striped cat fish (11.21±0.72 to 16.89±0.05 m^2g^{-1}, Singh and Benjakul 2016). The value of ESI was analogues to ESI of marine iguana and great barracuda (Devi et al.2016).

4.3.5.5. Foaming properties

A protein with good foaming properties has capacity to migrate quickly to the air water boundary as well as have ability of unfolding and rearrangement (Devi et al.2016). Foaming capacity (FC) and stability (FS) are important functional properties of protein. Gelatin is generally employed as protein foaming agent. Due to this property, it may stabilize foams speedily and efficiently at less concentration and therefore utilized as an essential foaming agent over the wide pH range in different food applications (Zakaria and Bakar 2015). Table no.4.4 depicted FC and FS (%) of 1%, 3% and 5% gelatin. FC was increases from 1.22±0.08% to 1.40±0.21% and it was higher for 5% gelatin while FS rises from 1.06±0.01% to 1.09±0.01% and more for 5% gelatin. The values were slightly less than control gelatin.

Interaction of protein molecule with air-water surface generates foam and it was controlled by transportation, dispersion and reformation of protein component. More concentration of protein generates thick and steady foam, thus FC and FS increases with increase in gelatin percentage. Foaming properties were influenced by different factors such as protein source, protein water interaction, protein surface characteristics, equilibrium rate of surface tension, bulk and superficial viscosities, steric steadiness and electrical repulsion between the two edges of foam lamella (Pavan kumar et al.2017). The results were analogues to gelatin extracted from cobia (2.28% and 1.93%) and cynoglossus (2.35% and 1.86%) (Kala and Mathew 2017) but was much less than gelatin from skin of striped cat fish (Singh and Benjakul 2016).

Sample	Emulsifying activity index	Emulsion stability index	Foaming capacity	Foam stability
	($m^2 g^{-1}$)	(min)	(%)	(%)
1 % gelatin	21.78±0.06	12.63±0.29	1.22±0.08	1.06±0.01
3 % gelatin	37.17±0.12	28.95±0.09	1.34±0.07	1.03±0.02
5 % gelatin	29.24±0.06	19.22±0.32	1.40±0.21	1.09±0.01
Control	49.25±0.08	47.20±0.38	3.77±0.09	3.41±0.13

Table 4.4.: Emulsifying and foaming properties of gelatin

4.3.5.6. Water holding and fat binding capacity

Water holding (WHC) and fat binding capacity (FBC) are important functional characteristics that are resembled to interaction between gelatin surface with the water, oil and other constituents. Number of hydrophilic amino acids has positive effect on WHC while high value of FBC was attained due to more amount of tyrosine present in gelatin (Ninan et al.2009). The WHC of gethar extracted gelatin was found to be 262.33±13.88% which is higher than cobia skin (179.26±3.30%) and cynoglossus skin (169.34±4.01%) (Kala and Mathew 2017) as well as rohu skin (184.33±3.30%) and common carp skin (176.00±4.90%) (Ninan et al.2009). The FBC of extracted gelatin was recorded as 407.66±14.83% which is lower than rohu skin (457.33±6.55%) but was higher than common carp skin (333.00±5.10%), cynoglossus (389.90±5.12%) and cobia (369.78±4.67%) (Kala and Mathew 2017; Ninan et al.2009).

The WHC of gelatin is a useful property for the preparation of sausages, custards and dough because these products include gelatin dispersed in water which is used as thickening agent as well as increases viscosity of product. More value of FBC is required to preserve flavour, increases tastiness and extends shelf life of meat products. FBC is assumed to be associated with the percentage of available hydrophobic, electrostatic and hydrogen linkages as well as mainly with hydrophobic amino acids like tyrosine, leucine, valine and isoleucine (Roy et al.2016).

4.3.5.7. Effect of pH on gelatin solubility

The effect of pH on gelatin solubility was observed at broad pH range of 2-14 as showed in figure no.4.13 and it was increased from pH 2.0 to 6.0 and reached to maximum solubility (99.55%) at pH 6 while very low solubility was obtained at pH 8 (81.36%). However, commercial gelatin has maximum solubility at pH 6 (99.60%). This difference in solubility may be due to alteration in molecular weight and unequal distribution of polar and non-polar group concentration in amino acid. The pH solubility is essential requirement for functional food used as a protein supplement in food industry (Ratnasari and Firlianty 2016). The results were closely resembled with gelatin of pangas catfish (pH 5.8) and walking catfish (pH 5.9) (Cheow et al.2007) but higher than gelatin from nile tilapia (pH 3.05) and black tilapia (pH 3.91) (Bakar and Harvinder 2002). The reduction in solubility at pH 8.0 is adjacent to its isoelectric point and it is due to greater electrostatic interaction. Gelatin is an amphoteric protein with an isoelectric point between pH 5.0 to 9.0 depending on raw material and method of extraction. At pH values below and above the isoelectric point, proteins possess more net charges, thus improving

hydration (Haddar et al.2011). Increased viscosity and higher pH were positively associated with each other, thus increasing viscosity results in rise of gelatin pH. The increasing pH from 2.0 to 14 causes decrease in viscosity of gelatin at pH range of 6.0-8.0 (Stainsby 1987b).

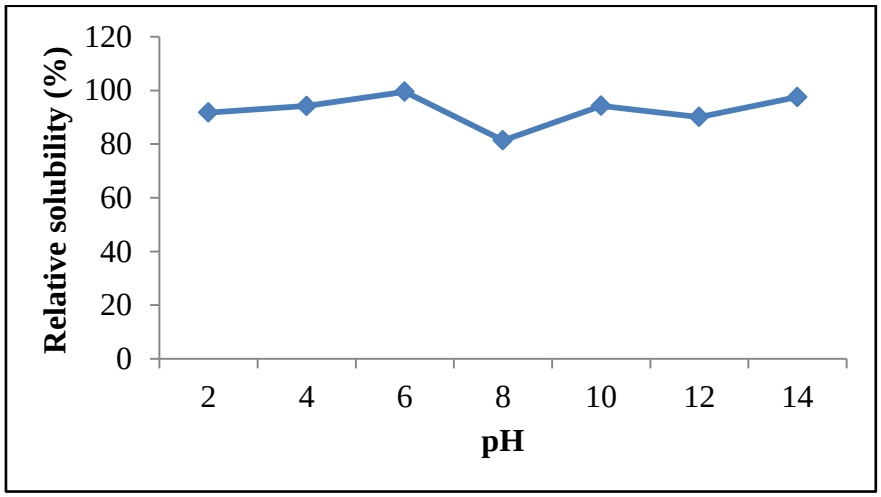

Figure 4.13.: Effect of pH on relative solubility of gelatin

4.3.6. Fatty acid profiling of fish oil

Gas chromatography followed by mass spectrometry was employed to detect and quantify the composition of fatty acids present in fish oils. Figure no.4.14 (a) and (b) elucidates the chromatogram of gethar fish head extracted oil before and after enzymatic action respectively. The triglycerides structure in gethar oil undergoes saponification to produce glycerol and free fatty acids. Then, methanol causes methylation of free fatty acids and derivatization in the methyl ester form and produces fatty acid methyl ester (FAME) (Iberahim et al.2018). The composition percentages polyunsaturated fatty acid is 3.18% EPA and 14.52% DHA before concentrated by enzyme while 1.74% EPA and 14.19% DHA was obtained after enzyme action which showed significant change and quantity of polyunsaturated fatty acid was reduced. This spontaneous change was observed due to auto-oxidation and photo-oxidation of fish oil.

It was studied that, polyunsaturated fatty acids more prone to oxidation than other. The oxidation of PUFA may release hydroperoxide and it will further oxidized into ketones or aldehydes compound (Iberahim et al.2018). The obtained results are in accordance with fish oil extracted from head of tropical little tuna in which 1.48% EPA and 15.7% DHA while in skipjack tuna 0.1% EPA and 18.8% DHA was found (Khoddami et al.2012; Chantachum et al.2000). The alteration in fatty acid compositions was occurred due to variations in the raw material utilized which has a lesser content of EPA and DHA than the cold water. The fatty acid composition of marine fishes varied on

the basis of lipid content of catch, water temperature, breeding season, age, geographical indices and nature of species (Ferdosh et al.2016).

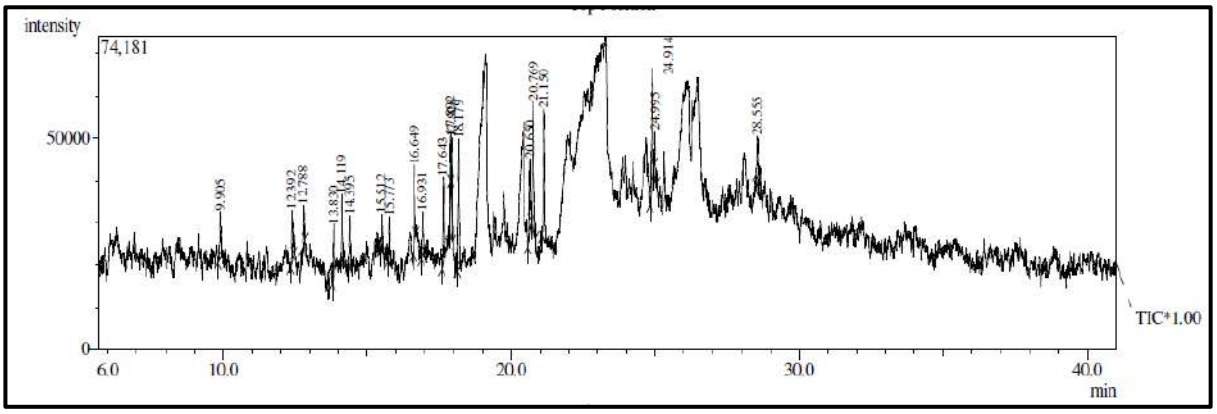

Figure 4.14 (a).: GCMS analysis of oil before enzymatic action

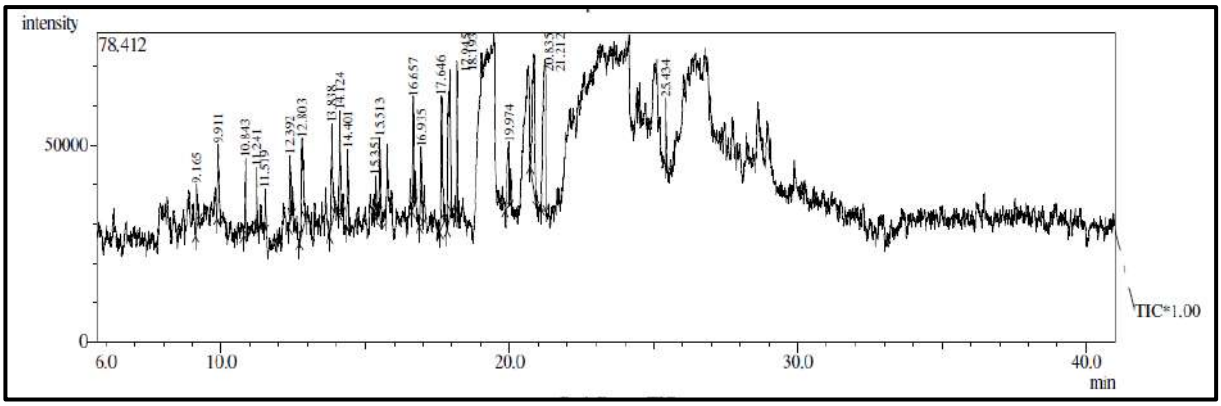

Figure 4.14 (b).: GCMS analysis of oil after enzymatic action

4.3.7. Functional properties of fish oil

4.3.7.1. Free fatty acid (FFA) content

The free fatty acid content determines the degree of disintegration of lipase activity and it was enhanced by heat as well as light. The lower value of FFA means oil is better quality because it has low lipase concentration (Aryee and Simpson 2009). About 2.25±0.20% FFA was present in extracted fish oil. The amount was higher than 0.7-1.7% of bigeye tuna (Ahmed et al.2017) but lower than 3.4% of tuna canning waste oil (Suriani and Komansilan 2019). Free fatty acids are vital component of hydrolytic degradation related to bad flavour and textural alterations. As per the International Association of Fish Meal and Oil Manufacturers (IFOMA 1981) the standard FFA value of fish oil ranges from 1%-7%. Above 3.5% FFA content of oil is inappropriate for edible purpose. The extracted oil has less than 3.5% FFA thus it may be suitable for edible purposes (Bako et al.2017).

4.3.7.2.Acid value of oil

The acid value determines the quantity of potassium hydroxide in milligram required to counterbalance the free fatty acid per one gm of oil. According to research, the lesser acid value means better the quality of oil while higher value is accompanying with the rancidity produced by hydrolysis of ester bonds as well as oxidation of double bonds (Ahmed et al.2017). The appropriate acid value of oil that can consume by human was lower than 5mg KOH/gm. The acid value was influenced by some factors including extraction method, freshness of raw resources and oil composition (Iberahim et al.2018). The acid value of gethar fish extracted oil was 2.57±0.36 mg KOH/gm of sample which was higher than 2.24mg KOH /gm of catfish oil (Iberahim et al., 2018) and lower than 13.63-16.94 KOH/gm of cobia liver oil (Santos et al.2016).

4.3.7.3. Iodine value

The quantity of unsaturated fatty acids present in fish oil was calculated using iodine value. The double bonds exhibited in unsaturated fatty acids would interact with iodine. More iodine value of oil indicated more number of unsaturated fatty acids. So it can easily bind with the iodine in large proportion (Pandiangan et al.2018). The iodine value of extracted oil was found to be 107.88±0.53 gm iodine/gm oil. The obtained value is related to the iodine value of crude, refined and hydrolyzed oil from different fish species and was reported as 117.7±0.2, 119±1.2 and 93.92±0.7 gm iodine/gm oil respectively (Nascimento et al.2015).

4.3.7.4. Saponification value

Saponification is the breakdown of neutral fat due to by alkali treatment into glycerol and fatty acids. The standard sap value for fish oil was 180-200 mg KOH/gm given by AOAC. The sap value of extracted oil was recorded as 78.53±0.49 mg KOH/gm oil which was much less than 295.4 mg KOH/gm oil of leaching fish waste as well as 177.8 mg KOH/gm of tilapia visceral oil (Norziah et al.2010; Oliveira et al.2013). It was observed that, crude oil comprises slight quantity of non-triglyceride constituents. Thus, higher sap value may be due to impurities that is unsaponifiable matter including sterols, glycerol ethers, hydrocarbons, fatty alcohols as well as less quantities of pigments and vitamins exhibited in crude fish oil (Bimbo and Crowther 1991; Norziah et al.2010).

4.3.7.5. Peroxide value

During the oxidation of oil hydrogen peroxides were generated and their quantity was determined by peroxide value. According to research, less peroxide value indicates good quality of oil. The gethar fish extracted oil has 25.72±0.45 Meq/gm oil peroxide

value which was higher than 2.5-5.5 Meq/gm oil of salmon waste (Jayasinghe et al.2013) and 20 Meq/gm of catfish oil (Iberahim et al.2018). There are various components that increases oxidation rate of oil. It includes moisture content, fatty acid concentration, light intensity, oxygen accessibility and temperature. Also greater amount of polyunsaturated fatty acid will cause fish oil enormously liable to oxidation (Iberahim et al.2018).

4.3.7.6. p-anisidine value

Anisidine value is an experimentally tested value which decides the progressive oxidative rancidity of oils and fats. The measure of α and β unsaturation due to secondary oxidation were determined by using p-anisidine value (Lee et al.2017). The p-anisidine value of gethar fish oil was recorded as 17.29±0.48 Meq/gm oil which was more than sardine fish (7.48 Meq/gm oil) and oyster oil (6.52-12.14 Meq/gm oil) (Hiremath et al.2018; Lee et al.2017).

4.3.8. Sensory analysis of gelatin and fish oil

The sensory analysis like appearance, odour, colour, texture and overall acceptability of pure and extracted gelatin were monitored using 9 point hedonic scale. The sensory analysis of extracted gelatin along with control was given in figure no.4.15. The gethar skin extracted gelatin has slight fishy odour but not easily perceivable. It has jelly like appearance with sticky nature and brownish yellow in colour. The colour and odour can be improved by utilizing more advanced technology for clarification of gelatin. Hence, good sensory characteristics of gelatin can be acceptable for various applications. The disparity in sensory characteristics of extracted gelatin was may be influenced by environmental living conditions of fish such as sea depth of fish habitat and habits, materials used for extraction, pollution level as well as variety of planktons around it (Jamilah and Harvinder 2002; Irwandi et al.2009).The sensory analysis was correlated to gelatin extracted from rohu, common carp and kerisi (white snapper) (Irwandi et al.2009; Ninan et al.2009). The sensory assessment of gethar fish extracted oil along with control (mackerel oil) was depicted in figure no. 4.16. The appearance, colour, fishy odour and overall acceptability were analysed using 5 point hedonic scale. The oil has slight fishy odour than control with reddish brown in colour as well as appeared same as control oil. Thus, due to good sensory properties it can be suitable for various applications. The similar analysis was observed for catfish and mackerel oil (Oladapo and Awojide 2015).

