UTILIZATION OF FISH SCALE BY MICROBIAL INTERVENTION

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by

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Dedicated to

My

Family, Friends

and

My Guide

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Dated: 30th June, 2019

CERTIFICATE

Certified that the dissertation entitled **"UTILIZATION OF FISH SCALE BY MICROBIAL INTERVENTION"** is a bonafide record of independent research work carried out by **Ms. Sangita Bora** during the period of study from August, 2018 to June, 2019 under our supervision and guidance for the degree of **Master of Fisheries Science (Post-Harvest Technology)** and that the dissertation has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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DECLARATION

I hereby declare that the dissertation entitled "**UTILIZATION OF FISH SCALE BY MICROBIAL INTERVENTION**" is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

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ABSTRACT

The present study was carried out to develop simple, cost-effective and eco-friendly methodologies for the utilization of fish scales by using microbes in order to overcome the problem of waste disposal. The protein and calcium content of carp scales varied from 21.27±0.74 to 24.10±0.86% and 3.11 ±0.35 to 4.70±0.43% respectively in the study. Out of 105 bacterial cultures isolated from the fish scale and scale disposed soil, only 28 were gelatinase positive. Out of 50 gelatinase positive cultures, only 28 showed different digestion patterns in RFLP done by using 2 restriction enzymes viz. MspI and AluI. Out of 10 halophilic cultures tested for gelatinolytic activity, only 4 were positive. Ten bacterial and three haloarchaeal cultures were selected to check their protein removal efficiency from carp scales. Bacterial cultures were more efficient as compared to haloarchaeal isolates. They removed up to 97.73% protein from carp scales within 28 days. Whereas, haloarchaeal cultures took 75 days to remove 96.54% protein. The microbes showed higher efficiency in the hydrolysis of carp scale protein viz. *Acinetobacter towneri* and *Lysinibacillus sphaericus* were selected for the production of fish scale protein hydrolysate by the microbial fermentation process. Fish scale protein hydrolysate prepared by using *Lysinibacillus sphaericus* showed higher protein content and a higher degree of hydrolysis than the hydrolysate prepared by using *Acinetobacter towneri.* Both the hydrolysates showed good antioxidant activity. Fish scale protein hydrolysate prepared by using *Lysinibacillus sphaericus* showed higher antioxidant activity as compared to the hydrolysate prepared by using *Acinetobacter towneri.* Both the hydrolysate showed antimicrobial activity against *Escherichia coli*, *Pseudomonas aeroginosa* and *Bacillus subtilis.* Carp scales were also used for the preparation of two value-added products, fish scale wafers and coated fish scales. Both the products obtained more than 7 scores for all the attributes which indicate that products are liked moderately by the panel members. Thus the present study demonstrates that microbes can be effectively used for the production of high valued products from fish scales as well as help to overcome the problem of their disposal.

सारांश

वर्तमान अध्ययन अपशिष्ट निपटान की समस्या को दूर करने के लिए सूक्ष्मजीवों का उपयोग करके मछली खाल के उपयोग के लिए सरल, लागत प्रभावी और पर्यावरण के अनुकूल तरीके विकसित करने के लिए किया गया था। अध्ययन में कार्प खाल की प्रथिन और कैल्शियम सामग्री क्रमशः २१.२७±०.७४ से २४.१०±०.८६% और ३.११±०.३५ से ४.७०±०.४३% तक परिवर्तित हुई। मछली के खाल और मिट्टी से अलग किए गए खाल के १०५ जीवाणु संवर्धन पृथक में से केवल २८ जिलेटिनस सकारात्मक थे। ५० जिलेटिनस सकारात्मक पृथक में से केवल २८ ने ही RFLP में अलग-अलग पाचनशक्ति स्वरूप दिखाया, जिसमें २ प्रतिबंध किण्वक का उपयोग किया गया; MspI और AluI। जिलेटिनोलिटिक गतिविधि के लिए परीक्षण किए गए १० हेलोफिलिक पृथक में से केवल ४ सकारात्मक थे। कार्प के खाल से उनकी प्रथिन हटाने की दक्षता की जांच करने के लिए १० जीवाणु संवर्धन और ३ हेलोआर्किया संवर्धन का चयन किया गया था। जीवाणु संवर्धन के हेलोआर्किया संवर्धन मुकाबले अधिक प्रभावी थीं। उन्होंने २८ दिनों के भीतर कार्प खाल से ९७.७३% प्रथिन को हटा दिया। जबकि, ९६.५४% प्रोटीन को हटाने के लिए हेलोआर्किया पृथक को ७५ दिन लगे। जीवाणु संवर्धन, कार्प खाल प्रथिन के हाइड्रोलिसिस में हेलोआर्किया आइसोलेट्स के मुकाबले अधिक कुशल साबित हुए। माइक्रोबियल किण्वन प्रक्रिया द्वारा मछली के खाल प्रथिन हाइड्रोलाइज़ेट के उत्पादन के लिए *Acinetobacter Towneri* और *Lysinibacillus sphaericus* को चुना गया था। *Lysinibacillus sphaericus* के द्वारा तैयार किए गए मछली के खाल प्रथिन हाइड्रोलाइज़ेट ने *Acinetobacter Towneri* के द्वारा तैयार किए गए हाइड्रोलाइज़ेट की तुलना में उच्च प्रथिन सामग्री और हाइड्रोलिसिस का उच्च स्तर दिखाया। दोनों हाइड्रोलिसिस ने अच्छी प्रतिउपचायक गतिविधि दिखाई। *Lysinibacillus sphaericus* का उपयोग करके तैयार किए गए मछली के खाल प्रथिन हाइड्रोलाइज़ेट में *Acinetobacter Towneri* का उपयोग करके तैयार किए गए हाइड्रोलाइज़ेट की तुलना में उच्च प्रतिउपचायक गतिविधि दिखाई दी। दोनों हाइड्रोलाइज़ेट ने *Escherichia coli, Pseudomonas aeroginosa* और *Bacillus subtilis.*के खिलाफ रोगाणुरोधी गतिविधि दिखाई। कार्प खाल का उपयोग दो मूल्य वर्धित उत्पादों- मछली खाल के वेफर्स और लेपित मछली खाल की तैयारी के लिए भी किया गया था। दोनों उत्पादों ने सभी मापदंडोंके आधार पर ७ से अधिक अंक प्राप्त किए जो दर्शाता है कि उत्पादों को पैनल के सदस्यों द्वारा पसंद किया गया था। इस प्रकार वर्तमान अध्ययन से पता चलता है कि मछली के खाल से उच्च मूल्यवान उत्पादों के उत्पादन के लिए रोगाणुओं को प्रभावी ढंग से इस्तेमाल किया जा सकता है और साथ ही उनके निपटान की समस्या को दूर करने में मदद मिलती है।