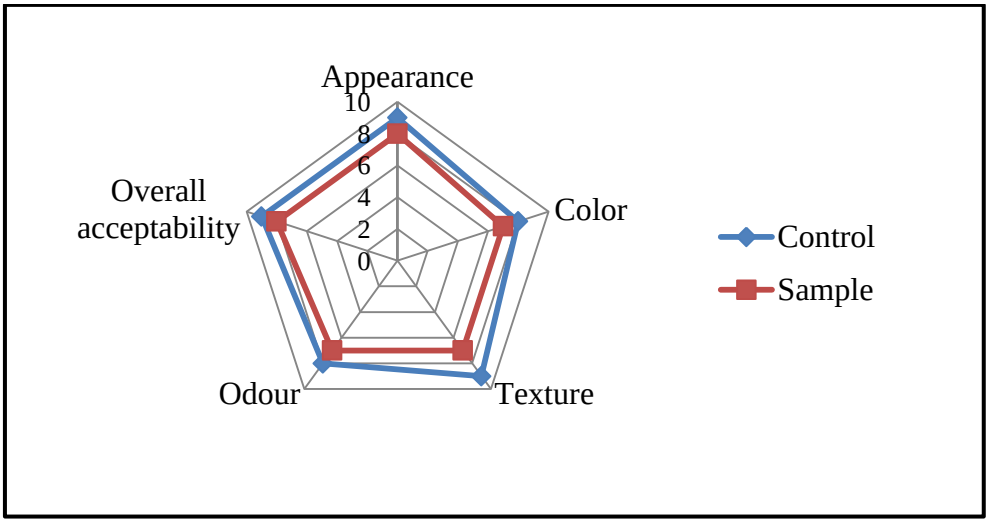

Figure 4.15.: Sensory analysis of extracted gelatin

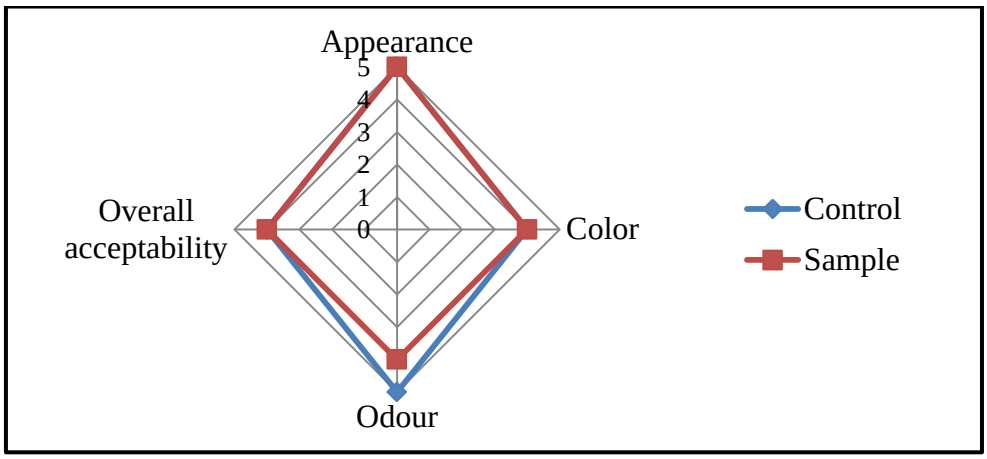

Figure 4.16.: Sensory analysis of gethar fish oil

4.4. Conclusion

In current study, proficient minimization and utilization of fish waste into value added products provides an attractive way to reduce some environmental problems by sustainable development. The fish waste which may acts as major environmental pollutant to disturb ecological balance was effectively utilized for extraction of gelatin and fish oil containing polyunsaturated fatty acids. Gelatin extracted from skin of gethar (*Sarda orientalis*) exhibited better yield with lactic acid treatment in less extraction time. It gives 0.63±0.03 gm/gm gelatin within 2 hr. The structural properties like UV-visible spectra, SDS-PAGE analysis, FTIR, XRD, DSC, zeta potential and particle size analysis confirmed that extracted gelatin was type A with pH 8.0 as isoelectric point. Extracted gelatin exhibited significant water holding capacity, fat binding ability, emulsifying and

foaming properties as well as good gelling and melting temperature. The good amount of lipid was found in proximate analysis of fish head so it can be useful for oil extraction. The methanol, chloroform and water are the best solvent system for oil extraction with 27.63±0.24% yield. The free fatty acid 2.25±0.20% was noted. The acid, iodine and saponification value of oil was found to be 2.57±0.36 mg KOH/gm of oil, 107.88±0.53 gm iodine/gm oil and 78.53±0.49 mg KOH/gm oil respectively. The oil possesses 25.72±0.45 Meq/gm oil peroxide and 17.29±0.48 Meq/gm oil p-anisidine value. Extracted oil exhibited good sensory properties with slight fishy odour. Gas Chromatography-Mass Spectroscopy was used to analysis the fatty acid composition of gethar fish oil. The analysis shows that there are two types of omega-3 polyunsaturated fatty acid found in gethar oil which is EPA (1.74%) and DHA (14.19%). Due to better characteristics gethar fish extracted gelatin and omega-3 fatty acid can be suitable for potential applications in nutraceutical as well as pharmaceutical fields. Therefore, there is a potential for exploitation of processing waste for gelatin and omega-3 fatty acid extraction from these species and also provides best health benefits.

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CHAPTER V

MICROBIAL DEGRADATION OF RESIDUAL FISH WASTE AND USE OF HYDROLYSATE AS PLANT GROWTH STIMULATOR

5.1. Introduction

From many decades, aquaculture and capture fisheries have contributed to the international economy by supporting livelihood as well as nutritional status. The subsequent rise in this area generates more than 60% by-products as waste which comprises scales, skin, head, frames, tail, fins and viscera. These unutilised wastes of fish are simply discarded which produces hazardous pollution and threat to the environment or can be used to prepare low market value products like fish meal, animal feed and fertilizers (Wangkheirakpam et al.2019). The fish releases non faecal components such as ammonia, urea, ortho-phosphate, and carbon dioxide into the surrounding water body. These wastes are harmful to the fish, if they are not removed and threat to environment (eutrophication and greenhouse effect), if they are released outside.

According to the research, fish waste contains some value added products including proteins like collagen and gelatin, amino acids, bioactive peptides, enzymes, oil and minerals (Kim and Mendis 2006; Blanco et al.2007; Ghaly et al.2013). More quantity of organic content was present in solid waste of fish released from seafood processing industries. Conventionally, fish wastes can be employed as fertilizer due to presence of its nutritive components (mainly N and P) and their quick disintegration. By using bioconversion practises, this waste can be converted into eco-friendly compost. The manufacturing of compost containing fish debris mainly obtained from aquaculture have been carried out in different regions of the world to find out feasible and sustainable procedures to convert fish waste into beneficial agricultural products. The microorganisms were utilized in this bioconversion helpful to accelerate the composting process. The produced compost is rich in nutrients as well as free from harmful elements. Thus it can be useful as organic fertilizer instead of commercially available chemical fertilizers. Composting method eliminates disease-causing organisms and flies larvae. Therefore, composting of fish waste is comparatively new, economically feasible, partially odourless method, environmentally safe and biologically favourable operation of fish waste disposal as compared to other discarding options (Lopez-Mosquera et al. 2011; Rajeswari et al.2018).

Protein hydrolysates like feather and fish hydrolysates are smaller group of plant biostimulants having phyto hormones like ability. Protein hydrolysates are made up of free amino acids and polypeptides as well as may consist of macro and micro-nutrients, polysaccharides and lipids which are present in original resources. The protein containing hydrolysate have capacity to improve physiological properties of crops that causes

growth which includes enhancement of yield and product quality, creating tolerance against environmental conditions like salinity, drought, thermal as well as nutrient stress and elevated soil pH (Fitriyah et al.2022).

Exploitation of waste containing organic matter in soil, is an appropriate way which naturally maintain the organic content in soil and has impact on soil fertility as well as provide essential nutrients required for plant growth (Ellyzatul et al.2018).The nutrients in fish waste hydrolysate stimulate development of plants through growth enhancing rhizobacteria, atmospheric nitrogen fixation and improving the uptake of necessary nutrients. Fish waste comprises vital minerals like calcium, phosphorus, potassium, sodium, magnesium, zinc, manganese and copper comparable to the nutritive value as fish so, it can acts as raw material for making of various nutritive and non-nutritive components. The quantity of protein, amino acids, calcium and phosphorus were found to be raised after the microbial degradation of the fish waste. These constituents are mainly utilized by plants for their growth and development (Thankachan and Chitra 2021).

Radziemska et al.(2018) stated that fertilizer from fish waste effectively utilized in agriculture. The study was carried out on ice lettuce (*Lactuca sativa L.*) to evaluate yield as well as macro and micro elemental composition of plant. The fish pond effluent was employed as organic fertilizer on growth of cucumber (*Cucumis sativus*) and helps to improve chemical properties of soil (Ndubuisi 2019). The fertilizer generated from fish and fish waste was effectively employed in organic farming to increase the properties of crop and horticultural plants (Ahuja et al.2020). There was little information about effect of protein hydrolysate derived from chicken feather waste and fish waste remaining after processing in agricultural crops (Fitriyah et al.2022).

The current study was designed for microbial degradation of residual fish waste remaining after valuable collagen recovery. The fish waste degrading micro-organism was isolated from waste dumping site and utilized for microbial hydrolysis of waste into protein hydrolysate. The nutrient rich protein hydrolysate was studied for its plant growth promoting potential on *Vigna radiata* as well as soil nourishment properties of hydrolysate was studied.

5.2. Materials and method

5.2.1. Sample collection

The soil samples were collected from fish waste dumping site of the Ichalkaranji region, Kolhapur, Maharashtra, India. The samples were collected in sterile autoclavable plastic bags and stored at RT for further studies.

5.2.2. Bacterial isolation

Approx. 1 gm of soil sample was added into minimal broth which consists of (gm/l): KH_2PO_4, 3; Na_2HPO_4, 6; NaCl, 5; NH_4Cl, 2; $MgSO_4$, 0.1; glucose, 8 and enrichment was carried out for 48 hr at 30ºC. The enrichment was repeated for 3 times. At the end of day, enriched sample was subjected to primary screening for isolation of micro-organisms.

5.2.2.1. Primary screening

The primary screening of enriched soil sample was carried out on nutrient agar. Serially diluted enriched sample were spread on media and incubated at 30ºC for 24 hr. Then, distinct isolates were selected and spread on casein agar media having composition 125 ml/l skimmed milk, 875 ml/l nutrient broth, 15 gm/l agar and pH was adjusted at 7.2 then after, plates were incubated at 30ºC for 24 hr and observed for zone of hydrolysis. Protease positive isolates were selected and used for secondary screening.

5.2.2.2. Secondary screening

To check potency of fish waste degradation ability, fish waste containing minimal broth was used. It was composed of (gm/l): NaCl, 0.5; $MgCl_2.6H_2O$, 0.24; K_2HPO_4, 0.3; NH_4Cl, 0.5; KH_2PO_4, 0.4; yeast extract,0.1; fish waste,10; pH 7.5 (Sekar et al.2015). 1 ml suspension of selected strains were inoculated into both and kept for 30ºC under constant stirring in shaking incubator. The cultures were supervised for fish waste degradation which lasts for 7 days. Degradation proficiency of micro-organism was calculated by using following formula;

$$\frac{\text{Weight of degraded fish waste}}{\text{Weight of fish waste}} \times 100$$

5.2.3. Enzyme activity assay

For enzyme activity assay, 0.2% gelatin was used as a substrate. The reaction mixture was prepared by mixing 0.3 ml of 0.2% w/v substrate, 0.2 ml culture supernatant and 0.5 ml 100 mM Tris-HCl buffer (pH 7.5). Incubation was carried out at 37ºC for 30 min and reaction was ceased by 0.5 ml TCA. Ninhydrin method was carried out to assess

liberated free amino acids (Mahesha 2012). One unit enzyme activity was defined as 1 μmol leucine released per ml of enzyme per min.

5.2.4. Effect for carbon and nitrogen sources on enzyme activity

To study effect of various carbon and nitrogen sources on enzyme activity, procedure of Pustake et al. (2019) was used. Carbon and nitrogen sources were screened to analyse their efficiency on the fish waste degradation activity of potent isolate. 1% w/v of numerous carbon sources (dextrose, fructose, lactose, CM cellulose, starch) as well as inorganic nitrogen sources (ammonium chloride, ammonium sulphate, urea, glycine, sodium nitrate) and organic nitrogen sources (soya peptone, yeast extract, beef extract, peptone) were applied to examine their impact on degradation activity.

5.2.5. Biochemical characterization of potent strain

The strain having effective waste degradation activity was selected and explored for biochemical characterization such as Gram staining, IMVIC test (Indole, Methyl Red, Voges Proskauer and Citrate), starch hydrolysis, gelatin liquefaction, urease activity as well as effect of NaCl concentration, pH and temperature were studied.

5.2.5.1. Gram staining

Gram staining was executed according to method of Abiola and Oyetayo (2016) with slight alterations. On a clean grease free slide, a thin smear of 24 hr old bacterial culture was prepared and fixed it by passing through gentle flame. Firstly, smear was stained by crystal violet for 60 second and cleaned with water. Then, smear was flooded with Gram's iodine for 60 second and again rinsed with water. It was decolorized by using ethanol for 30 second and rinsed with water. At the last, safranin was applied as counter stain for 60 second and again rinsed with water. The smear was allowed to air dry and observed under oil immersion lens of microscope using cidar wood oil. Gram positive bacteria appeared as violet color while Gram negative showed pink colour.

5.2.5.2. IMVIC analysis

IMVIC test consist of indole (I), methyl red (MR), vogues proskauer (VP) and citrate utilization (C). The tests were performed according to method of Hussain et al. (2013) with some changes. In indole test bacteria were incubated in tryptophan broth at 30ºC for 24 hr after that Kovac's reagent (0.5 ml) were added to it and observed for pink red ring formation. For MR test, micro-organism were incubated in MRVP broth at 30ºC for 24 hr and after incubation 5 drops of methyl red were added to it and observed for formation of red colored ring. VP test was performed by using MRVP broth and bacteria were incubated in it at 30ºC for 24-48 hr. After incubation, 0.6 ml of alpha-naphthol

solution (VP-A reagent) and 0.2 ml of potassium hydroxide (VP-B reagent) were added to it and observed for pink red color. For citrate utilization, micro-organism were streaked on citrate agar and observed for color change of media from intense blue to deep green within 24-72 hr at 30ºC.

5.2.5.3. Catalase activity

Catalase activity of isolate was performed according to Syahri et al. (2019) using 3% hydrogen peroxide (H_2O_2). Bacterial suspension was taken on glass slide and 100 µl of 3% H_2O_2 were added onto it. Slide was observed for formation of bubbles which indicates positive catalase activity of bacteria.

5.2.5.4. Urease test

Urease test was performed to determine the ability of micro-organism to split urea through urease enzyme. For this purpose, (gm/l): urea, 20; NaCl, 5; KH_2PO_4, 2; peptone 1; dextrose, 1; phenol red, 0.012; agar 15 gm; and pH was adjusted to 6.7. Bacterial culture were spread on plate and incubated at 35-37ºC for 48 hr and observed for colour change. The formation of pink colour indicates positive urease activity (Isenberg 1992).

5.2.5.5. Starch hydrolysis

Starch hydrolysis test of micro-organisms was done to check their capability to degrade substrates with carbon composites and able to grow on it. It was performed as per method of Abiola and Oyetayo (2016) with slight modifications. 2 gm of starch was solubilized in nutrient agar and plates were prepared. Bacterial suspension was spread on it and incubated at 30ºC for 24 hr. After growth, plates were flooded with Gram's iodine. Clear zone around colony indicates hydrolysis of starch due to alpha amylase activity of bacteria while unhydrolyzed starch forms blue colour in presence of iodine.

5.2.5.6. Gelatin liquefaction

Gelatin hydrolysis test was carried on nutrient gelatin media. For this, 23 gm/l nutrient agar and 8 gm/lit gelatin were used. Nutrient gelatin plates were inoculated with bacteria and incubated at 30ºC for 24 hr. After adequate microbial growth, plates were flooded with saturated ammonium sulphate to precipitate unhydrolyzed gelatin and observed for clear zone around bacterial colony within 5-10 min (Isenberg 1992).

5.2.5.7. Phenylalanine deaminase

The aim of this experiment is to determine ability of bacteria to oxidatively deaminate phenylalanine to phenylpyruvic acid. The media containing yeast extract 3 gm; NaCl 5 gm; Phenylalanine 2 gm; Na_2HPO_4 1 gm; agar 15 gm and D/W 1000 ml were used. The plates were spread with bacterial suspension and incubate at 30ºC for 24

hr. After incubation, plates were flooded with 10% ferric chloride and observed for light to deep green colour (Tille and Forbes 2014).

5.2.5.8. Effect of NaCl concentration, pH and temperature

The influence of NaCl concentration, pH and temperature on growth of micro-organism was studied by using method of Hussain et al. (2013) with slight modification. For this purpose, salt concentration from 1% to 10%, pH ranging from 2.0 to 10 and temperature varying from 10 to 50ºC were studied.

5.2.6. Microbial strain identification and phylogenetic analysis

DNA was isolated from the potent bacterial strain. Its quality was evaluated on 1.0% Agarose Gel, a single band of high-molecular weight DNA has been observed. Fragment of 16S rDNA gene was amplified by 27F and 1492R primers. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyser. Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the database of NCBI GenBank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 7.

5.2.7. Estimation of liberated end compounds

Fish waste degradation by micro-organism results in discharge of some useful end products like proteins as well as amino acid. Assessment of these products was carried out till complete degradation was attained. Protein estimation was carried out by Lowery method using BSA as standard (Waterborg 1996) while amino acid content was calculated by Ninhydrin method using Leucine as standard (Mahesha 2012).

5.2.8. Antibiotic susceptibility test of micro-organism

After 16 S rDNA sequencing, identified isolate was tested for antibiotic susceptibility on nutrient agar according to method of Begum et al. (2017) with some modification. Disc diffusion method was performed and commercially available seven antibiotic discs of penicillin (P, 10 mcg), chloramphenicol (C, 10 mcg), streptomycin (S, 10 mcg), gentamycin (GEN, 10 mcg), azithromycin (AZM, 15 mcg), amoxicillin (AMC, 30 mcg) and clarithromycin (CLR, 15 mcg) was employed for this investigation. Above pre-treated antibiotic plates were incubated at 37ºC for 24 hr. Sensitivity of micro-

organism against each antibiotic was determined by measuring zone of inhibition (mm) on next day.

5.2.9. Plant growth promotion by fish waste hydrolysate

After degradation of fish waste by micro-organisms, the residual hydrolysate was tested for their capacity of plant growth stimulation. The mung seeds (*Vigna radiata*) were used for this study.

5.2.9.1. Plantation and dose application

To evaluate plant growth promotion, fish waste hydrolysate was utilized. The study was carried out during month of September to November using pot method. The mung seeds were surface sterilized by 70% ethanol and further washed appropriately with D/W and sowed at the start of (3^{rd} Sept.) September. 5ml of fish hydrolysate was supplemented to test plant; with 5 days of interval and continued for one month until germination was achieved. The water was supplied to both plants test and control while control was excluded of hydrolysate.

5.2.9.2. Evaluation of morphological parameters of plants

Both control and hydrolysate treated plant were monitored for above stipulated time and at the middle of November, plants were fully developed into mature plant and attained maximum growth. After that plants were analysed for its biochemical as well as morphological parameters including, shoot, root length, root hairs, number of leaves, flowers, both chlorophyll a and b pigment, protein, phenolic and flavonoid content were investigated.

5.2.9.3. Biochemical analysis of plants

5.2.9.3.1. Chlorophyll estimation

Chlorophyll content was estimated as per protocol of Arnon (1949) with few modifications. Thoroughly washed 1g fresh leaves of mung were crushed in (80 % v/v) acetone to prepare slurry and make the final volume 20ml. The extract was refrigerated overnight followed by centrifugation and absorbance of supernatant was measured spectrophotometrically at 645 nm and 663 nm (Shimadzu UV-1800, Japan).

$$\text{Chlorophyll a } (\mu g/L) = (12.7 \times A663) - (2.69 \times A645)$$

$$\text{Chlorophyll b } (\mu g/L) = (22.9 \times A645) - (4.68 \times A663)$$

$$\text{Total Chlorophyll } (\mu g/L) = (20.2 \times A645) + (8.02 \times A663)$$

5.2.9.3.2. Estimation of free proteins and amino acids

The procedure of Gurav and Jadhav (2013) was followed to determine the free proteins and amino acids. For sample preparation, 0.5gm of vegetative part of it

homogenised in 80% ethanol and 100 mM phosphate buffer (pH 7.0) respectively. Extracts were centrifuged and estimated for protein and amino acids by using Lowry (Lowry et al.1951) and Ninhydrin method respectively (Moore and Stein 1954).

5.2.9.3.3. Determination of total phenolics and flavonoid

The total phenolics content was calculated by Folin-Ciocalteu's reagent (Singleton and Rossi 1965). Reaction mixture were prepared and incubated for 90 min in dark. After incubation, the absorbance was measured at 765 nm and total phenolics were calculated by using standard gallic acid calibration curve. The total flavonoid was determined by measuring absorbance at 415 nm (Shimadzu UV-1800, Japan) by using standard quercetin curve (Chang et al.2002).

5.2.9.4. Chemical analysis of soil

Chemical analysis of sample soil was carried out to study the effect of fish waste hydrolysate on plant growth. The Kjeldahl's method and TOC analyser was used to determine total nitrogen and carbon content respectively. Similarly, samples were prepared to check its P, K, Ca, Mg and Zn content by the method of Hseu (2004). Diluted samples were analysed for the presence of P, K (flame photometry), Zn, Mn, Ca and Mg (atomic absorption spectrophotometer).

5.2.10. Statistical analysis

All the experimental sets were performed in triplicates (n=3). The statistical analysis was carried out on the basis of mean and standard derivation (SD).

5.3. Result and discussion

5.3.1. Isolation of potent bacterial strain from soil

In current work, it was observed that the enriched minimal media containing micro-organisms exhibiting fish waste degradation potential. Total 13 micro-organisms were potent and are selected on the basis of their growth on nutrient agar and named as PSD 1 to PSD 13.

5.3.2. Proteolytic activity

Isolates selected from serially diluted sample were listed in table no.5.1 and checked for their proteolytic activity on casein agar. Out of which, eight isolates displayed significant and prominent hydrolytic zone (figure no.5.1). Isolate PSD 11 showed remarkable casinolytic activity and screened for its degradation ability as well.

Figure 5.1.: Plates showing zone of casein hydrolysis by eight isolates during screening

No.	Strains	Growth on nutrient agar	Caseinase activity	Zone of hydrolysis (mm)	Fish waste degradation activity
01	PSD 1	+	+	11	+
02	PSD 2	+	+	12	+
03	PSD 3	+	+	8	-
04	PSD 4	+	-	-	-
05	PSD 5	+	+	7	+
06	PSD 6	+	-	-	-
07	PSD 7	+	-	-	-
08	PSD 8	+	+	5	-
09	PSD 9	+	+	2	-
10	PSD 10	+	+	10	-
11	PSD 11	+	+	15	+
12	PSD 12	+	-	-	-
13	PSD 13	+	-	-	-

Table 5.1.: Strains isolated using synthetic casein medium

5.3.3. Fish waste degradation activity

Isolates was selected on the basis of their ability to hydrolyse fish waste in minimum period. Figure no.5.2 represents the residual fish waste remaining after collagen recovery; utilized for degradation studies. This study was carried out by using a minimal broth containing fish waste as sole carbon and nitrogen source. Entire fish waste was accompanied in media to study waste degradation by visual identification (figure no.5.3). The maximum degradation of waste showed by PSD 11 than PSD 1, PSD 2 and PSD 5, this noticeable change was observed in 7 days while rest of three strains were delay in biochemical process.

The obtained results were closely related to feather waste degradation by keratinolytic bacteria (Nagarajan et al.2017; Bhange et al.2016) and fish waste degradation by *B. cereus* (Bhagwat et al.2018). The results were further assisted by their considerable increase in turbidity of media which indicates that fish waste was utilized for microbial growth (Reyes et al.2018) and therefore, PSD 11 was explored for further characterization. Fish waste degrading efficiency of bacterium under suitable conditions was found to be 64% (weight of initial fish waste: - 0.5 gm/50 ml and degraded one: - 0.32 gm).

Figure 5.2: Fish waste remaining after collagen extraction

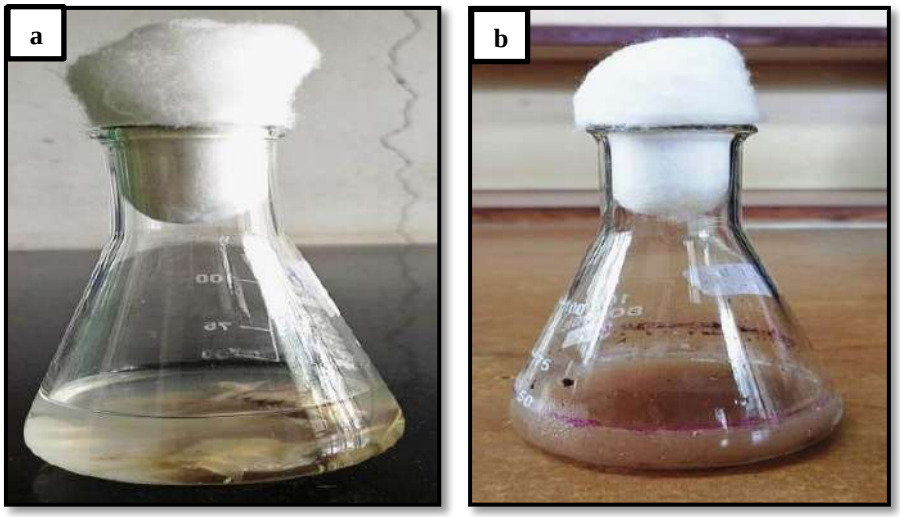

Figure 5.3.: (a) Control without micro-organism; (b) Test sample with micro-organism

5.3.4. Enzyme activity

Fish waste degradation capability of PSD 11 was continuously monitored during microbial growth. It was observed that, the activity was enhanced day by day (Govinden and Puchooa 2012) and the change was significant. Activity was minimum on zero day (8.24±0.27 µg/ml/min) while at the end of seventh day maximum activity was attained. The figure no. 5.4 indicated the increase in enzyme activity within seven days and after that it was ceases. In current study, it was observed that activity was increased up to 72.41±0.21 µg/ml/min in same period which was more significant in comparison of earlier studies.

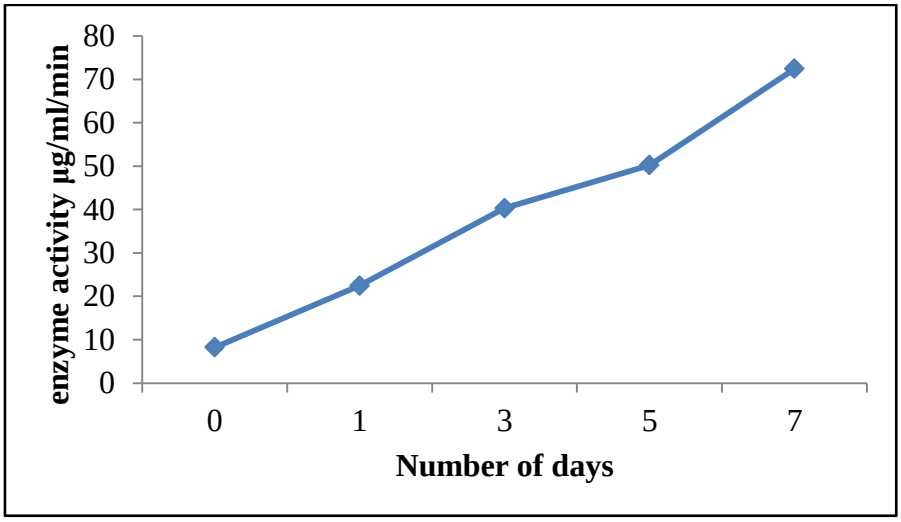

Figure 5.4.: Enzyme activity with respect to number of days

5.3.5. Enzyme activity in presence of carbon and nitrogen sources

Carbon and nitrogen source plays important role in any media formulation. Simple and easily available sources are usually preferred. Enzyme activity may be increased or decreased in presence of these sources and it depends on nature of sources used. Enzyme activities were analysed with respect to various simple to complex carbon and nitrogen sources (organic as well as inorganic). Among these different carbon sources used, enzyme activity was maximal in presence of dextrose (77.35±0.85 µg/ml/min) while least in starch (44.97±0.69 µg/ml/min). In comparison with organic and inorganic nitrogen sources; yeast extract (organic source) and ammonium chloride (inorganic source) exhibited 46.13±0.45 µg/ml/min and 63.06±0.25 µg/ml/min enzyme activity respectively. Thus, dextrose, ammonium chloride and yeast extract was employed as carbon and nitrogen source for efficient fish waste degradation and enzyme activity with respect to these components were illustrated in figure no.5.5 and 5.6.

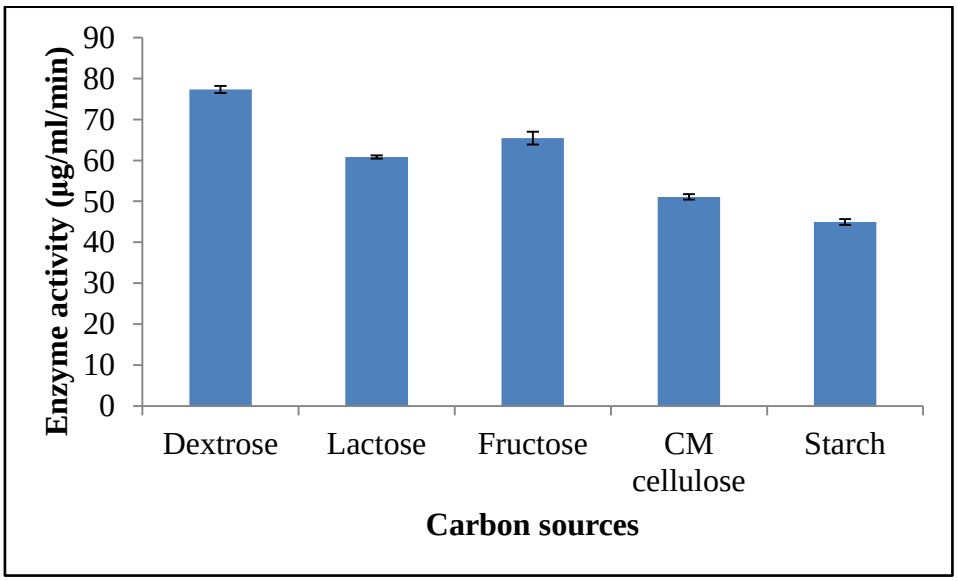

Figure 5.5.: Effect of carbon sources on enzyme activity

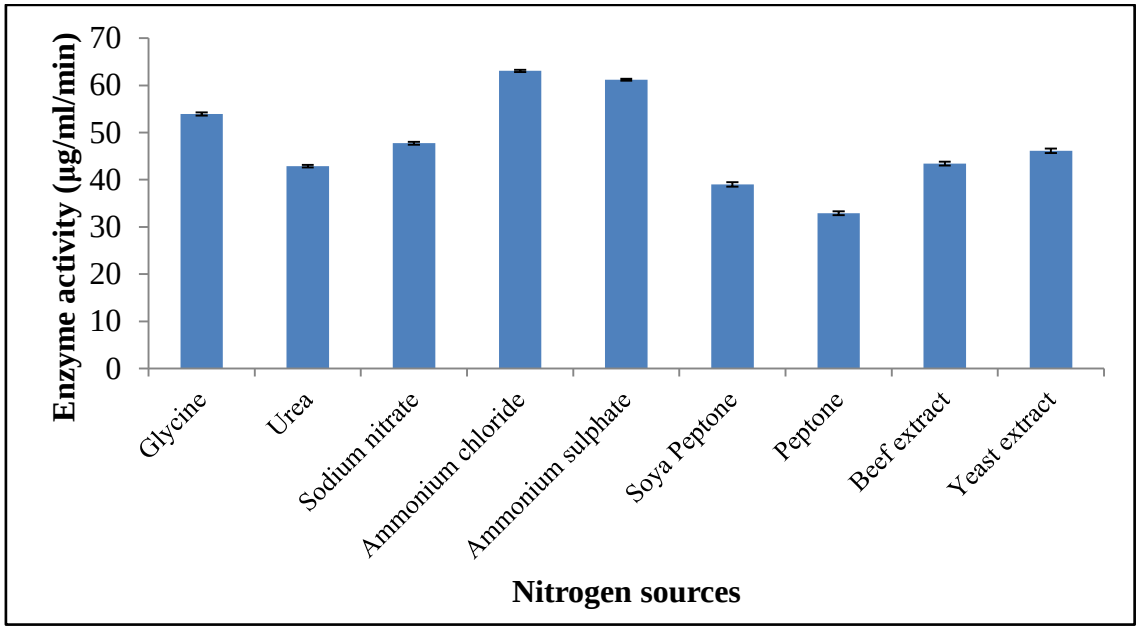

Figure 5.6.: Effect of nitrogen sources on enzyme activity

5.3.6. Microscopic and biochemical assay

The strain PSD 11 was characterized by biochemical and microscopic studies. The colony characterization and biochemical assays were listed in table no.5.2 and 5.3 respectively. Figure no.5.7 showed growth of PSD 11 on nutrient agar which was further identified by 16 S rDNA sequencing.

Size	Shape	Margin	Elevation	Color	Opacity	Consistency
3 mm	Circular	Regular	Convex	White	Opaque	Mucoid

Table 5.2.: Colony characteristics

No.	Test	Result
01	Gram nature	Gram positive
02	Morphology	Clustered Cocci
03	Motility	Motile
04	Indole	+
05	Methyl red	+
06	Voges-Proskuer	-
07	Citrate utilization	-
08	Catalase	+
09	Urease	+

10	Starch hydrolysis	+
11	Casein hydrolysis	+
12	Gelatin liquefaction	+
13	Phenylalanine deaminase	-
14	NaCl (%)	
	1.0	+
	2.0	+
	4.0	W
	6.0	W
	8.0	-
	10.0	-
15	Growth pH	
	2	-
	4	-
	6	-
	8	+
	10	W
16	Growth temperature (ºC)	
	10	+
	20	+
	30	+
	40	W
	50	-

Table 5.3.: Biochemical characterization of PSD 11 (+: positive; -: negative; W: weak)

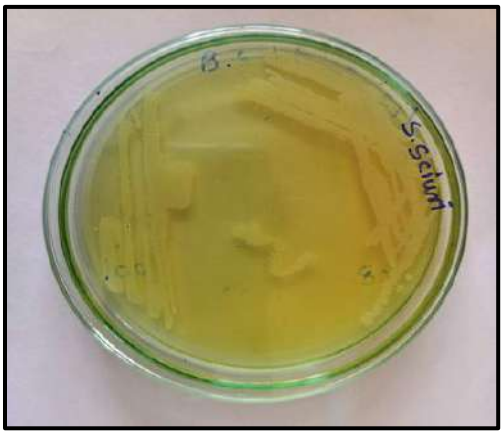

Figure 5.7.: Growth of *S. sciuri* on nutrient agar

5.3.7. Identification by 16S ribosomal DNA sequencing

The 16S ribosomal DNA sequencing of PSD 11 was carried out. From sequencing, it was revealed that isolate PSD11 showed highest similarity with *Staphylococcus sciuri* strain DSM 20345 based on nucleotide homology and phylogenetic studies. The PCR amplification of genomic DNA along with ladder was depicted in figure no.5.8.(a). The molecular phylogenetic analysis was carried out by using MEGA7 software and results were given in figure no.5.8.(b). BLAST similarity alignment of micro-organism with respect to NCBI GenBank was given in figure no. 5.9. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura 1980). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed (Felsenstein 1985).