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LIST OF ACRONYM

FPH	Fish protein hydrolysate
%	Percentage
µl	Microlitre
bp	Base pair
DNA	Deoxyribonucleic acid
CFU	Colony forming unit
dNTP	Deoxyribonucleotide triphosphate
Fig.	Figure
Tab.	Table
g.	Gram
Kb	Kilobase
Min	Minute
mL	Milliliter
mM	Millimolar
OD	Optical density
MHA	Mueller Hinton Agar
RFLP	RFLP (Restriction fragment length polymorphism) analysis

DPPH	1,1-diphenyl-2-picryl-hidrazyl) Radical Scavenging Activity
ANOVA	Analysis of variance
FAO	Food and Agricultural organization
MPEDA	Marine Products Export Development Authority
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) Radical Scavenging Activity
FRAP	Ferric reducing antioxidant power
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
DH	Degree of hydrolysis

1. INTRODUCTION

Global fish production has been increasing steadily over the last decade and this trend is expected to continue. The world fish production has reached to approximately 171 million metric tons (FAO, 2016). India is the second largest fish producing country contributing about 6.4% of the world's fish production (MPEDA, 2017). As a highly perishable commodity, fish needs to be processed and preserved quickly. About 60-70% of fish is processed before final sale, resulting in the generation of 20-80% of fish waste depending on the level of processing and type of fish (Ghaly *et al*., 2013). According to MPEDA (2017) data, there are about 525 fish processing units in India with a daily processing capacity of 25000 metric tons. These processing plants generate a very high amount of organic waste which is either land-filled or used as manure or even dumped into the river or sea. The organic components of the waste have a high biological oxygen demand when released to the sea and, if not managed properly it will create an unbalanced and unhealthy condition for the aquatic flora and fauna. Therefore, proper management of the waste is critical for environmental reasons.

The processing of fish generates solid wastes as well as liquid waste. Solid fish processing waste consists of head, skin, scale, fins, bones, gut, internal organs etc., which accounts for 50-80% of the original raw material (Zynudheen, 2010). Scales contribute a significant proportion of the total solid waste generated from the processing of fish. In the case of freshwater fish, scales generally constitute 1-2% of the body weight (Zynudheen, 2010). Scales are biodegradable waste but it takes a long time to degrade due to its hardy nature and constitution (Pal, 2017). Therefore, it causes hurdles in the complete utilization of mixed waste.

Fish scale is a source of numerous organic and inorganic components. The organic components of fish scale account for 40%-55% which includes collagen, scleroprotein, lecithin, fat and variety of vitamins, etc. On the other hand, the percentage of inorganic components was about 7%-25%, including hydroxyapatite and calcium phosphate (Suo-Lian *et. al*., 2017). Scales are the source of many

commercially important products such as hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) and type I collagen (Gil-Duran *et al.,* 2016) which have many applications in food, biomedical, pharmaceutical, cosmeceutical industries. Therefore, there is a need for the development of technologies to effectively utilize fish scales. Most of the collagen available in the market is extracted from the mammalian sources. Since mammalian tissue collagen proteins can transmit diseases such as bovine spongiform encephalopathy, avian and swine influenzas, and tooth-and-mouth disease, the attention is shifted to fish collagen. Hydroxyapatite is another important component of fish scale which has biomedical and pharmaceutical applications. Due to its similarity to human hard tissues, it is suitable for the dental and orthopedic field (Mustafa *et al.,* 2015).

Several chemical and enzymatic methods have been developed to extract these compounds from the fish scale. But the main problem associated with these methods is the use of hazardous chemicals. The disposal of these chemicals to the environment may cause so many problems. Some chemicals may not have an immediate, visible impact. But they can enter into the food chain and accumulate or persist in the environment for many years. The use of microbes is a promising area where microbial activities can be used to extract these commercially important components of fish scale. Several studies have been conducted on the collagenolytic activity of microorganisms and it is found that *Zygosaccharomyces rouxii, Bacillus subtilis* FS-2*, Clostridium histolyticum, Streptomyces* sp., *Pseudomonas* sp*., Vibrio vulnificus* etc. show collagenolytic activity. (Mandl, 1953; Smith, 1982; Endo *et al*., 1987; Ok and Hashinaga, 1996; Nagano and To, 2000; Gautam *et al*., 2017). These microbes can effectively release commercially important compounds from the fish scale. So, the main aim of this study is the application of microbes to utilize fish scales for the production of high valued products. In addition, this will alleviate the problem of waste disposal. With this background the following objectives were set for the present study:

1) To analyze the composition of scales.
2) To select microbes targeting specific component of fish scales.
3) To apply selected microbes for the utilization of fish scales.