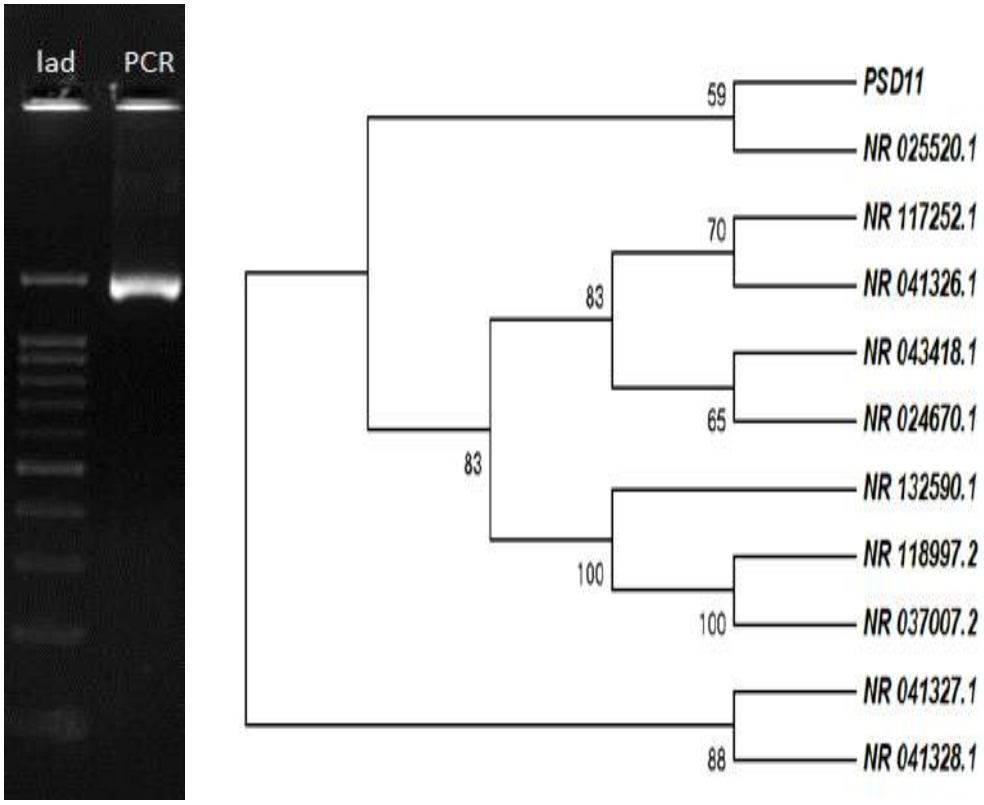

Figure 5.8.: (a) PCR amplification of DNA fragments with ladder; (b)Molecular phylogenetic analysis of PSD 11(*S. sciuri*) by Maximum Likelihood method

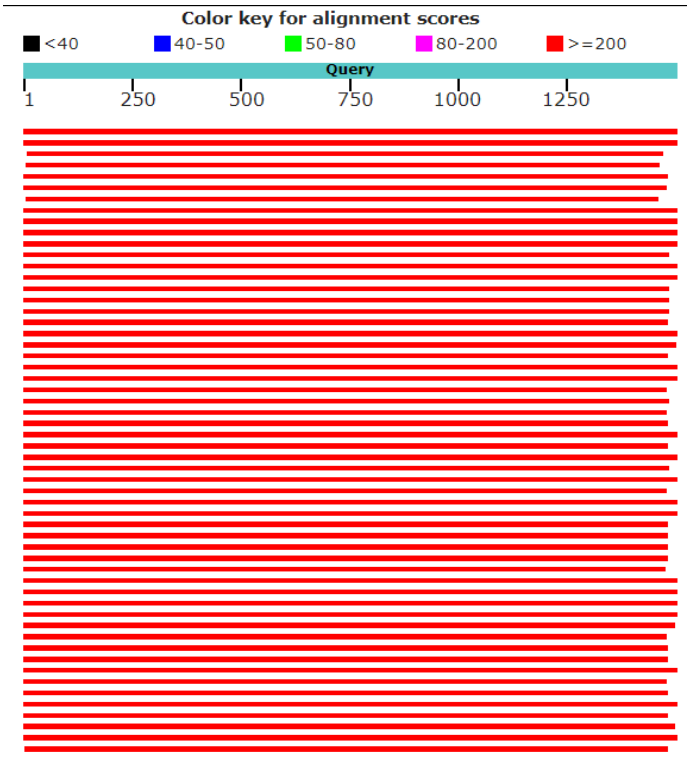

Figure 5.9.: Alignment view of *S.sciuri* using combination of NCBI GenBank

5.3.8. Analysis of liberated end products

It was found that *S.sciuri* isolated from fish waste dumping site has good capacity to degrade waste. It utilizes fish waste as whole carbon and nitrogen source therefore, results in generation of free proteins and amino acids. Figure no. 5.10 depicted, rise in amount of free amino acids and proteins during waste degradation by *S.sciuri* which indicated that fish waste was utilized by micro-organism for growth.

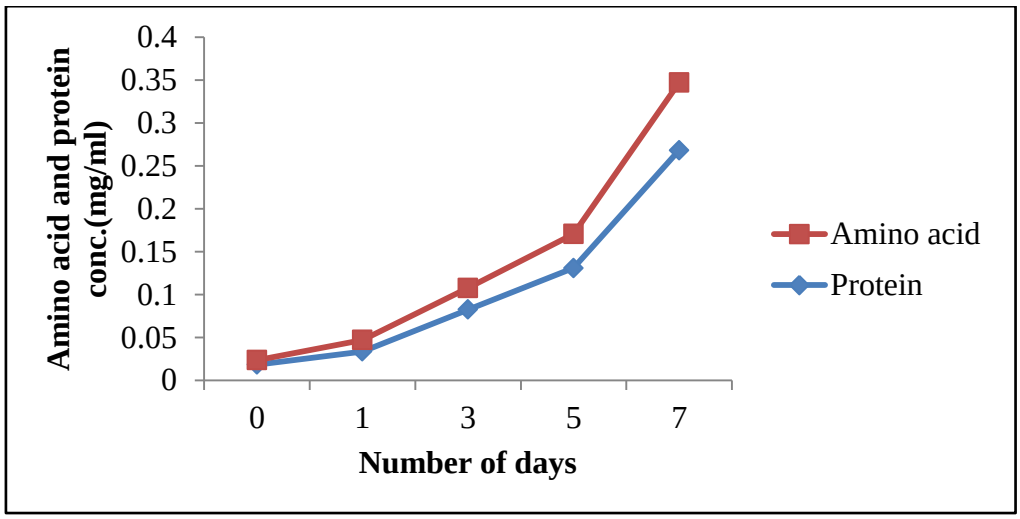

Figure 5.10.: Analysis of degraded end products during fish waste degradation by *S.sciuri*

5.3.9. Antibiotic susceptibility analysis

Antibiotics are the vital secondary metabolites synthesized by bacteria during stationary phase of growth. More than 80% antibiotics were extracted from soil micro-organisms. In various bacterial infections antibiotics perform an essential role but several bacteria were resistant to these antibiotics. This resistance was occurred due to impermeable cell membrane, genetic transformation, alteration of plasmids and the generation of beta- lactamases (Odusanya 2002). *S.sciuri* isolated from fish waste dumping site was tested for antibiotics sensitivity against commercially available antibiotics. It was observed that, isolate was susceptible to penicillin, streptomycin, gentamycin, chloramphenicol, azithromycin, amoxicillin and clarithromycin. *S.sciuri* was more sensitive to amoxicillin and less sensitive to streptomycin with zone of inhibition about 31 mm and 19 mm respectively (Begum et al.2017) while marginal changes was observed for other antibiotics. Antibiotics susceptibility of *S.sciuri* against commercial antibiotics was depicted in figure no. 5.11 and 5.12. Due to susceptibility against various antibiotics it can be concluded that, isolated micro-organism is non-pathogenic and can

be explored for different applications in agricultural field and as a probiotics in poultry as well as aquaculture feed.

Figure 5.11.: Zone of inhibition by *S.sciuri* against antibiotics

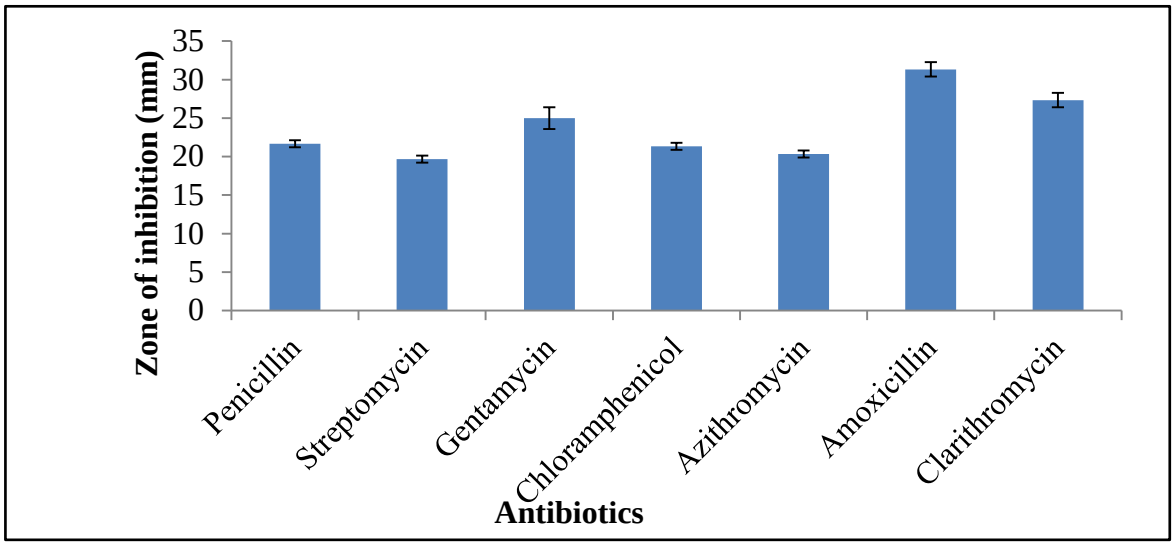

Figure 5.12.: Antibiotics susceptibility of *S.sciuri*

5.3.10. Effect of degraded feather waste hydrolysate on plant growth

The following morphological and biochemical analysis of plants and soil supplemented with fish waste hydrolysate were carried out.

5.3.10.1. Plant growth promotion study

It was observed that, there is significant enhancement of phytochemicals in case of test while marginal change was observed in control plant. This confirms, fish waste hydrolysate was rich in nutritional factors that act as effective plant growth stimulator so it has positive effect on test plants. Besides this, time duration study revealed that, mung plant nourished with fish waste hydrolysate showed germination after 2 days and healthy growth of all vegetative parts were observed after one week, but in control plant delayed in germination as well as all phytochemical parameters; no significant change was observed.

5.3.10.2. Effect of hydrolysate on morphological parameters of plants

The organic components of fish waste hydrolysate were utilized by mung plant. Figure no.5.13 depicted the results of waste hydrolysate on morphological parameters of plant. The hydrolysate treated plants exhibited better morphological characters than control one (table no.5.4). Test plant showed 36±0.84 cm and 2.9±0.24 cm shoot and root length respectively while in control it was decreased in shoot and root length (25±0.81 cm and 2.46±0.12 cm respectively). The increases in number of leaves, root hairs, root nodules, number pods as well as seeds were observed in treated plant as compared to control (figure no.5.14). Early fruiting was detected in test plant (2 days) while control (3 days) was delayed in fruiting. The pod colour of test plant was black while control showed brown colour, thus it indicated the good quality of seeds as well as weight of seeds also increases (figure no.5.15).

Other morphological parameters such as stem and leaf width as well as length of leaves and pod were increased in test plant than control plant. This significant change was detected due to microbial degraded fish waste hydrolysate. Similar results were observed after application of feather hydrolysate on *Vigna radiata* (Paul et al.2013; Bhange et al.2016) and fish waste hydrolysate (Bhagwat et al.,2018) on *C. arietinum* (Bhagwat et al.2018), *Amaranthus dubius* and *Trigonella foenum- graecum* (Thankachan and Chitra 2021).

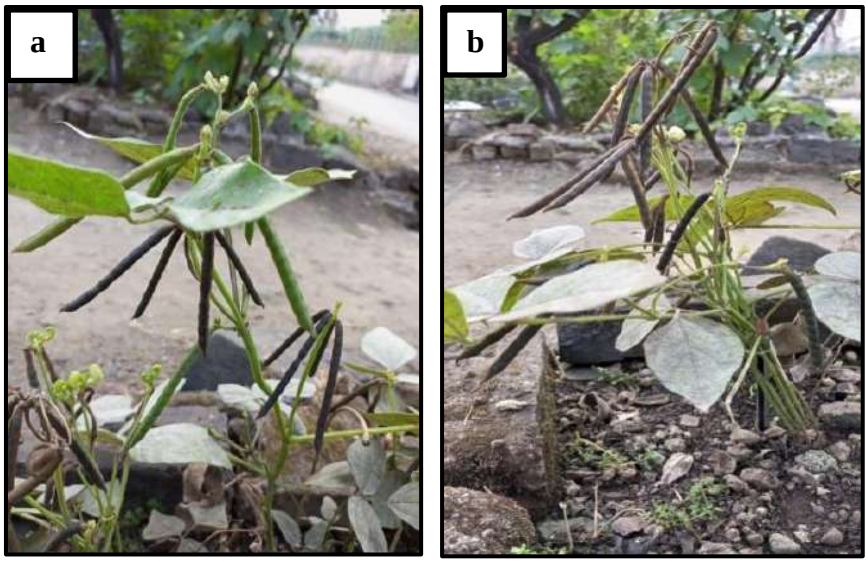

Figure 5.13.: Plant study of hydrolysate (a) Control; (b) Sample

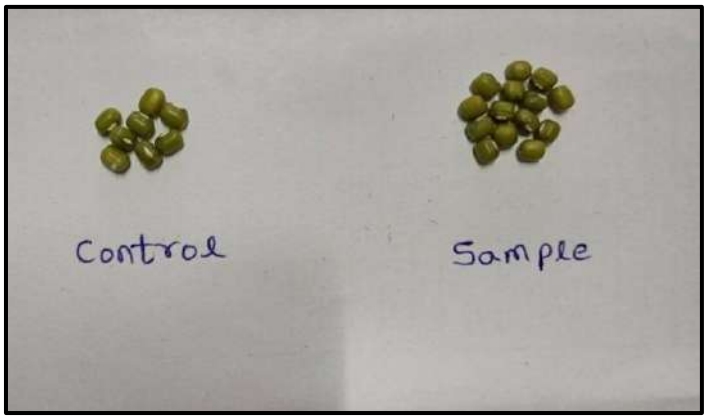

Figure 5.14.: Seeds of control and treated mung plant

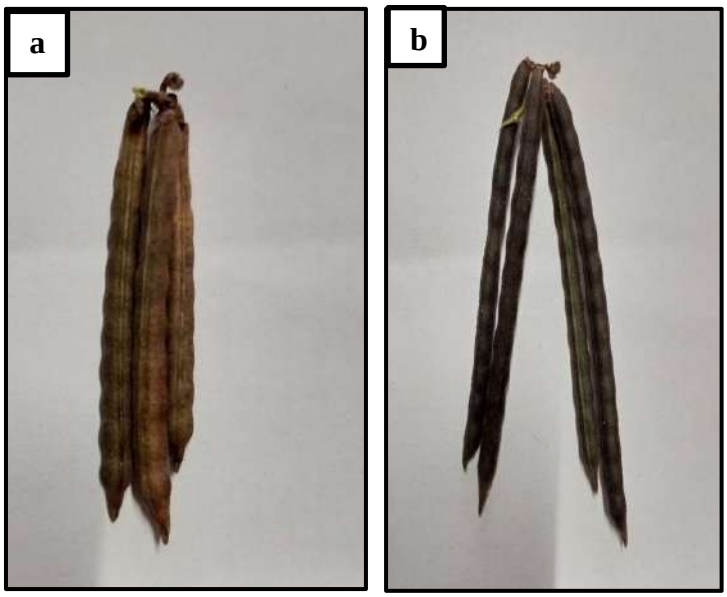

Figure 5.15.:Pod study (a) Control; (b) Sample

Parameters	Control	Sample
Germination time	3 days	2 days
Flowering time	10 days	7 days
Fruiting time	3 days	2 days
Stem length	25±0.81 cm	36±0.84 cm
Stem width	0.5±0.081 cm	0.6±0.04 cm
Leaf width	5.63±0.12 cm	7.06±0.12 cm
Leaf length	6.93±0.12 cm	7.3±0.16 cm
Root length	2.46±0.12 cm	2.9±0.24 cm
No.of leaves/plant	8±0.14	11±0.47
No.of root hairs	05±0.47	08±0.41
No.of root	03±0.47	04±0.86

nodules/plant		
No.of pods/plant	03	04
Colour of pod	brown	black
Length of pod	6.83±0.23 cm	7.5±0.40 cm
No.of seeds	05±0.47	08±0.94
Weight of seeds	0.047±0.14 gm	0.091±0.14 gm

Table 5.4.: Morphological characters of control and treated mung plant ((n=3)

Addition to that, development of root nodules was detected in *both control and test V.radiata* (figure no.5.16), representing that amendment of fish waste hydrolysate could help to colonize and elevate population of nitrogen fixing bacteria in soil. Nitrogen fixers assimilate nitrogen and show rapid invasion of root cortex that stimulates nodulation using protein rich hydrolysate (Paul et al.2013).

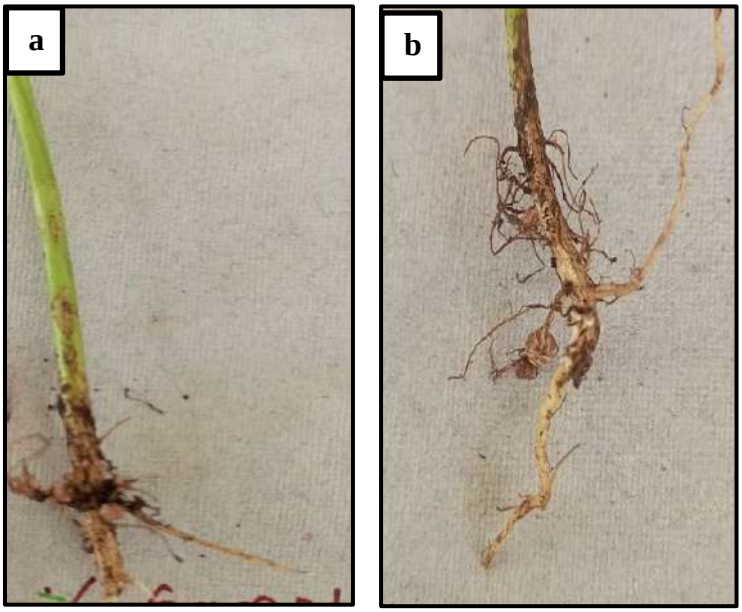

Figure 5.16.: Root study (a) Control; (b) Sample

5.3.10.3. Biochemical parameters of plants

The hydrolysate of fish waste degradation by microbes contains important nutrients which act as plant growth stimulant so it has potential applications in agriculture particularly horticulture field which has great commercial value. It is potent source of small peptides and amino acids that involved in protein synthesis, secondary metabolism and signalling mechanism of organism. Some amino acids in waste hydrolysate act as precursor of plant growth hormones thus; it may helpful to develop

tolerance in various stress conditions (Colla et al.2015). Hence, plants treated with fish waste organic hydrolysate exhibited effective increase in biochemical characters of plants like concentration of photosynthetic pigments, proteins, amino acids, flavonoid and phenolic. Some amino acids in waste hydrolysate may acts as precursor for plant growth hormone thus helpful to develop tolerance in various stress as well as atmospheric seasonal conditions.