2. REVIEW OF LITERATURE

2.1 Fish Production Status

The world fish production is increasing with the growing demand for fish and seafood throughout the world. The world fish production has reached about 171 million tons in 2016, with aquaculture representing 47% of the total fish production. India is the second largest fish producing country contributing 6.4% to global fish production (FAO, 2016). India's fish production has reached 12.6 million metric tons in 2017-18, of which nearly 65%2 is from the inland sector (FAO, 2014). Freshwater aquaculture contributes to over 95 percent of the total aquaculture production of the country.

Carp culture forms the backbone of freshwater aquaculture of India. Freshwater aquaculture of the country is dominated by the culture of Indian Major Carps which includes *Labeo rohita, Catla catla and Cirrhinus mrigala*. The Indian major carps, namely catla (*Catla catla*), rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*) is the most important group contribute the bulk of production up to 70 to 75 percent of the total freshwater fish production (FAO, 2014). The remaining 25 to 30 percent is contributed by silver carp (*Hypophthalmichthys molitrix*), grass carp (*Ctenopharyngodon idella*), common carp (*Cyprinus carpio*), catfishes forming the second important group (FAO, 2014). They occupy an important place in aquaculture due to their high consumer preference. High domestic consumption results in the generation of a high amount of waste which poses a different level of waste disposal problems.

2.2 Impact of Fish Processing Waste on Environment

The most important negative impact of an industry is pollution due to waste they generate as a result of various production activities. All the food processing industries generate a huge amount of waste which may lead to a long-term negative

impact on the environment. The most important environmental issues related to the food industry are wastewater discharge and disposal of both solid organic and inorganic wastes. Wastewater discharge may cause the following problems in the receiving water bodies-increase in biological oxygen demand (BOD), total suspended solids (TSS) along with eutrophication due to excess level of nitrogen and phosphorous content. Solid organic wastes include seeds, rinds of fruit; skin, heads, scales, fins, viscera of fish etc. results from processing operations. Inorganic wastes include-plastic, glass, metal etc. (Shanahan *et al*., 1998).

The fish processing industry is one of the major food industries in the world. Every processing operation generates some amount of waste. More than 50% of the fish tissues including head, fins, scales, viscera etc. are considered as waste. As a result, every year discards from world fisheries exceed 20 million tons which is equivalent to 25% of the total production of marine capture fishery and include non-target species, fish processing waste and by-products (FAOSTAT, 2001)

2.3 Composition of Fish Scale

Fish scale is an important part of the fish body which has a variety of functions ranging from protection to aqua dynamics. It consists of both organic and inorganic components. The percentage of organic components is about 40-55% which includes collagen, fat, lecithin, scleroprotein and variety of vitamins etc. The inorganic components constitute 7-25% of fish scale and comprised of hydroxyapatite and calcium phosphate (Suo-Laian *et al,* 2017). Collagen is a fibrous protein that contributes as a major structural protein in the fish scale. Mehboob *et al*. (2015) studied the proximate composition of *Catla catla* and *Cirrhinus mrigala* scale and reported that the proximate composition of the fish scale differs significantly between different weight groups. The protein content of the catla scale was varied between 22.26% and 23.90% on a wet weight basis. On the other hand, the protein content of the mrigal scale was varied between 20.36% and 21.77% on a wet weight basis. According to Sankar *et al*. (2008), fish scale contains 41-84% protein and the remaining is calcium salts on a dry weight basis.

2.4 Utilization of Fish Scales

The scale is an important component of fish processing waste which is often discarded as waste. But, the components of fish scales have so many important potential uses which are as follows-

2.4.1 Fish Collagen

The fish scale is an important source of type I collagen. About 70% of organic components of scale is consist of only collagen protein. Collagen has a wide range of applications in food, biomedical and pharmaceutical industries. Collagen derived from fish scales has been effectively used in various biomedical applications such as wound healing. It improves blood and lymphatic vessel formation, thereby improving tissue repair and regeneration. It also acts as a carrier of drugs that can enhance wound healing. Fish collagen also has applications in cosmetics and it is used in anti-aging and anti-wrinkle creams.

Fish collagen has also been used in the food and agriculture industries. Bhagwat and Dandge (2016) developed paneer by in-corporation of the collagen extracted from *Cyprinous carpio* scales. Paneer was found to be acceptable with good textural and sensorial attributes. Residual scales were further treated enzymatically and the released metabolites were used to promote the growth of plant *Vigna radiata*. The plant showed excellent growth.

Generally, bovine collagen is the main source of type-I collagen, but the outbreak of Bovine Spongiform Encephalopathy (BSE), Foot and Mouth Disease (FMD), Transmissible Spongiform Encephalopathy (TSE) in cattle, pigs have resulted in anxiety among users of collagen from these sources (Jongjareonrak *et al*., 2005). Therefore, other sources such as fish processing wastes including skin, bone or scale have been used as alternative sources of collagen extraction (Ogawa, *et al*., 2004; Nagai *et al.,* 2006). Pati *et al*. (2010) reported that collagen extracted from freshwater fish scale have denaturation temperature (Td) of 36.5^{o}C, which close to mammalian collagen.

2.4.2 Fish Gelatin

Denatured collagen is known as gelatin. It is a colorless, flavorless food ingredient derived from collagen. It is commonly used as a gelling agent in food, medications, drug and vitamin capsules, photographic films, papers and cosmetics. In the food industry, gelatin is used as an ingredient to improve the elasticity, consistency and stability of foods such as ice-creams, desserts, gummy candies, marshmallows, dips, yogurts etc. In the pharmaceutical industry, it is used for weight loss formulations and for treating osteoarthritis, rheumatoid arthritis, and brittle bones (osteoporosis) (Su and Wang, 2015).It is used as water-soluble capsules and coating materials for oral drugs ((Su and Wang, 2015). It is also used as a stabilizer of photo-sensitive reagents in photographic films, adsorbent for diluted chemicals and adhesive agents. Weng *et al.,* 2014 used gelatin from the tilapia scale as an edible food packaging material. Nowzari *et al.,* 2013 studied the effect of chitosan–gelatin composite and bilayer coating on the quality of refrigerated rainbow trout. The composite showed antibacterial and antioxidant properties and had the potential to be used as active packaging material for the preservation of fish under refrigerated storage.

2.4.3 Hydroxyapatite

Hydroxyapatite (HAp) is a naturally occurring mineral form of calcium apatite usually written $Ca_{10}(PO_4)_6(OH)_2$. It is similar to the human hard tissues in morphology and composition. Therefore, hydroxyapatite is a kind of bioceramic that has so many applications i.e. for bone tissue engineering, bone void fillers for orthopedic, spine and dental surgery, orthopedic and dental implant coating, endodontic treatment like pulp coating, remineralizing agent in toothpaste, drug and gene delivery by using nano-hydroxyapatite etc. (Mondal *et al.,* 2018) Pon-On *et al. (*2016) reported that hydroxyapatite derived from freshwater fish scale (*Probarbus jullieni*) are biologically better than the chemically synthesized Hydroxyapatite and have the potential for use as a bone scaffold or as regenerative materials. Kongsri *et al.* (2013) reported that nanocrystalline hydroxyapatite prepared from fish scale waste (FHAp) using the alkaline heat treatment method possessed a good ability to adsorb

selenite. They concluded that hydroxyapatite nanopowders from the fish scale waste can be used as a natural adsorbent with high adsorption efficiency. Liu *et al.* (2017) studied the synthesis of hydroxyapatite scaffold from the fish scale and utilized it for the removal of lead ion. They reported that fish scale-derived hydroxyapatite scaffolds can be used for heavy metal ion removal and wastewater treatments.

2.4.4 Protein Hydrolysate/Bio-Peptides from Fish Scales

Fish protein hydrolysate is an excellent source of amino acids, dipeptides and polypeptides which is produced by the hydrolysis of protein using chemicals, enzymes or microbes. It serves as the main source of bioactive peptides and they become more active after the hydrolysis (Gill *et al.,* 1996). Lu *et al.* (2016) utilized the tilapia fish scale to prepare protein hydrolysates which are usually discarded as solid waste. They reported that peptides present in the hydrolysate have the calcium-binding ability and that peptide-calcium complex showed higher calcium uptake in Caco-2 cells. Therefore, it can be used in food industries as a calcium supplement. Another study conducted by Zeng *et al*. (2015) and reported that collagen hydrolysate produced by using enzymes possess antioxidant activity in vivo and in vitro assays. They suggested that it can be used as a good source of desirable antioxidant peptides for nutraceutical and pharmaceutical applications. The antioxidant properties of protein hydrolysate could be attributed to the fact that there are peptide sequences with the ability to transfer H-atom/electron, thereby terminating the free radical-induced chain reaction (Elavarasan *et al.*, 2014). Song *et al. (*2010) added fish scale collagen hydrolysate in cake and sausage. It was found that the textural and sensory quality of cake and sausage enhanced due to the addition of hydrolysate. Huang *et al*. (2015) reported that collagen peptides have an iron-binding ability. They utilized scales *Lates calcarifer, Mugil cephalus, Chanos chanos*, and *Oreochromis* sp. for their study. Collagen peptides from *Chanos chanos* showed the highest Fe (II)-binding activity, followed by those from *Lates calcarifer* and *Mugil cephalus; Oreochromis* sp. exhibited the lowest ability. They concluded that collagen peptides from fish scales can be applied in the industry as a bioresource. Hussain *et al*. (2017) used alkali (NaOH) for the preparation of fish protein hydrolysate from *Cyprinus carpio* scales. The hydrolysate showed

antimicrobial activities against different pathogens viz. *Escherichia coli, Margenella morganii*, *Haemophilus influenzae*, *Klebsilla pneumoniae*, *Pseudomonas aeruginosa*, and *Actenobactor baumannii*. They suggested that the antimicrobial properties of protein hydrolysate could be due to the presence peptide sequences with inhibitory effects towards the selected microbes. Fahmi *et al.* (2004) extracted the angiotensin-I converting enzyme inhibitory peptides from sea bream scale hydrolysate produced by *using an* alkaline protease.