5.3.10.3.1. Chlorophyll content

There was increase in chlorophyll a and b pigment concentration of treated plant than control. Table no.5.5 illustrated the change in chlorophyll content. This significant change was observed because along with soil macro and micronutrients, hydrolysate provides additional nutrients which enhance the growth, development and improve in chlorophyll content as well (Neales 1956). The rise in photosynthetic pigments may cause rapid synthesis of carbohydrates thus improve yield in plants (Paradikovic et al.2011). Chlorophyll a content in treated plant was found to be 3.481±0.49 µg/ml while 1.836±0.45 µg/ml for control. The chlorophyll b content was found to be 11.117±0.53 µg/ml and 15.294±0.49 µg/ml for control and treated mung plant respectively. The overall chlorophyll content was recorded as 17.123±0.53 µg/ml in treated 14.593±0.37 µg/ml in control. The results were analogous to *T. aestivum* and *V. radiata* treated with feather and fish waste hydrolysate respectively (Bhise et al.2017; Bhagwat et al.2018).

Variants	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Total Chlorophyll (µg/ml)
Control	1.836±0.45	11.117±0.53	14.593±0.37
Test	3.481±0.49	15.294±0.49	17.123±0.53

Table 5.5.: Concentration of chlorophyll

5.3.10.3.2. Free protein and amino acids

The absorption of macro and micronutrients in hydrolysate causes improvement in amino acid and protein synthesis in treated plants than control (table no.5.6) which may help to the plant for easy absorption, results in healthy growth of plants. Fish hydrolysate acts as rich source of L-amino acids and peptides which are simply absorbed by test plants. Similarly this improved uptake of macro and micronutrients increases protein as well as amino acid synthesis in plants (Colla et al.2015). The concentration of

free proteins and amino acids in test plant was significantly higher than control one. Hence, fish waste hydrolysate promotes rapid growth and development of treated plants.

Variants	Protein (mg/g fresh weight)	Amino acid (mg/g fresh weight)	Total phenolics (mg GAE /g fresh weight)	Total flavonoids (mg Que /g fresh weight)
Control	1.052±0.13	0.176±0.34	4.942±0.23	3.973±0.39
Test	1.885±0.23	0.256±0.37	6.105±0.27	5.635±0.40

(GAE- gallic acid equivalent; Que- quercetin equivalent; n=3±SD)

Table 5.6.: Biochemical analysis of vegetative tissue in control and treated plants

5.3.10.3.3. Total phenolics and flavonoid

The natural antioxidants, polyphenols are present in all plants and have vital function in defence mechanism of it. Besides this, polyphenols have effective health benefits in human beings also (Mahesha 2012). The vegetative parts of plants contains high amount of phenolic and flavonoid. The treatment of fish waste hydrolysate to test mung plant showed little bit increase in total phenolics and flavonoid content (table no.5.6) as compared to control plant. These results are in accordance with Gurav and Jadhav (2013) and Bhise et al. (2017) who reported effects of feather hydrolysate on banana and wheat.

5.3.10.4. Chemical analysis of soil

Soil composition and its chemical parameters are crucial factors for growth and development of any crop. The pre-treatment of hydrolysate on soil and observed its effect on plants and results showed that tremendous positive change was occurred in all constituents of soil tabulated in table no 5.7. The increased concentration of macro and micronutrients were observed in test soil. The current study showed enrichment of C and N percentage which indicates raise in soil fertility. With the supplement of hydrolysate, it was found that, other macro and micro components of soil drastically increased viz; Mg, P, Zn, K, Mn, Ca, Fe, N, K, Cu and proteins. Hydrolysate treated soil contains increasing amount of total nitrogen, phosphorus, potassium of about 22±0.02 %, 22±0.02 mg/l and 65±0.02 mg/l respectively. Thus, this micro-organism degraded fish waste can be considered as a best source of nutrients which improve soil in terms of soil fertility or

quality and crop productivity. The high amount of carbon and nitrogen causes increase in plant biomass. Therefore, fish waste hydrolysate efficiently employed as soil conditioner which elevates the water holding and buffering ability of soil (Barrow 1960; Haynes and Naidu 1998).

Sample	**P (mg/l)**	**K (mg/l)**	**Ca (mg/l)**	**Mg (mg/l)**	**Mn (mg/l)**	**Zn (mg/l)**	**Total C (%)**	**Total N (%) Kg/ hector**
Control	9.52± 0.02	52.5 ±0.02	197.5± 0.016	11.05± 0.01	BDL	4.892± 0.05	1.18± 2.07	235.2± 0.30
Sample	22± 0.02	65± 0.02	1050± 0.06	13.68± 0.01	1.971 ±0.28	1.971± 0.28	3.01± 0.05	282.2± 0.17

(Test: - 5ml hydrolysate; n=3±SD)

Table 5.7.: Chemical analysis of control and sample soil

5.4. Conclusion

In present study, complete utilization of fish waste including skin, fins and tail was carried out which was remaining after the recovery of valuable collagen. Previous study was restricted to collagen extraction only, but remaining waste has gained increasing attention for bioprocessing in agricultural field and could become vital factor for fish waste management. *Staphylococcus sciuri* (PSD 11) has potential to degrade fish waste within 24 hours hence successfully exploited for production of organic nutrient rich hydrolysate. Similarly, fish waste hydrolysate serve as an organic liquid fertilizer with significant plant growth stimulator. Amendment of liquid fertilizer results in improved morphological, biochemical parameters in mung (*Vigna radiata)* plant. Therefore, application of nutrient rich hydrolysate from *S.sciuri* PSD 11 has capability to degrade fish waste and could increase the yield of crops by ameliorating growth of plants. Hence, this work efficiently employed as novel, eco-friendly procedure to overcome environmental pollution problems as well as those arise from agriculture associated with chemical fertilizer.

5.5. References

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CHAPTER VI

APPLICATIONS OF MACROMOLECULES DERIVED FROM WASTE MATERIAL OF GETHAR (*Sarda orientalis*)

6.1. Introduction

Pollution due to waste is key negative factor produced by an industry and human being. The manufacturing processes carried out in all types of industries generate more or less amount of waste. The environmental impact caused by chemical wastes is immediately detected when it released beyond allowable limits. Generally, food processing trades creates organic wastes and its effect on environment is not easily identified until it reached to unmanageable proportion. One of the main food industry which has large impact on environment is seafood processing industry. It generates more pollution due to its coastal specific location (Sasidharan et al.2013). The retrieval of merchantable by-products from fish wastes is an important waste minimization approach. The waste material contains significant amount of bioactive constituents with varied pharmaceutical and biotechnological applications. It includes proteins and bioactives like (enzymes, collagen and gelatin), protein hydrolysates, lipids, astaxanthin, chitin. Also, oil from fish waste comprises more amount of polyunsaturated fatty acids (PUFAs) particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). It has increasing demand because of its great profitable value as well as probable applications in pharmaceutical and nutraceutical sectors. Fish waste and its hydrolysate can be employed as organic fertilizer to improve crop productivity. However, more research was carried out to convert waste from marine sources into beneficial products. Also it generates extra revenue as well as decreases the cost required for its proper disposal (Mathew 2010; Caruso 2015).

Inside the natural protein, bioactive peptides are in inactive state. The digestion of native protein by physical, chemical or enzymatic method cleaves peptides from it which has various beneficial effects. Collagen is one of the abundant source of biologically active peptides and these peptides possess different physiological properties which are important to maintain good health (Paul et al.2019). The gelatin is produced from triple helical collagen after thermal denaturation which changes molecular configuration of amino acids. Gelatin from mammalian sources is mostly utilized for industrial purpose. But, there are some ethical concerns behind the use of this gelatin. The fish waste is the most prominent alternative source for gelatin extraction. The extracted gelatin exhibits better functional properties so can be useful in biomedical sector (Yusof et al.2019). The EPA and DHA are mainly found oil from marine fishes and it has valuable health benefits as they are used in treatment of arteriosclerosis and hyperlipemia as well as

cardiovascular disease. Thus, it can be useful in prevention of various health related diseases and acts as potential therapeutic agent (Byun et al.2008).

The current research work was designed to synthesize collagen peptides from extracted collagen by enzymatic hydrolysis. The biological macromolecules derived from marine fish gethar (*Sarda orientalis*) were studied for its applications in biomedical and agriculture field as well as utilized for functional food preparations.

6.2. Experimental methodology

6.2.1. Chemicals and reagents

The chemicals including lactic acid, sodium hydroxide (NaOH), phosphate buffer, agarose, acrylamide, bisacrylamide, ammonium persulphate, coomassaie brilliant blue, sodium dodecyl sulphate (SDS), methanol, ascorbic acid and dimethyl sulfoxide (DMSO) were procured from Hi-Media and Sisco research laboratory, India. Also, egg albumin, standard acarbose, Diclofenac sodium and α-amylase enzyme from fungal diastase were purchased from Sigma-Aldrich, USA. The reagents like 3,5, dinitro salicyclic acid (DNSA), phosphate buffered saline (PBS), Dulbecco's modified eagle medium (DMEM), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 3-(4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide) (MTT) were obtained from Sigma-Aldrich, USA. All the chemicals and reagents utilized for current study were of analytical grade. The food grade components were used for functional food preparations.

6.2.2. Formation of collagen peptides

6.2.2.1. Extraction of collagen hydrolysate

For the extraction of collagen hydrolysate from gethar fish waste methodology of Kumar et al. (2018) was used with slight modification. The waste material was washed with cold tap water followed by cold D/W and cut into small pieces. Then, it was treated with 0.1 M NaOH for 24 hr at 4ºC to remove non-collagenous impurities. After that, it was washed with cold D/W till neutral pH attained. The pre-treated sample was placed in 0.2 M lactic acid (1:30, w/v) at 4ºC for swelling and then homogenized with 50 mM phosphate buffer (pH 7.0) for 5 min (1:2, w/v). The 1 ml suspension of *Staphylococcus sciuri* (*S.sciuri*) was mixed into mixture and kept for constant stirring (150 x g) at 4ºC for 24 hr. The protease enzyme from bacteria hydrolyses collagen; reaction was ceased by adding 4 ml of 6.0 N HCl and centrifuged at 6000 x g for 15 min at 4°C. The supernatant was collected and neutralized with the help of 1 N NaOH and passed through 0.2 μm millipore syringe filter to obtain collagen hydrolysates.

6.2.2.2. Fractionation of collagen peptides

Fractionation of collagen peptides was carried out on the basis of their molecular weight cut-offs (MWCOs). The 30 kD biopeptide fractions were acquired by using ultra-membrane filters with MWCO <30kD (Amicon membrane filters). These fractions were utilized to determine bioactive characteristics.

6.2.2.3. SDS-PAGE analysis of peptides

Enzymatic hydrolysis of collagen results into peptides was confirmed by SDS-PAGE analysis. The 3 mg/ml standard as well as test (30 µl each) was mixed with gel loading buffer (30 µl) and about 50 µl of each sample were loaded into wells. The separation was carried out by using 4% stacking and 8% separating gel. The gel was stained by coomassie brilliant blue to visualize separated fragments (Laemmli 1970).

6.2.3. Biomedical applications

6.2.3.1. In vitro anti-diabetic assay of peptide

An in vitro anti-diabetic potential of collagen peptides was determined by using α-amylase inhibition assay as per protocol of Mccue and Shetty (2004) with some modifications. For this activity, 1 mg/ml, 3 mg/ml and 5 mg/ml of collagen peptide was used from stock solution. The 500 µl of each sample was mixed with 500 µl of 0.1 M phosphate buffer (pH 6.9) containing α-amylase from fungal diastase (0.5%). The reaction was incubated at 25ºC for 10 min and 500 µl of 0.1 M phosphate buffer (pH 6.8) containing 1% starch was added to it. The resulting mixture was incubated at 25ºC for 10 min followed by addition of 1 ml dinitro salicylic acid (DNSA). The reaction mixture was kept in boiling water bath for 10 min and cooled. After cooling, absorbance was measured at 540 nm using UV-visible spectrophotometer (Shimadzu UV-1800, Japan). 0.2 M lactic acid was used as control while standard acarbose was taken as positive control. The percentage inhibition of α-amylase enzyme was calculated using following formula;

$$\text{Inhibition (\%)}=\frac{\text{A(control)}-\text{A(sample)}}{\text{A(control)}}\times 100$$

6.2.3.2. Cytotoxic activity of peptide

The cytotoxic activity of collagen peptide was studied by using MTT assay according to method of Chang et al. (2015) with slight modifications. The activity was checked on breast cancer cell line (MCF-7, NCCS Pune). Collagen peptides 1, 3, 5 mg/ml from stock solution were utilized. Then cells were incubated at a concentration of

1×10^4 cells /ml in DMEM culture medium for 24 hr at 37ºC and 5% CO_2. Cells were seeded at a concentration (70 µl) 10^4 cells /ml in 100 µl culture medium and 100 µl of each sample was added into 96 well micro plates. Control wells were incubated with DMSO (0.2% in PBS) and cell line. Control was maintained to determine the control cell survival and percentage of live cells after culture. Cell cultures were incubated for 24 hr at 37°C and 5% CO_2 in CO_2 incubator (Thermo scientific BB150, USA). After incubation, the medium was completely removed and 20 µl of MTT reagent (5mg/ml PBS) was added to it. After addition of MTT, cells were incubated at 37ºC for 4 hr in CO_2 incubator. The cells were observed for formazan crystal formation under electron microscope. The viable cells reduce yellowish MTT into dark coloured formazan. The medium was removed completely and 200 µl DMSO was added to it (kept for 10 min) and incubate at 37ºC (wrapped with aluminium foil). Triplicate samples were analysed by measuring the absorbance of each sample by microplate reader (BeneSphera E21, Avantor USA) at 550 nm wavelength.

6.2.3.3. Antioxidant activity

The antioxidant activity of fish waste extracted macromolecules was performed according to method of Chang et al. (2013) with some modification. The DPPH (2,2-diphenyl-1-picrylhydrazyl, Sigma-Aldrich, USA) was employed for this study. The DPPH (24 mg) was diluted in methanol until 1.1±0.02 absorbance was obtained at 517 nm. Ascorbic acid (vitamin C) was utilized as standard (100 µg/ml). 20-100 µl of standard vitamin C and 10, 50, 100 µl of each collagen peptide, gelatin and omega-3 fatty acid was added to test tube. The volume was adjusted to 100 µl with D/W. Into each tube 3 ml of DPPH was added and incubated in dark for 30 min. The absorbance at 517 nm was measured using UV-visible spectrophotometer (Shimadzu UV-1800, Japan). The free radical scavenging activity (%) of each sample was calculated using following formula;

$$\% \text{ inhibition} = \frac{A(\text{control}) - A(\text{sample})}{A(\text{control})} \times 100$$

Where;

A (control):- initial DPPH absorbance

A (sample):- absorbance of each sample

6.2.3.4. Antimicrobial activity

Antimicrobial efficacy of collagen peptide and gelatin was evaluated against Gram positive *Staphylococcus aureus* NCIM 2654 (*S.aureus*) and *Bacillus subtilis* NCIM

2635 (*B.subtilis*) as well as Gram negative *Escherichia coli* NCIM 2832 (*E.coli*) and *Proteus vulgaris* NCIM 2813 (*P.vulgaris*). Sterilized nutrient agar media plates were prepared by using standard procedure. 100 µl of each bacterial suspension was spread with the help of sterilized borer. The 10-100 µl of 1 mg/ml of collagen peptide and gelatin were added into respective well. The prepared plates were incubated at 37ºC for 24 hr and observed for zone of inhibition. The 0.2 M lactic acid was used as control and antimicrobial activity was studied as per same method.

6.2.3.5. Anti-inflammatory activity of omega-3 fatty acid

The in vitro anti-inflammatory activity of omega-3 fatty acid was investigated by using protein denaturation method as per method of Nayaka et al.(2021). From stock, 1.5 and 3 mM concentration of omega-3 fatty acid was used for this study. The reaction mixture contains, 0.4 ml egg albumin (from fresh hen's egg), 5.6 ml PBS (pH 6.4) and 100 µl of sample. The double distilled water was employed as control. The incubation of resulting mixture was carried out at 37ºC in incubator for 15 min and further heated for 5 min at 70ºC. The absorbance of both control and sample was recorded at 660 nm. Diclofenac sodium was utilized as reference and its absorbance was determined as per same protocol. The percentage inhibition (IC 50) of protein denaturation was calculated by using following equation,

$$\% \text{ inhibition} = \frac{C - T}{C} \times 100$$

Where,

T:- absorbance of test

C:- absorbance of control

6.2.4. Agriculture applications

6.2.4.1. Seed germination by collagen

The extracted collagen was tested for its efficacy to stimulate seed germination.The seeds of mung (*Vigna radiata*), masoor (*Lens culinaris*) and mataki (*Vigna aconitifolia*) were surface sterilized with 70% ethanol and washed thoroughly with D/W. The seeds were placed in petri plate and various concentrations of collagen (1%, 3% and 5%) were supplemented to it. The radicle emergence of all three types of seed was recorded after 24 and 48 hr. The deionized water was used as control. The experiment was carried out in triplicate on 30 seeds with 10 ml of each solution.

6.2.5. Preparation of functional foods supplemented with collagen and gelatin

6.2.5.1. Raggi balls

For preparation of raggi balls incorporated with collagen, 100 gm fine raggi flour, 50 gm jaggary, 75 gm ghee and 2% (w/w) collagen was used. Raggi flour and jaggary were taken in a bowl and mixed properly. The mixture was poured into ghee containing pan and continued with further addition of remaining ghee on low gas flame. Mix all components appropriately to avoid clumping and continue it till flour attains brown colour. At last, collagen was added to it and balls were prepared while control balls were made without addition of collagen. Physicochemical and nutritional characteristics were studied according to method of Kim et al., (2012).

6.2.5.2. Gelatin jelly

The jelly incorporated with gelatin was prepared as per protocol of Choi and Regenstein (2000) with some changes. The 2.5 gm of extracted gelatin was soaked in hot water. The sugar syrup was prepared by keeping it on low gas flame followed by addition of 2 ml lemon juice. The soaked gelatin was poured into sugar syrup and boiled for 5 min for proper mixing. The prepared mix was transferred into large plate and 15 ml mango juice as well as few drops of yellow food colour (Asian Food products, Maharashtra, India) were mixed together. The resulting solution was kept for maturation at 4ºC for 4 hr. After incubation, the gelatin jelly was cut into pieces for its nutritional and physicochemical analysis. The market jelly was used as control (Choi and Regenstein 2000).