2.4.4.1 Antioxidant Mechanism of Bio-Peptides

In general amino acid composition, molecular size, sequence and hydrophobicity of peptides believed to play an essential role in antioxidant activity (Harnedy and FitzGerald, 2012). Xing *et al*. (2016) reported that hydrophobic amino acids including methionine and proline in peptide sequences are believed to play an important role in scavenging free radicals because their large hydrophobic group can help them to facilitate the interactions with hydrophobic radical species. Acidic and basic amino acids play a critical role in HO- scavenging and metal ion chelating activities of the peptide. These activities are related to carboxyl and amino groups in their side chains (Murota, *et al*., 2014; Sila and Bougatef, 2016). Chang *et al*. (2013) found that basic (arginine) and acidic (Glutamic acid and aspartic acid) amino acid residues in the specific sequences of NTDGSTDYGILQINSR and LDEPDPLI were critical for their antioxidant activities. Antioxidant activities of peptides also depend highly on their molecular size. Shorter size peptides especially peptides with 2–10 amino acid residues are evidenced to show stronger lipid peroxidation inhibition and radical scavenging activities than long-chain peptides and their parent native proteins (Matsui *et al.,* 2018).

2.4.4.2 Mechanisms of Action of Antimicrobial Bio-Peptides

Antimicrobial peptides (AMPs) are low molecular weight peptides that have a net positive charge and are amphiphilic (Abuine *et al.,* 2019).Most antimicrobial peptides (AMPs) work directly against microbes through a mechanism involving membrane disruption and pore formation which allows reflux of essential ions and nutrients (Taha *et al.,* 2013). The molecular mechanism of membrane permeation may vary for different peptides depending on a number of parameters, such as the amino acid sequence, membrane lipid composition and peptide concentration (Shai, 2002). AMPs are able to bind to the cytoplasmic membrane which leads to a disruptive effect. Intracellular targeting of cytoplasmic components crucial to proper cellular physiology is another mechanism that may responsible for the antimicrobial activity of peptides (Harris *et al*., 2009; Yeaman and Yount, 2003). Thus, the initial interaction between the peptides and the microbial cell membrane would allow them to penetrate into the cell to bind intracellular molecules which results in the inhibition of cell wall biosynthesis and DNA, RNA and protein synthesis.

2.4.5 Other Potential Uses of Fish Scales

Fish scales have been used for the production of bio-plastic which is bio-degradable in nature. Thammahiwes *et al.* (2017) studied the properties of wheat gluten-based bioplastics with fish scales. They reported that the fish scale powder improved the tensile strength of wheat gluten-based bio-plastic due to the good adhesion between the wheat gluten matrix and the fish scale powder. They also reported that the rate of degradation of the wheat gluten-based bio-plastic was affected by the interaction between the wheat gluten matrix and fish scale powder. Fish scales have also been used for the green synthesis of metal nanoparticles. Sinha *et al.* (2014) reported that silver-nanoparticles synthesized by using fish scales of *Labeo rohita* can be used as catalysts for the reduction of aromatic nitro compounds. Durairaj and Hemalatha (2018) reported that silver nanoparticles prepared by using fish scales of *Catla catla* showed larvicidal activity against Anopheles larval strain in stage IV and Culex larval strain in stage III. As a natural adsorbent fish scales have the potential to

be used in wastewater treatment plants. Uysal *et al.* (2017) investigated the color removal efficiency of a column reactor filled with scales of European Seabass (*Dicentrarchus labrax*) which are released from the fish markets in huge amounts as waste. Othman *et al.* (2016) efficiency of fish scale in removing zinc (Zn) ion and ferrum (Fe) ions in domestic wastewater. Stevens and Batlokwa (2017) studied the efficiency of fish scales as a sorbent material for removing heavy metal ions (lead and zinc) from wastewater. Chowdhury *et al.* (2012) reported that the fish scales have the potential to be used as a low-cost biosorbent for the removal of hazardous Malachite Green (MG) dye from aqueous solutions.

2.5 Collagenolytic/Gelatinolytic Microorganisms

There are so many microorganisms known to produce collagenase and gelatinase enzymes. The bacterial collagenase secreted by *Clostridium histolyticum* was the first bacterial collagenase to be discovered and studied and was reported first by MacLennan *et al.* in 1953.Evans and Wardlaw (1953) studied Gelatinase and Collagenase Production by certain species of Bacillus. They found that some strains of B. cereus and *B. anthracis* produced a true collagenase which completely disintegrated the collagen substrate, while strains of *B. subtilis, B. licheniformis, B. brevis and B. megaterium* were either completely inactive or produced only partial disintegration. Gautam and Azmi (2017) isolated collagenase producing microorganisms from protein waste. Out of 27 collagenase producing bacteria *Pseudomonas* spp. was found the most efficient collagenase producer. Merkel *et al.* (1975) studied the collagenolytic activity of some marine bacteria using reconstituted, acid-extracted calfskin collagen. Savita and Arachana (2015) studied the production of collagenase by *Bacillus* KM369985 isolated from the leather sample. Suphatharaprateep *et al.* (2011) studied the production and properties of two collagenases enzyme extracted from a Gram-positive bacteria *Bacillus cereus* CNA1 and a Gram-negative bacteria *Klebsiella pneumoniae* CNL3 isolated under alkaline and acidic conditions respectively. Smith and Merkel (1982) studied the collagenolytic activity of *Vibrio vulnificus* and its potential contribution to its invasiveness. Ok and Hashinaga (1996) studied the production of the extracellular collagenolytic enzyme

from yeast *Zygosaccharomyces rouxii.* Cevahir *et al*. (2008) detected gelatinase activity in 14% isolates of *Acinetobacter baumannii.* Balan *et al*. (2012) reported gelatinase production in *Bacillus* spp isolated from the sediment sample Coastal sites. Maiti *et al*. (2013) reported high gelatinase activity in a newly isolated polycyclic aromatic hydrocarbon-degrading bacteria *Bacillus weihenstephanensis* strain AN1. Nakamura *et al.* (2004) reported that *Cerasibacillus quisquiliarum* strain BLx^T degrades gelatin. Sekowska *et al*. (2006) detected that 17.5% strains of *Klebsiella oxytoca* produced proteases hydrolyzing gelatin and in case of K. pneumoniae 8.9% strains were positive for gelatinase test.