6.2.6. Sensory analysis of functional foods

The sensory properties of functional foods prepared from collagen and gelatin were carried out. The characteristics like food appearance, odour, colour, texture and overall acceptability were examined by non-trained 9 participants from Food Science and Technology department, Shivaji University, Kolhapur by using 9 point hedonic scale (from 1:- dislike extremely; 2:- dislike very much; 3:- dislike moderately; 4:- dislike slightly; 5:- neither like nor dislike; 6:- like slightly; 7:- like moderately; 8:- like very much and 9:- like extremely).

6.3. Result and discussion

6.3.1. Enzymatic hydrolysis of peptide

Use of proteolytic enzymes for the enzymatic hydrolysis of proteins is commonly used in food sector. As compare to acids and alkalis, enzymes hydrolyse proteins more softly. They do not need high temperature and generally breakdown the specific peptide

linkages, so it was easy to predict the peptide profile of protein. Collagen peptides with low molecular weight are expected to possess improved bioactivities than their larger parts (Hong et al.2019). The various commercial enzymes were employed for collagen peptide or hydrolysate preparation. It includes papain (Hong et al.2017; Sasaoka et al.2017), alcalase, pepsin (Cheung and Chan 2017), α-chymotrypsin (Ngo et al.2011), neutrase, flavourzyme (Chen et al.2016), trypsin (Abdollahi et al.2018), pronase E (Kim et al.2001) and collagenase (Liu et al.2011).

6.3.2. Molecular weight distribution of collagen peptide

The collagen peptides of extracted collagen were generated by enzymatic hydrolysis from protease of *S.sciuri* PSD 11 and it was depicted in figure no.6.1. It was observed that, the fractional breaking of β chain was occurred due to enzyme within 24 hr. The both α chains cleave into fragments of 10-60 kD. The similar peptide pattern was observed for standard calf skin collagen. However, complete hydrolysis of collagen was not happened because it was resistant to enzymatic hydrolysis.

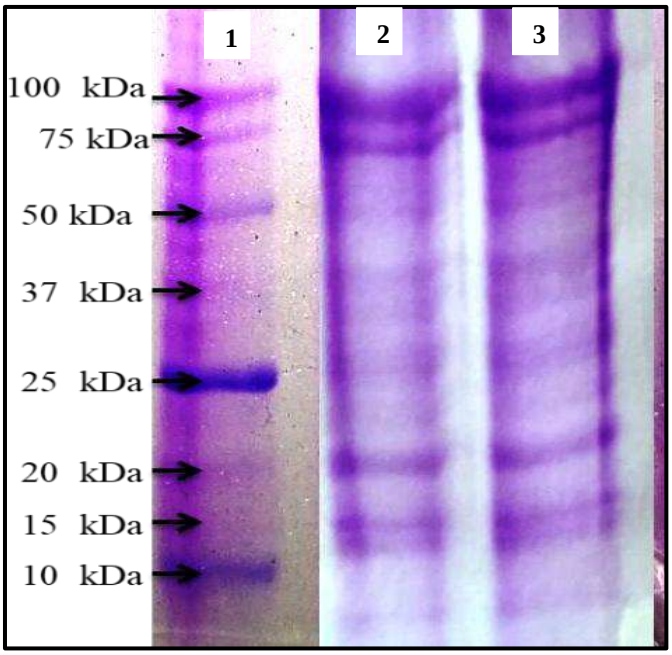

Figure 6.1.: Peptide mapping of collagen: lane 1:-molecular weight marker; lane 2:-peptides of waste collagen; lane 3:-peptide of standard calf skin collagen

6.3.3. Biomedical applications

6.3.3.1. Anti-diabetic activity of peptide

The interaction between genetic and environmental elements is the main reason of diabetes and is identified by absence of insulin secretion and resistance leads to metabolic illnesses of fat, protein and carbohydrates (Kumanan et al.2010). In presence

of calcium, α-amylases carry out hydrolysis of α-1,4 glycosidic bond. Due to more α-amylase action and insulin scarcity, the blood glucose level raises results in type II diabetes (Agarwal and Gupta 2016). In current study, the α-amylase inhibition assay was implemented to evaluate in vitro anti-diabetic ability of collagen peptides. The percentage (%) inhibition of α-amylase is increased as the concentration of peptide increases from 1 to 5 mg/ml (figure no.6.2;a).The maximum inhibition was recorded for 5 mg/ml (65.59±0.10%).

The standard acarbose exhibited 73.64±0.53% inhibition which is more than synthesized collagen peptide. The 1, 3 and 5 mg/ml collagen peptides displayed 6.51, 6.78 and 7.13 mg/ml IC50 values respectively while acarbose has 7.75 mg/ml (figure no.6.2;b). The anti-diabetic capability was absent in 0.2 M lactic acid. The obtained % inhibition and IC50 of collagen peptides were more or less similar to the red porgy, common pandora, annular seabream and unicorn leatherjacket (Fernandez et al.2001; Kumar et al.2018). The potency of α-amylase inhibition by collagen peptides is influenced by substrate, peptide configuration as well as hydrolysis temperature (Kumar et al.2018).

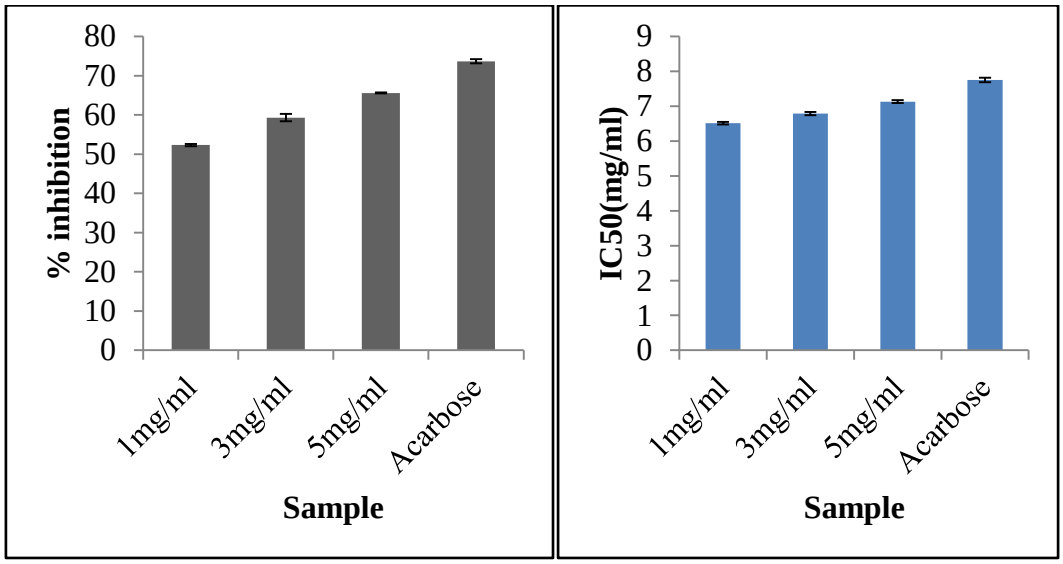

Figure 6.2.: Anti-diabetic activity of collagen peptide (a) % inhibition; (b)IC50

6.3.3.2. Cytotoxic activity of peptide

The cytotoxic activity was performed by membranolytic and non-membranolytic mechanism. In membranolytic action, initially peptide binds parallel to the membrane at low concentration while connects perpendicularly as concentration increases. Besides this, higher peptide to lipid ratio causes peptide insertion into bilayer results in formation of trans membrane pores. The non-membranolytic mechanism involves stimulation of

apoptosis, activation of extrinsic apoptotic pathways and inhibition of angiogenesis (Oelkrug et al.2015).

MTT assay was performed to study the influence of collagen peptides on growth, and viability of MCF-7 (Breast cancer cell line). The 1-5 mg/ml collagen peptides were used and from above stock solutions, 10, 40 and 100 µg/ml concentration was used. The cytotoxic activity of peptides ranges from 24.63 to 80.46% (figure no.6.3;a). It was found that, 1 mg/ml displayed 24.57±0.44%, 49.19±0.57% and 65.47±0.49% inhibition while 3 mg/ml showed 35.59±0.40%, 40.02±0.549% and 54.56±0.57% inhibition at 10, 40 and 100 µg/ml concentration respectively. Also, 5 mg/ml exhibited 70.80±0.49%, 71.97±0.65% and 80.40±0.46% inhibition at 10, 40 and 100 µg/ml concentration respectively. The positive control cyclophosphamide exhibited 75.29±0.51, 83.81±0.42 and 86.54±0.49% inhibition at same concentration. The observed values are very close to standard cyclophosphamide. The cytotoxic activity of peptides increases subsequently with increase in sample concentration from 1 mg/ml to 5 mg/ml. IC50 value is the measure of half maximal inhibitory concentration of a drug.

The figure no.6.3 (b) depicted the improvement in IC50 with respect to increase in concentration. The IC50 for 1, 3 and 5 mg/ml collagen peptide was reported as 37.0, 47.0 and 58.59 mg/ml respectively. Comparatively less IC50 was detected for cyclophosphamide (32.91 mg/ml). The control 0.2 M lactic acid does not show cytotoxic effect. In some cases, only collagen from bluefin tuna skin may reduce the growth of HepG2 and HeLa cells (Han et al.2011). The obtained results were analogues with the collagen peptides from salmon, milk fish, tilapia and unicorn leatherjacket which showed anticancer activity against HepG2, HeLa and HCT-166 cells, human fibro sarcoma and COLO320 cancer cells Han et al.2011; Baehaki et al.2016; Chen et al.2009; Kumar et al.2018). This results indicated that, the decline in cell growth by collagen or collagen peptides was influenced by type of cancer cell, fish species used and sometimes degree of hydrolysis. The enzymatic hydrolysis converts triple helical collagen into its disintegrated structure with low molecular weight. So, peptides can easily adhere to the surface of cancer cells resulting in inhibition of their growth and proliferation (Kumar et al.2018).

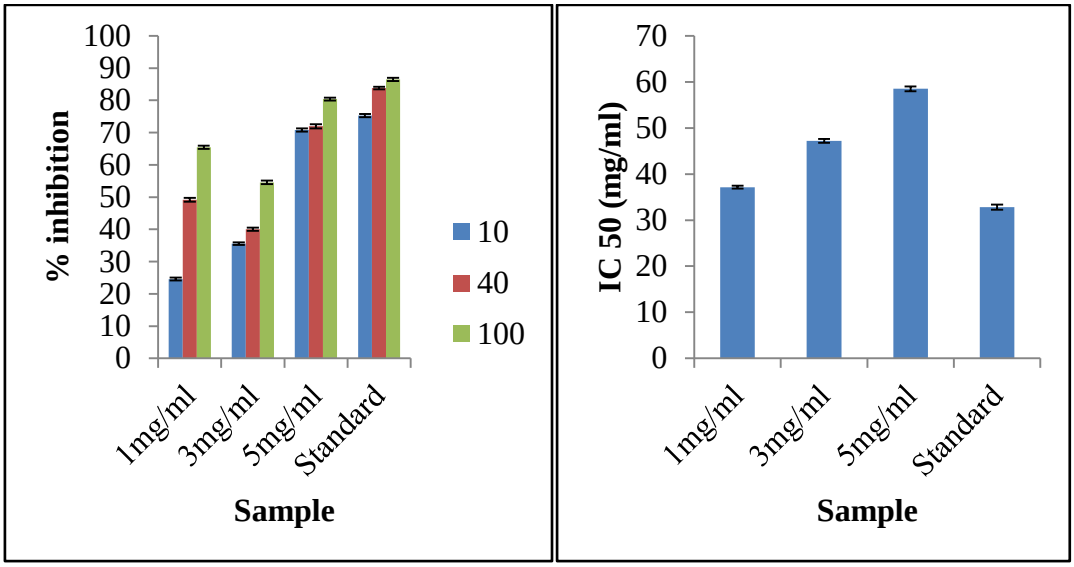

Figure 6.3.: Cytotoxic activity of collagen peptide (a) % inhibition; (b)IC 50

6.3.3.3. Antimicrobial activity of collagen peptide

The collagen peptide was assessed for its antimicrobial activity against Gram positive (*B. subtilis* and *S. aureus*) and Gram negative (*E.coli* and *P. vulgaris*) bacteria by agar well diffusion technique. The observation of activity was given in figure no.6.4. The highest 32.66 mm zone of inhibition was observed against *S.aureus* for 100 µl and 24.33mm zone was detected against *B.subtilis*. In case of Gram negative bacteria, *E.coli* and *P.vulgaris* showed 23.83mm and 27.83mm zone of inhibition respectively. The collagen peptide from marine fish gethar has good antimicrobial activity against Gram negative than Gram positive micro-organism. Collagen from marine sources exhibited improved quantity of peptides having antimicrobial potential. These peptides mainly synthesized by enzymatic hydrolysis so safe for various applications related to human health. The similar activity was observed by Ennaas et al. (2016).

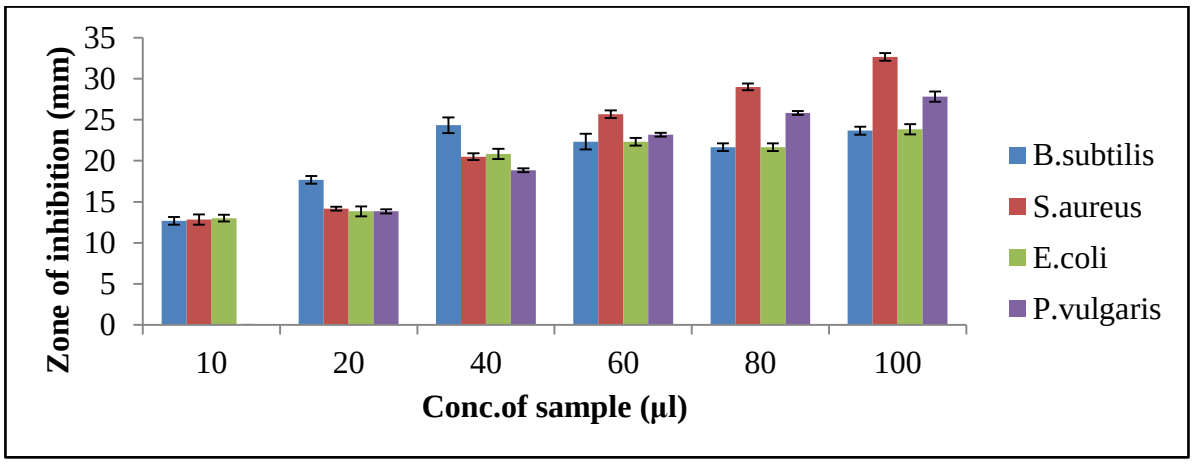

Figure 6.4.: Antimicrobial activity of collagen peptide

6.3.3.4. Antimicrobial activity of gelatin

The antimicrobial compound has ability to damage cell wall which leads to disruption of cell membrane, cytoplasmic leakage, cell lysis and ultimately causes cell death. The damage of cell membrane leads to reduction in pH results in loss of regulation of cellular metabolism and other activities such as ATP biosynthesis, DNA transcription as well as protein synthesis (Kavoosi et al.2013). Antimicrobial activity of extracted gelatin was studied by agar well diffusion method. The results were given in figure no.6.5. In case of Gram positive bacteria, 21mm zone of inhibition against *S.aureus* was observed for 100 µl while 19mm zone was detected against *B.subtilis* for same concentration. Both Gram negative bacteria *E.coli* and *P.vulgaris* showed 18mm zone of inhibition for 100µl concentration. The 10µl sample was ineffective against all four micro-organisms.

It was noticed that, 60µl concentration of gelatin effectively inhibits the growth of micro-organisms by generating 14, 19, 14 and 15 mm zone of inhibition against *B.subtilis*, *S.aureus*, *E.coli* and *P.vulgaris* respectively. It was found that; gelatin from skin of marine fish gethar exhibited good antimicrobial potential against Gram positive and negative micro-organisms while it was more useful in inhibiting growth of Gram positive than Gram negative bacteria. The results were similar to the gelatin from skin of unicorn leatherjacket incorporated with essential oils (Ahmad et al.2012). Due to good antimicrobial potential gelatin can be acts as a suitable food packaging material.

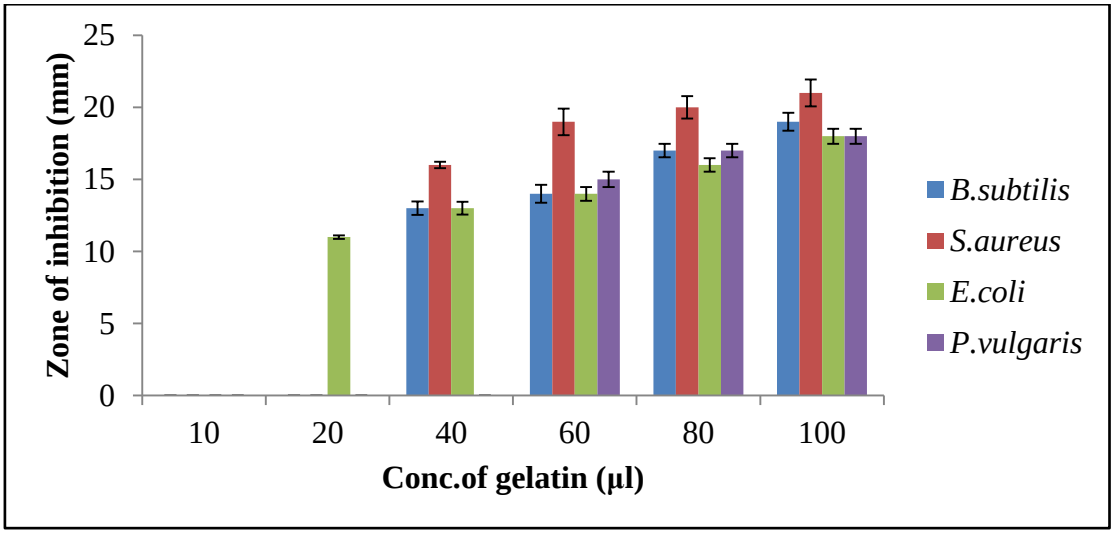

Figure 6.5.: Antimicrobial activity of gelatin

6.3.3.5. Antioxidant activity of collagen peptide, gelatin and omega-3 fatty acid

The radical scavenging capacity of collagen peptides, gelatin and omega-3 fatty acid was examined to determine their hydrogen atom or electron transfer property. The amount and sequence of amino acid, degree of hydrolysis and molecular weight of peptides has effect on antioxidant activity of peptides. The low molecular weight hydrolysate has greater antioxidant ability than high molecular weight. (Hong et al.2019; Sionkowska et al.2021). DPPH is a cell permeable stable radical and therefore usually utilized for rapid assessment of antioxidant properties of compounds. The reaction between DPPH and antioxidant molecules generates analogous hydrazine while purple colour of solution gets reduced (absorbance at 517 nm) (Yang et al.2018).

Gomez-Guillen et al.(2010) reported that, repeating motif Gly-Pro-Hyp of peptides is the prime sequence responsible for antioxidative property of peptides. The antioxidant activity of studied collagen peptide and gelatin was related to peptide from porcine and bighead carp skin (Hong et al.2019; Sionkowska et al.2021) as well as hoki skin and giant squid muscle gelatin respectively (Mendis et al.2005a; Rajapakse et al.2005b). The obtained antioxidant activity of omega-3 fatty acid was in accordance with EPA and DHA of fish oil capsules (Kotue et al.2019). The antioxidant activity of collagen peptide, gelatin and omega-3 fatty acid was depicted in figure no.6.6. The results showed that, increase in sample concentration from 10-100 µl improve the antioxidant potential of each sample. The maximum % inhibition was obtained at 100 µl. For collagen peptide, inhibition was 23.51±0.58% while 13.51±0.76% and 6.27±0.31% was obtained for gelatin and omega-3 fatty acid respectively.

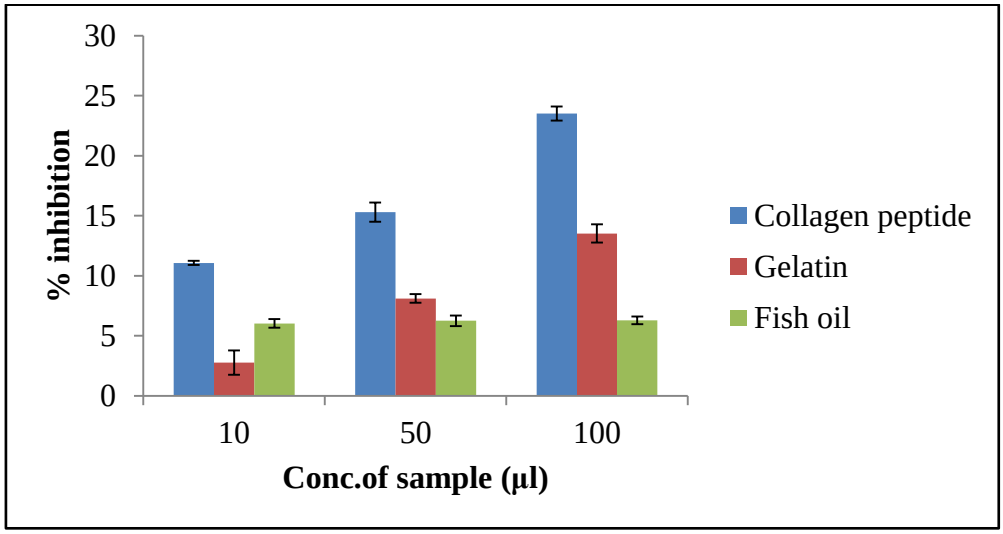

Figure 6.6.: Antioxidant activity

6.3.3.6. Anti-inflammatory activity of omega-3 fatty acid

Protein denaturation involves damages of protein's secondary and tertiary structure due to the interference of external pressure or compounds, including strong acid or base, organic solvents, concentrated inorganic salts or heat. Numerous biological proteins fail to perform their biological action after its degradation. The disintegration of tissue proteins is one of the important causes of inflammation. Hence, protein denaturation is useful screening test for the determination of anti-inflammatory potential of compounds without exploitation of animals. The anti-inflammatory activity of polyunsaturated fatty acids was confirmed by various studies. PUFA supplemented diet has been advantageous in treatment of irritable bowel disease (IBD), psoriasis, eczema, rheumatoid arthritis, ulcerative colitis as well as helps to reduce mucosal damage (Fratianni et al.2021).

The n-3 fatty acids especially EPA and DHA from fish oil found to inhibit different inflammation processes like leucocyte chemotaxis, adhesion molecule communication and leucocyte-endothelial adhesive connections. The anti-inflammatory mechanism of n-3 fatty acid is associated with the variation in phospholipid concentration of cell membrane, interruption of lipid rafts and reduced expression of inflammatory genes by inhibiting activation of the pro-inflammatory transcription factor nuclear factor kappa B (Calder 2012). The EPA and DHA provide protection to egg albumin against heat induced denaturation. The gethar fish oil exhibited less % inhibition than pure fish oil and Diclofenac sodium which was taken as control. The % inhibition of 1.5mM and 3mM of oil was recorded as 25.62±0.47% and 57.70±0.34% respectively (figure no.6.7). The obtained results were related to the % inhibition by n-3 fatty acids from seeds of apricot, peach, cherry and plum (Fratianni et al.2021).

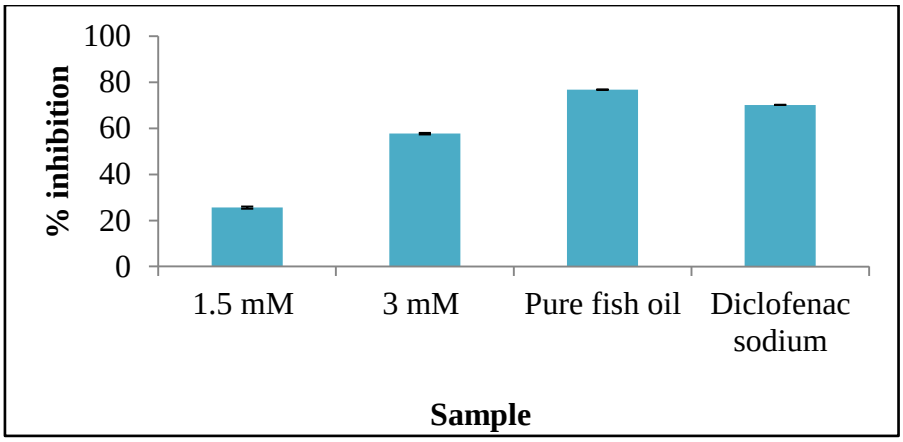

Figure 6.7.: Anti-inflammatory activity of fish oil containing omega-3 fatty acid

6.3.4. Agriculture applications

6.3.4.1. Seed germination by collagen

Collagen and its hydrolysate widely applied as plant growth stimulator because they have ability to promote plant growth by increasing shoot and root length, plant biomass as well as its productivity (Luziatelli et al.2016). The good amount of amino acids in collagen supplies energy during germination phase and hence enhances germination index (Niculescu et al.2017). In present work, 1 to 5% collagen was tested for seed germination enhancement against mung (*Vigna radiata*), masoor (*Lens culinaris*) and mataki (*Vigna aconitifolia*) for 24 and 48 hr.

The results were depicted in figure no.6.8 (a), (b) and (c) respectively. It was observed that, maximum sprout length was detected for 3% collagen in all seeds mentioned above. The *V.radiata* possessed highest sprout length as 22.33±2.62mm for 48 hr as compared to other seeds. The similar results was reported for chicken feather and protein hydrolysate which promotes the growth of wheat and lettuce respectively (Bhise et al.2017; Luziatelli et al.2016). All three concentrations have good germination effect as compared to control (D/W). Thus, collagen extracted from fish waste can be employed as alternative to chemical biofertilizer for various agricultural applications.

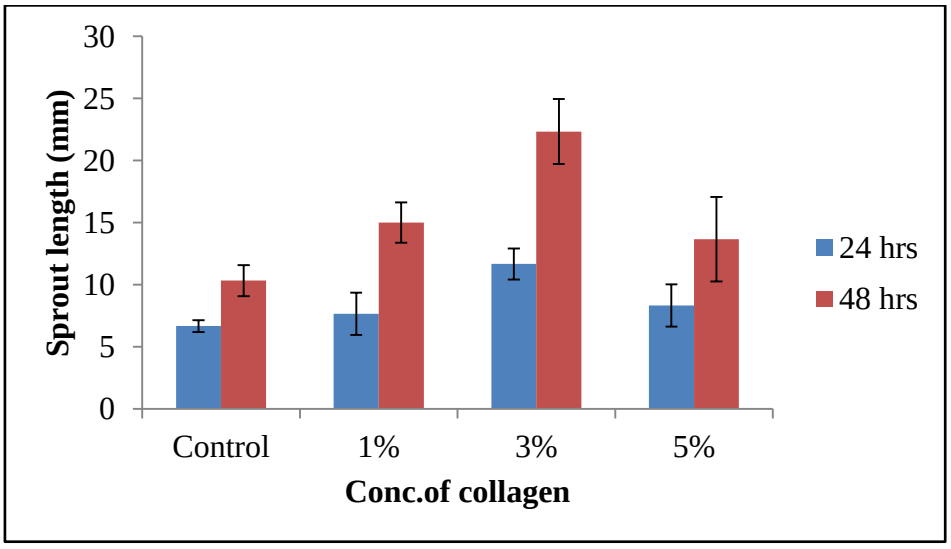

Figure 6.8 (a).: Seed germination analysis of *V.radiata*

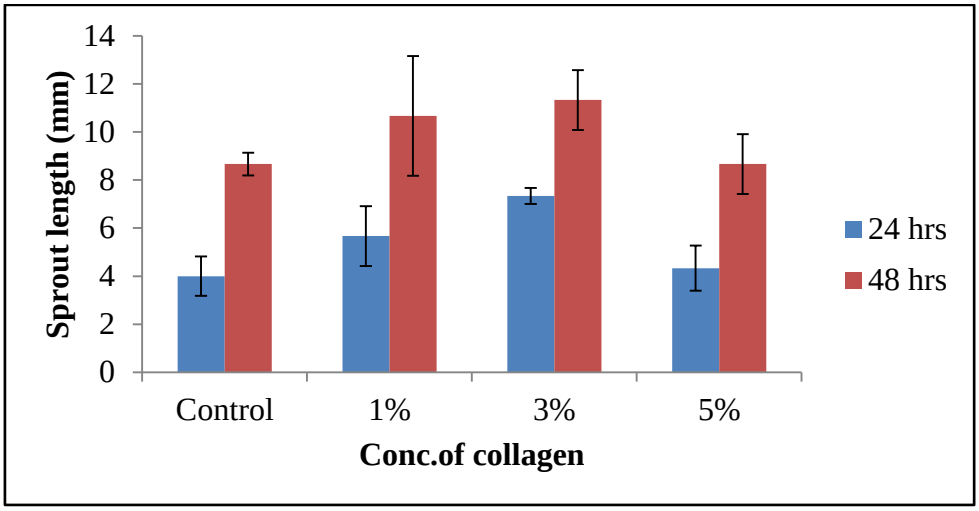

Figure 6.8 (b).:Seed germination analysis of *L.culinaris*

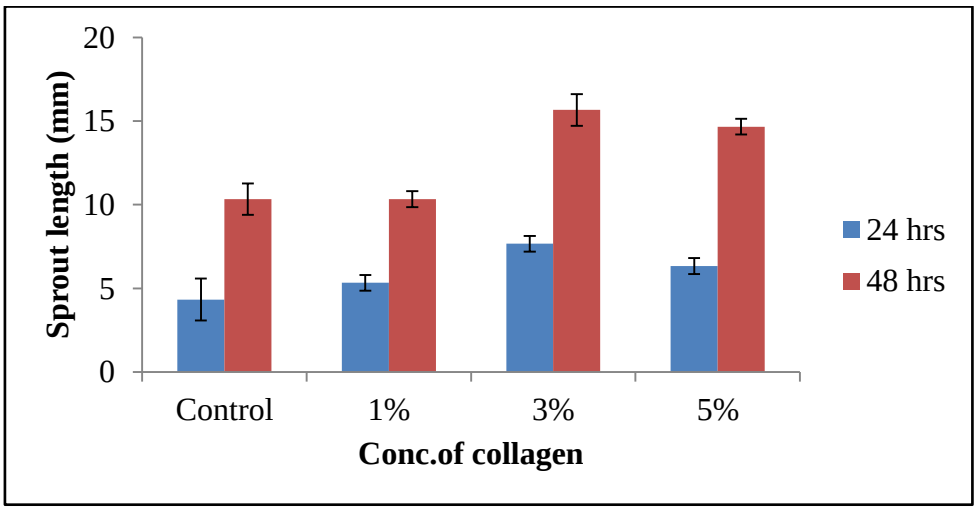

Figure 6.8 (c).: Seed germination analysis of *V.aconitifolia*

6.3.5. Functional food preparations

In this modern era, human beings are very conscious about health, so development of nutritious diet has increasing demand to fulfil the body need. The collagen content in body decreases due to aging so collagen supplementation in diet maintains its level. So, collagen becomes an important component for the preparation of functional foods by combining it with other nutritional components. Gelatin is the hydrolysed form of collagen. It has good sensory characteristics thus, useful in the preparation of jelly and other food products.

6.3.5.1. Raggi balls incorporated with collagen

Raggi is useful in natural weight loss. It is rich source of iron so, its consumption minimizes problem of anaemia. Also, it has good amount of protein so raggi containing

supplements along with collagen provides essential health benefits. The nutritional and physicochemical analysis of raggi balls was tabulated in table no.6.1. As per analysis, it was detected that, very less moisture (%) and fat content was present in both control and test. The amount of protein was increases in test (8.33±0.61 gm) than control (6.99±0.80 gm) due to incorporation of 2% collagen. Nearly same quantity of carbohydrates was noticed in both control and test while small difference was observed in energy (533.43±0.53 Kcal for control and 533.89±0.54 for test). The amount of calcium, iron and potassium was slightly reduced in test than control one.

The figure no.6.9 depicted the raggi balls incorporated with collagen while sensory analysis of it by using 9 point hedonic scale was given in figure no.6.10. The analysis was carried out with respect to appearance, colour, odour, texture and overall acceptability. The sample raggi ball possesses slight fishy odour but not easily perceivable, this is due to marine source of collagen which can be removed by utilizing more effective purification techniques. This is the first report on preparation of raggi balls incorporated with collagen. Therefore, the overall nutritional and physicochemical characteristics of raggi balls were increased due to supplementation of collagen.

Parameters/100gm	Control	Test
Moisture (%)	5.04±0.90	5.13±0.44
Fat (gm)	1.92±0.48	1.77±0.28
Protein (gm)	6.99±0.80	8.33±0.61
Total ash (gm)	1.12±0.41	1.13±0.65
Carbohydrates (gm)	64.89±0.31	64.91±0.89
Energy (Kcal)	533.43±0.53	533.89±0.54
Salt as NaCl	Nil	Nil
Calcium (mg)	279.48±0.57	273.56±0.69
Iron (mg)	5.36±0.44	5.43±0.19
Potassium(mg)	17.59±0.50	17.52±0.99

Table 6.1.: Nutritional and physicochemical analysis of raggi balls

Figure 6.9.: Raggi balls incorporated with collagen

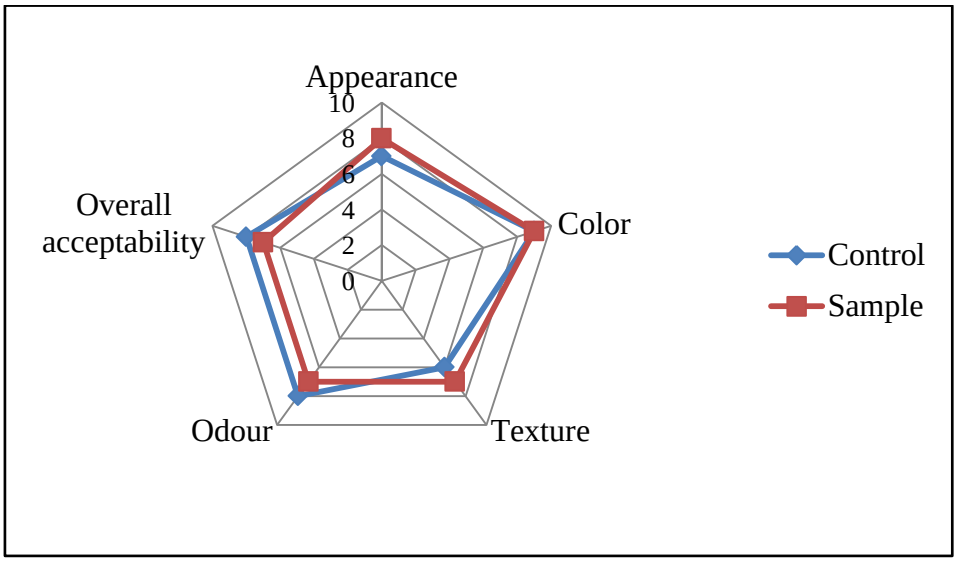

Figure 6.10.: Sensory analysis of raggi balls

6.3.5.2. Jelly incorporated with gelatin

Jelly is the common product that used for decoration of food components like cake and ice-cream. It is a soft elastic component made up from gelatin, boiled sugar and fruit juice. Gelatin is extensively used in confectionery because of its gel like nature and solidifies into small pieces that dissolve slowly in mouth to create sweet sensation. The nutritional and physicochemical analysis of jelly incorporated with gelatin was presented in table no.6.2. Jelly is one of the high moisture containing food and it was prime factor to define shelf lifespan and pureness of protein. The moisture content of control and test gelatin was recorded as 84.89±0.23% and 86.24±0.53% respectively. The ash content has no significant effect on jelly quality (Chukwu and Abdullahi 2015). The slight variation was detected in carbohydrate and fat concentration. The quantity of calcium, iron and

potassium was detected as 6.13±0.41 mg, 0.168±0.97 mg and 52.11±0.25 mg respectively in test which was more than control. The calorie obtained from control was 266.25±0.18 Kcal but in test it was enhanced as 271.14±0.07 Kcal.

The figure no.6.11 showed the jelly incorporated with gethar skin extracted gelatin. The sensory evaluation of jelly was carried out using 9 point hedonic scale with respect to appearance, colour, odour, texture and overall acceptability was given in figure no.6.12. The jelly incorporated with gelatin has slightly rough texture than control because of crystallization of sugar. The colour alteration is due to use of different food colours. Thus, jelly integrated with fish gelatin is acceptable along with control for various applications in nutraceutical. The similar results were reported by Yusof et al. (2019) for jelly combined with halal gelatin.