2.6 Collagenase Enzyme

Collagenase enzymes come under the category of Metrix Metallopeptidase (MMPs) known to degrade collagen (Elliott and Cawston, 2001). Collagen is the most abundant protein that occurs in the extracellular matrices of animals making 25% to 35% of the whole-body protein content (Yang and Shu, 2014). Because of its special triple helix structure, collagen is resistant to most common proteases and can be degraded only by a few types of proteases. These protease enzymes capable of degrading one or more types of collagens are regarded as collagenases or collagenolytic proteases (Zhang *et al.,* 2015). This group of enzymes cleaves the collagen triple helix at a defined site near its N-terminus (Zhang *et al.,* 2015).

Bacterial collagenases from pathogens have been of concern mainly because of their potential as virulence factors. In a review by Popoff and Bouvet (2009), it is documented that the collagenases from several pathogenic *Clostridium* species are to be involved in the degradation of the specific cell membrane or extracellular matrix components. Smith and Merkel (1982) reported that the collagenase of *Vibrio vulnificus* has a potential contribution to the invasiveness of the bacteria into human tissue through open wounds. Many collagenases from *Clostridium* and *Vibrio* pathogens are supposed to have similar roles. Kim *et al.,* 2006 reported that serine collagenase plays an important role in the pathogenesis of *Acanthamoeba* sp.

and it is the main component of virulence factor. Besides pathogens, a lot of collagenolytic bacteria have been isolated from terrestrial soil and marine sediments. Collagenolytic proteases from various environmental bacteria play an important role in the release of fixed nitrogen into the global nitrogen cycle by the degradation of collagen protein (Zhao *et al.,* 2008). Bacterial collagenases also have biotechnological and medical applications. *Clostridium* collagenase has been used in laboratories to dissociate tissues and isolate cells (Suggs *et al.,* 1992). It is also used as a therapeutic drug for the removal of necrotic wound tissues (Mandl, 1982). It is successfully applied in the treatment of third-degree burns (Vrabec *et al.,* 1974).

2.7 Gelatinase Enzyme

Gelatinase is a protease enzyme capable of hydrolyzing gelatin, collagen, casein, hemoglobin, and other bioactive peptides (Cevahir *et al.,* 2008). Gelatinases are the most studied enzymes of the group matrix metalloproteinases, which also contains collagenases. They require Ca^{2+} for activity and are involved in the degradation of type IV, V, and denatured collagen (gelatin). There are two types of gelatinases: 72kDa gelatinase (gelatinase A) or MMP-2 and 92kDa gelatinase (gelatinase B) or MMP-9 (Baragi *et al*., 2000). Gelatinase has been reported to be virulence factors of *Enterococci* (Kanemitsu *et al.,* 2001). Vergis *et al*. (2002) reported that gelatinase producing strains of *Enterococcus faecalis* have been associated with the virulence of endocarditis in an animal model. Cevahir *et al*. (2008) detected gelatinase activity in 14% isolates of *Acinetobacter baumannii.* Shimoi *et al.* (2010) showed that gelatinolytic bacteria inhibiting spore adhesion by phytopathogenic fungi can be used as a biological control for rice blast disease. Sathya and Ushadevi (2014) isolated industrially important enzymes producing *Streptomyces* species from mangrove sediments. Among 35 morphologically distinct *Streptomyces* species, 21% were exhibited positive results for Gelatinase enzyme which can be used as a source of gelatinase to compensate current industrial demand.

2.8 Utilization of Fish Scale by Physical, Chemical and Enzymatic Methods

There are so many chemical and enzymatic methods available that have been extensively used to extract the important components from the fish scales. Collagen is the most important component of fish scale. Huang *et al.* (2016) extracted and characterized fish collagen from the tilapia (*Oreochromis* sp.) scale by a novel extrusion–hydro-extraction process. Zhang *et al*. (2011) prepared collagen from freshwater fish scales using acetic acid and pepsin. Wang *et al*. (2014) studied the physical-chemical properties of collagen from the skin, scale, and bone of grass carp (*Ctenopharyngodon idellus*). Mahboob (2015) extracted and characterized collagen from fish waste material viz. skin, scales and fins of *Catla catla* and *Cirrhinus mrigala*. Gelatin is another important product can be extracted from fish scales. It is the denatured form of collagen. Wangtueai and Noomhorm, (2009) optimized different combinations of NaOH concentration, treatment time, extraction temperature and extraction time to extract the gelatin from lizardfish (*Saurida* spp.) scales. Das *et al*. (2017) extracted and characterized gelatin from *Labeo rohita* scales by using alkali and used and concluded that fish scale is a cost-effective and environmentally friendly source of gelatin which can be an alternative of currently used synthetic polymeric materials in various industrial applications. Huang *et al*. (2017) extracted the gelatin from the scales of bighead carp (*Hypophthalmichthys nobilis*) by using an ultrasound purifier instrument and studied the rheological and structural properties of the extracted gelatin. Mohammad *et al.* (2015) studied the optimum conditions required for the preparation of gelatin hydrolysate by enzymatic hydrolysis of tilapia (*Oreochromis* spp.) scale gelatin.