Parameters/100gm	Control	Test
Calories (Kcal)	266.25±0.18	271.14±0.07
Fat (gm)	0.023±0.04	0.024±0.003
Carbohydrates (gm)	69.95±0.41	68.73±0.82
Protein (gm)	0.157±0.48	0.175±0.32
Calcium (mg)	7.04±0.36	6.13±0.41
iron (mg)	0.191±0.03	0.168±0.97
Potassium (mg)	54.01±0.08	52.11±0.25
Ash (%)	0.25±0.08	0.29±0.01
Moisture (%)	84.89±0.23	86.24±0.53

Table 6.2.: Nutritional and physicochemical analysis of jelly

Figure 6.11.: Jelly incorporated with gelatin

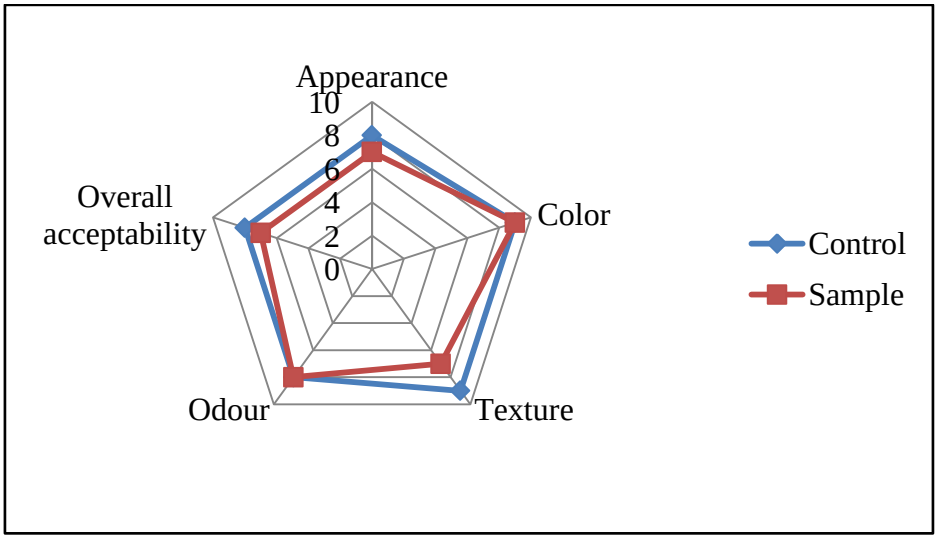

Figure 6.12.: Sensory analysis of jelly

6.4. Conclusion

The biological macromolecules and peptide from fish waste material of gethar (*Sarda orientalis*) was extracted and evaluated for various applications. The enzymatic hydrolysis of collagen generates peptide of less than 30 KDa. This peptide was effectively prevent growth of MCF-7 cancer cells and also has good anti-diabetic as well as antioxidant potential. It also inhibits growth of Gram positive and negative micro-organisms. The amino acids present in collagen provide nutrition during seed germination of *Vigna radiata, Lens culinaris and Vigna aconitifolia*. The extracted gelatin has ability to scavenge free radicals as well as good antimicrobial ability. The EPA and DHA present in fish oil have anti-inflammatory property. As collagen and gelatin are proteins, they incorporated in functional food preparations such as raggi balls and jelly, so this the major avenue to fulfil the need of protein as protein supplement in sports, bodybuilders and malnourished peoples in all age groups. Thus, total exploitation of fish waste was carried out which helps to diminish environmental pollution caused by these waste. The compounds derived from it have potential applications in pharmaceutical, nutraceutical and agricultural industries.

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CHAPTER VII

SUMMARY AND CONCLUSION

7.1. Summary

The continuous development in fishing industries from last few years produces more amount of waste. But till date, there is no proper disposal strategy was available for this. The improper disposal causes hazardous impact on environment as well as on health. The fish waste comprises scale, skin, fins, tail and head. Fish and its waste contains good amount of biomolecules such as proteins, lipids, enzymes, vitamins, macro and micronutrients. Among these biomolecules, the valuable collagen, gelatin and omega-3 fatty acid containing oil has increasing demand. Thus, marine species provides an alternative and safe resource for the extraction of these components with minimum ethical and health issues. The fish waste remaining after recovery of these valuable components was degraded by micro-organism. The formed organic liquid hydrolysate was applied as plant growth stimulator as well as soil conditioner to improve crop productivity and soil fertility. This study provides a creative idea for the utilization of fish waste in a proper technological way which helps in management of fish waste. Henceforth, this research work compromises stepwise and complete exploitation of fish waste. It will help to decrease environmental pollution problems and small scale industry can be developed by utilizing these techniques to help the entrepreneurs. The collagen, gelatin and omega-3 fatty acid from this waste has diverse applications in pharmaceutical, nutraceutical and agriculture sector.

7.2. Conclusion

- The waste material of marine fish gethar (*Sarda orientalis*) was utilized for extraction of collagen, gelatin and omega-3 fatty acid.
- For collagen extraction, the protocol was optimized to find out suitable organic acid for maximum production of collagen.
- The 0.2 M lactic acid gives 34% collagen yield within 40 hr and further it was utilized to study its different structural and functional properties.
- The collagen showed UV absorption spectra at 269 nm. The SDS-PAGE revealed that extracted collagen was type I with two α and one β chain.
- The various functional groups attached to collagen were detected by FTIR spectroscopy while fibrous nature of it was observed in SEM analysis.
- The good thermal stability of collagen was determined using DSC analysis. The zeta potential was found as -0.345 mV and the mean particle size ranges from 3.12-14.51 d.nm.
- The collagen contains high amount of glycine, proline and alanine amino acid. Also good amount of hydroxyproline was detected.
- The extracted collagen possesses good functional properties. It has 0.229±2 turbidity, 57.23±0.37% solubility and 2.18±0.04 cP viscosity. Also 19.7µl/mg water holding capacity and 12.2±0.21 gm/gm oil absorption capacity was detected. It exhibits better emulsifying and foaming characteristics.
- The collagen has maximum solubility at pH 2.0 (99.36%) while forms more precipitation at 2 M NaCl (99.96%).
- The sensory analysis score of colour, appearance and overall acceptability of extracted ASC was more than 3 hedonic points. It has slight fishy odour but is not easily detected and can be removed by using more specific purification techniques.
- The skin and head part of gethar was utilized for extraction of gelatin and fish oil containing omega-3 fatty acids respectively.
- The gelatin was extracted using lactic acid solubilisation method and chloroform: methanol: water solvent system was utilized for extraction of omega-3 fatty acid.
- The 0.63±0.03 gm/gm gelatin was obtained within 2 hr from skin of gethar.
- The gelatin showed UV absorption spectra at 280 nm and its molecular distribution was studied using SDS-PAGE.

- The FTIR spectroscopy was used to analyse different functional groups attached to gelatin. It exhibits 2θ = ~19º in XRD analysis which is occurred mainly due to gelatin.
- In DSC studies, gelatin forms single broad endothermic peak and three smaller peaks. It has 2.24 mV zeta potential. The particle size of gelatin rages from 30-600 r.nm.
- Gelatin contains more amounts of glycine and alanine followed by imino acids (proline and hydroxyproline).
- The extracted gelatin possesses goof functional properties. It is hot water soluble and has 6.67±0.19 cP viscosity. Clarity and turbidity value of gelatin was found to be 41.1±1.31 and 257.66±6.54 FTU respectively. It exhibits 10.75±0.65ºC gelling and 29.88±0.41ºC melting temperature.
- The 3% gelatin showed good emulsifying properties while 5% gelatin has good foaming characteristics. The water holding and fat binding capacity was recorded as 262.33±13.88% and 407.66±14.83% respectively.
- The extracted gelatin from gethar skin was type A with pH 8.0 as isoelectric point but it has maximum solubility at pH 6.0 (99.55%).
- The 27.63±0.24% oil was extracted from gethar fish head waste.
- The fatty acid profiling of fish oil was carried out using gas chromatography-mass spectroscopy. The analysis shows that there are two types of omega-3 polyunsaturated fatty acid found in gethar oil which is EPA (1.74%) and DHA (14.19%).
- The functional characteristics of fish oil were evaluated to study its quality. It contains 2.25±0.20% free fatty acids. The acid, iodine and saponification value was recorded as 2.57±0.36 mg KOH/gm of oil, 107.88±0.53 gm iodine/gm oil and 78.53±0.49 mg KOH/gm oil respectively. The peroxide and p-anisidine value was found to be 25.72±0.45 Meq/gm and 17.29±0.48 Meq/gm.
- The isolation and characterization of fish waste degrading micro-organism from fish waste dumping site was carried out and named as *Staphylococcus sciuri* PSD 11.
- The microbial hydrolysis of waste remaining after extraction was carried out by *S.sciuri* and degrades waste within 7 days with liberation of amino acids and proteins.

- The organic liquid hydrolysate was generated after degradation was studied for its plant growth promotion ability on *Vigna radiata*.
- The improvement in physical and biochemical characteristics of *V.radiata* was observed. Thus, fish waste hydrolysate effectively acts as plant growth stimulator.
- The collagen, gelatin and omega-3 fatty acids were extracted from various waste materials of gethar (*Sarda orientalis*) using suitable extraction procedures.
- The collagen peptides of molecular weight less than 30 KDa were synthesized using enzymatic hydrolysis technique. These peptides were explored to study its bioactive properties like anticancer, anti-diabetic, antioxidant and antimicrobial.
- These peptides inhibit growth of MCF-7 cancer cells with 58.59 mg/ml IC50. It also has anti-diabetic potential and IC50 value ranges from 6.51 to 7.13 mg/ml. It has ability to scavenge free radicals with 23.51±0.58% inhibition. Also acts as antimicrobial agent against Gram positive and negative micro-organisms.
- The different amino acids present in collagen supplies nutrition during seed germination of *Vigna radiata, Lens culinaris and Vigna aconitifolia*. All seeds form good sprout length within 24 and 48 hr.
- The extracted gelatin was tested for its antioxidant and antimicrobial potential. It has capacity to scavenge free radicals with 13.51±0.76% inhibition. Also has antimicrobial activity against Gram positive and negative micro-organisms.
- The EPA and DHA present in fish oil have better anti-inflammatory and antioxidant property with 57.70±0.34% and 6.27±0.31% inhibition respectively.
- The extracted collagen and gelatin was utilized in functional food preparations like raggi balls and jelly respectively. The sensory analysis of these foods showed that, incorporation of collagen and gelatin from fish source is moderately acceptable. Both functional foods have better nutritional and physicochemical characteristics. Thus, helps to provide potential health benefits.
- This was the first report in which waste material of marine fish gethar was utilized for extraction of collagen, gelatin and omega-3 fatty acid.
- Collagen is a rich source of amino acids. It's supplementation in functional foods helps to fulfil the protein demand of health. Also provides protection against some diseases. It was the first report in which collagen was incorporated into raggi balls.
- The complete utilization of fish waste was carried out to extract biologically important macromolecules. The residual waste remaining after extraction was

degraded by micro-organism to generate organic liquid fertilizer which is used to improve crop productivity. This will helps to reduce use of chemical fertilizers in agriculture.

- This research work successfully employed as innovative and sustainable technology to minimize the environmental pollution caused by improper disposal of fish waste.
- The efficient extraction of collagen, gelatin and omega-3 fatty acid from waste material decreases the cost of waste management and the extracted macromolecules have increasing demand in food, pharmaceutical and agricultural sectors.

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- **Kirdat P.N.**, Dandge P.B. (Dec 2021).Structural properties of gelatin extracted from marine fish Gethar (*Sarda orientalis*). International Journal of Innovative Science, Engineering and technology, volume 8, issue 12.ISSN:- 2348-7968.
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- **Kirdat P.N.**, Dandge P.B., Hagwane R.M., Nikam A.S., Mahadik S.P., Jirange S.T. (Dec 2020). Synthesis and characterization of ginger (*Zingiber officinale*) extract mediated iron oxide nanoparticles and its antibacterial activity. Materials Today: Proceedings, 43(1), pp.2826-2831. DOI no.10.1016/j.matpr.2020.11.422. IF=1.46, citations: 6.

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- **Pranoti N. Kirdat**, Padma B. Dandge. Microbial degradation of residual fish waste and use of hydrolysate as plant growth stimulator. Two days online international conference on advances in science and technology (ICAST-2022). Rajarshi Chhatrapati Shahu College, Kolhapur, 9-10 March 2022.
- **Pranoti N. Kirdat**, Sandip S.Kale, Padma B. Dandge. Structural and Functional properties of gelatin extracted from fish waste. International E-conference on sustainable development in chemistry and scientific applications (SDCSA-2021) organized by Sadguru Gadage Maharaj College, Karad, 16-17 December 2021.
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STUDIES ON BIOCHEMICAL ANALYSIS OF FISH WASTE AND ITS APPLICATIONS

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BY

Miss. PRANOTI NAGESH KIRDAT
M. Sc.

UNDER THE GUIDANCE OF

Dr. (Mrs.) PADMA B. DANDGE
M.Sc. Ph.D.

ASSOCIATE PROFESSOR
DEPARTMENT OF BIOCHEMISTRY,
SHIVAJI UNIVERSITY, KOLHAPUR,
MAHARASHTRA, INDIA

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Chapter “80 Recommendations”

1. Recommendation

Chapter I:-

It gives brief information about fish waste, its management and useful components present in it. The literature review describes the details regarding sources, extraction methods and applications of collagen, gelatin and omega-3 fatty acid present in fish waste.

Chapter II:-

It represents the various experimental procedures carried out for extraction of useful components from fish waste. The different characterization techniques utilized to characterize samples were also described in it.

Chapter III:-

The waste material of marine fish gethar (*Sarda orientalis*) was collected from fish industries and local market. The extraction, purification and characterization of collagen were carried out in this. The structural and functional properties of collagen were studied.

Chapter IV:-

The waste material of marine fish gethar (*Sarda orientalis*), mainly skin and head was collected from fish industries and local market. The extraction, purification and characterization of gelatin and omega-3 fatty acid from skin and head were carried out in this. The structural and functional properties of gelatin and omega-3 fatty acid were studied. The GCMS analysis revealed the presence of omega-3 fatty acid in fish oil.

Chapter V:-

Isolation and characterization of micro-organism *Staphylococcus sciuri* PSD 11 from fish waste dumping site was done in this study. The fish waste remaining after extraction of useful components was subjected to microbial degradation by isolated micro-organism. The generated liquid hydrolysate was employed as plant growth stimulator for *Vigna radiata*.

Chapter VI:-

The collagen peptides were synthesized from extracted collagen using enzymatic hydrolysis. It was then studied for anticancer, anti-diabetic, antioxidant and antimicrobial activity. The extracted collagen was used to study seed germination of *Vigna radiata, Lens culinaris* and *Vigna aconitifolia.* The extracted gelatin was studied for its antioxidant and antimicrobial potential. Omega-3 fatty acid was subjected to study their antioxidant and anti-inflammatory potential. The extracted collagen and gelatin were employed for preparation of functional foods like raggi balls and jelly respectively.

2. Conclusion

The waste material of marine fish gethar (*Sarda orientalis*) was utilized for extraction of collagen, gelatin and omega-3 fatty acid. The 0.2 M lactic acid gives 34% collagen yield within 40 hr and further it was utilized to study its different structural and functional properties. The gelatin was extracted using lactic acid solubilisation method and chloroform: methanol: water solvent system was utilized for extraction of omega-3 fatty acid. The 0.63±0.03 gm/gm gelatin was obtained within 2 hr from skin of gethar. The 27.63±0.24% oil was extracted from gethar fish head waste. The fatty acid profiling of fish oil was carried out using gas chromatography-mass spectroscopy. The analysis shows that there are two types of omega-3 polyunsaturated fatty acid found in gethar oil which is EPA (1.74%) and DHA (14.19%). The isolation and characterization of fish waste degrading micro-organism from fish waste dumping site was carried out and named as *Staphylococcus sciuri* PSD 11. The microbial hydrolysis of waste remaining after extraction was carried out by *S.sciuri* and degrades waste within 7 days with liberation of amino acids and proteins. The organic liquid hydrolysate was generated after degradation was studied for its plant growth promotion ability on *Vigna radiata*. The collagen peptides of molecular weight less than 30 KDa were synthesized using enzymatic hydrolysis technique. These peptides were explored to study its bioactive properties like anticancer, anti-diabetic, antioxidant and antimicrobial. The extracted gelatin was tested for its antioxidant and antimicrobial potential. It has capacity to scavenge free radicals with 13.51±0.76% inhibition. Also has antimicrobial activity against Gram positive and negative micro-organisms. The EPA and DHA present in fish oil have better anti-inflammatory and antioxidant property with 57.70±0.34% and 6.27±0.31% inhibition respectively. The extracted collagen and gelatin was utilized in functional food preparations like raggi balls and jelly respectively. The sensory analysis of these foods showed that, incorporation of collagen and gelatin from fish source is moderately acceptable. Both functional foods have better nutritional and physicochemical characteristics. Thus, helps to provide potential health benefits. This was the first report in which waste material of marine fish gethar was utilized for extraction of collagen, gelatin and omega-3 fatty acid.

3. Summery

The continuous development in fishing industries from last few years produces more amount of waste. But till date, there is no proper disposal strategy was available for this. The improper disposal causes hazardous impact on environment as well as on health. The fish

waste comprises scale, skin, fins, tail and head. Fish and its waste contains good amount of biomolecules such as proteins, lipids, enzymes, vitamins, macro and micronutrients. Among these biomolecules, the valuable collagen, gelatin and omega-3 fatty acid containing oil has increasing demand. Thus, marine species provides an alternative and safe resource for the extraction of these components with minimum ethical and health issues. The fish waste remaining after recovery of these valuable components was degraded by micro-organism. The formed organic liquid hydrolysate was applied as plant growth stimulator as well as soil conditioner to improve crop productivity and soil fertility. The collagen, gelatin and omega-3 fatty acid from this waste has diverse applications in pharmaceutical, nutraceutical and agriculture sector.

4. Future Findings

To minimize the environmental pollution caused due to fish waste.

Utilization of fish waste, to extract more amount of collagen, gelatin and omega-3 fatty acid.

To develop antimicrobial food packaging film from gelatin.

Preparation of functional food incorporated with collagen to provide healthy diet for malnutritious and anaemic patients.

To produce anticancer and anti-diabetic drug by using collagen peptides.

To generate anti-inflammatory product by using omega-3 fatty acid.

Synthesis of nanoparticles from these components and their various applications.

To develop organic liquid biofertilizer for horticulture and agriculture.