2.9 Disadvantages of Chemical and Enzymatic Methods

Chemical hydrolysis is carried out by either acid or alkali. It is achieved by cleaving the bonds between different peptide groups in the protein by chemical agents. Since these processes require extreme working conditions such as the high concentration of acid or alkali, high temperature etc., the process of hydrolysis is almost uncontrollable in

chemical methods. Due to lack of control, the nutritional and functional properties of the fish protein hydrolysate (FPH) are reduced (Loffler 1986; Webster *et al.* 1982). Thus, FPH produced by chemical methods has a very limited spectrum of utilization (Petrova *et al.,* 2018). A number of amino acids are sensitive to acid or alkali. Amino acid serine and threonine are destroyed during alkaline hydrolysis (Pasupuleti and Braun 2010). The most important problem associated with the use of chemicals is their harmful effects on the environment.

Enzymatic hydrolysis is carried out at mild conditions; slightly elevated temperatures (generally around 35-65^{o}C) and at a certain pH according to the optimal requirements of the enzyme. Enzymes of microbial origin are considered to have greater temperature and pH stability (He *et al.*, 2013). Enzymatic methods allow control over the process of hydrolysis. The limitations of enzymatic processes are the low yield, high cost, need for special treatment in order to deactivate the enzyme etc. (He *et al.,* 2013; Kristinsson and Rasco, 2000)

2.10 Microbial Utilization of Fish Scales

Collagenase or gelatinase enzyme-producing Microorganisms have the ability to degrade collagen-based fish waste and. Ghanem *et al*. (2010) used a mutant strain of *Aspergillus terreus* isolated from fishery polluted waste for the biodegradation of parrot fish scale waste. He concluded that protease production was influenced by the culture medium and enzyme production decreased with the decreased sizes of fish-scales; larger sizes (normal, non-grinded) were the best for enzyme production than the finest ground scales. Basu and Banik (2005) studied the biodegradation of fish scale powder by using the crude protease of a mutant strain of *Aspergillus niger.* Pal (2017) studied the fermentative production of extracellular bacterial collagenase from *Bacillus* spp. using fish scale powder as a substrate.

3. MATERIALS AND METHODS

As described in the chapter1, this work is based on the utilization of fish scales. In order to utilize the scales, one should know what kinds of high-value nutrients can be extracted. In addition to this, the evaluation should be made to know if the scales can be directly utilized. Since it has been proposed to segregate this recalcitrant material from mixed fish waste, the scales should be easily disposable after microbial treatment. With these overall aims, efforts were made for direct consumption of scales by gelatinizing the collagen in a product development mode. Further, the scales were microbiologically treated to develop high valued hydrolysates from collagen protein. In the whole process of doing so, the following materials and methods were followed.

13.1 Analysis of the composition of carp scales

The compositional analysis of carp scales was determined by the method as described in AOAC (2005).

3.1.1 Moisture (A.O.A.C, 2005)

Moisture content in carp scales was determined by using hot air oven. About 5 gm of the prepared sample was weighed and kept in a pre-weighed Petri plate. The sample was dried in a hot air oven at 100°C±2°C for 16-18 hours. After that, the dish was taken from the oven and cooled in a desiccator to room temperature. The final weight was taken by the dish with the sample.

$$\text{Moisture (\%, wet weight)} = \frac{(W1-W2)}{(W1-W)} X100$$

Where, W = weight in grams of the empty dish

W1 = weight in grams of the dish with the material before drying

W2= weight in grams of the dish with the material after drying

3.1.2 Total Nitrogen (TN)/Crude Protein content (A.O.A.C, 2005)

About 2 g of the sample was taken in a Kjeldahl digestion flask. A pinch of digestion mixture (5 parts K2SO4 and 1 part CuSO4) and 10 ml concentrated sulphuric acid was added to the flask. Then it was digested over a heating mantle while the temperature and time were set at 250°C-15 min, 380°C-15 min and 420°C-60 min until the solution turned colorless. The digested sample was made up to 50 ml with distilled water. 2ml of the digested sample was taken in a distillation flask and 4 ml of 40% NaOH solution was added to the flask. Distillation was done by the Micro-Kjeldahl distillation apparatus. Distillation was prolonged for 6-7 minutes and ammonia liberated was absorbed into 2% boric acid containing a drop of Tashiro's indicator thereby turning it into light greenish color. The amount of ammonia liberated was determined by titrating with 0.1 N sulphuric acid. Crude protein content was calculated by multiplying total nitrogen content with the conversion factor of 6.25 and expressed as a percentage.

Total nitrogen (%, wet weight) = $\frac{14*N*X*100}{1000*V*W}$ X100

3.1.3 Crude Ash content (A.O.A.C, 2005)

10 g sample was taken in a dry and pre-weighed silica crucible and heated at 100±5°C using a Bunsen burner until the samples are completely dried. Then the crucibles containing charred samples were transferred into muffle furnace held at a temperature of 600±5 and incinerated until white ash was obtained (about 5-6 hour). The crucible was cooled down and transferred to desiccators, and the final weight was taken. The percentage of ash was calculated based on the initial weight of the sample.

Crude ash (%, wet weight) = $\frac{W2-W}{W1-W}$ X 100

Where, W = weight of the empty crucible in grams

W1 = weight of the crucible with sample before ashing in grams

W2 = weight of crucible with ash in grams

3.1.4 Mineral content by flame photometry

The flame photometer requires calibration with a series of standard solutions of the ion to be tested. Therefore, standard solutions for calcium were prepared for different concentrations of viz. 10ppm, 20ppm, 30ppm 40ppm, 50ppm, 60ppm were prepared using calcium carbonate($CaCO_3$).1g of the moisture-free sample was weighed in a crucible. The weight of the crucible was also taken. The sample was kept on the heater to char it. The charred material was kept inside the muffle furnace at 600°C temperature for 6 hours till white or grayish-white ash was obtained. Then the crucible was allowed cooled in Desiccators. To the sample, a few drops of concentrated HCl were added for dissolving. Distilled water was added to make the total volume up to 100 ml. Then the content was filtered through ashless filter paper (Whatman 41, 125 mm Ø). 2 ml of filtered sample was taken and 18 ml distilled water was added into it (total volume 20ml. The sample was analyzed using Flame photometer. The result was calculated using the following formula:

$$\text{Mineral content (gm \%)} = \frac{\text{Reading X l00 X l0 X l00}}{\text{1000 X 1000 X Weight of sample}}$$

Where, 100 = Made up sample

10 = Dilution factor

Weight of sample= 1g

3.1.5 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

10% acrylamide gel was prepared according to the standard procedure described by BIO-RAD lab (2000) and run the till the dye crossed the stacking gel margin. The unit was switched off when the dye front reached the bottom of the running gel and then transferred the gel to distilled water. Then the gel was placed in a

small plastic box and cover with the staining solution Coomassie blue R-250. The gel was shaken slowly for 3 hours or more on a rotary rocker. After that, it was transferred to the destaining solution until the bands became clear and the background completely destained.

3.2 Isolation of microbes from fish scales

Fish scale and scale deposited soil samples were collected from different fish markets of Mumbai. All the bacteriological media used in the study were purchased from Hi-media Laboratories Pvt. Ltd. Mumbai is given in Appendix-1. All the media were sterilized by autoclaving at 15psi pressure for 15 minutes. Nutrient agar plates and 0.85% saline tubes were prepared. Samples were suspended in normal saline (0.85%), serially diluted and then spread nutrient agar plates. Plates were incubated at 37^{o}C for 24 hours. Then different colonies on agar plate were selected and streaked on Luria Bertani plates to get pure isolates. Plates were incubated at 37^{o}C for 24 hours. Morphologically different colonies were picked up from the agar plate and tested for gelatinase activity.

3.2.1 Screening of bacteria for gelatinolytic activity (Suphatharaprateep *et al.*, 2011)

Overnight grown pure cultures were spot inoculated on gelatin agar plates (containing 1.5% agar and 1% gelatin). The incubation temperature was 37^{o}C for 24 hours. After incubation, plates were flooded with acidic mercuric chloride to check the zone of hydrolysis around the colonies.

3.2.2 Screening of extremely halophilic microorganisms for gelatinolytic activity (Birbir *et al.,* 2004)

Extremely halophilic bacterial and archaeal cultures were collected from the repository of QC Laboratory, CIFE, Mumbai. The media used for the gelatinase test had the following composition per liter: 3g trisodium citrate, 10g yeast extract, 7g tryptone, 15g agar 20 g of $MgSO_4$. $7H_2O$, 0.036g $FeCl_2$, 0.036g $MnCl_2.4H_2O$, 250 g of

NaCl and 2% gelatin (pH 7.2±0.2). The cultures were spot inoculated on halophilic gelatin agar plates. Acidic mercuric chloride was used to check the zone of hydrolysis around the colonies.

3.3 Identification of bacteria

DNA isolation

The bacterial cultures were grown overnight in Luria Bertani agar plates. 1.5ml capacity sterile Eppendorf tubes were taken. To each tube 200µl, 1X TE buffer was added. The loopful culture was suspended in 1X TE buffer and mixed properly by vortexing. The tubes were kept in a dry bath at 98ºC for 15 minutes. Then they were kept ice for 10-15 minutes. After that centrifugation was done at 10000 rpm for 50second.The supernatant contains isolated DNA

Amplification of DNA by polymerase chain reaction (PCR) (Marchesi *et al*., 1998)

PCR amplification of the 16S rRNA gene was performed to get sufficient copies of the gene. PCR Master Mix of Thermo fisher scientific was used. The 14kb fragment (16S rRNA gene) was amplified using 10pmoles of the primers; forward primer: 5'-AGAGTTTGATCCTGGCTCAG-3' and Reverse primer: 5'-GGTTACCTTGTTACGACTT-3' under the following amplification profile: Initial denaturation at 95ºC for 1 minute, 30 cycles 15 each consisting of a denaturation step at 95ºC for 1 minute, an annealing step at 50ºC for 1 minute, and extension step at 72ºC for 1 minute and a final extension at 72ºC for 7 minutes.

Table 1.Composition of PCR master mix

Components	Amount (µl)
Sterile MilliQ water	14.2
10x taq buffer	3
MgCl2	1.8
dNTP mix (10pmoles/µl)	2
Forward primer (10pmoles/ µl)	3
Reverse primer (10pmoles/ µl)	3
Taq DNA polymerase	1
Template DNA	2
Total	30

Agarose Gel Electrophoresis

The first step was to prepare a tray to hold the gel matrix. A gel comb was used to create wells in the gel. The gel comb was placed in the tray. 1.2% agarose was measured and dissolved in 1X tris acetate (TAE buffer) by heating the mixture until the agarose dissolved completely. The gel was allowed to cool to 50-60ºC. Then 8µl ethidium bromide was added to it. Then the gel was poured over the tray and allowed to set. After setting the gel comb was removed and DNA samples were loaded in wells after mixing with loading dye. The cover was placed over the gel. Then the electrical current was applied. DNA fragments migrated through the gel at various rates, depending on their size. When the dye marker indicated that DNA fragments have

moved through the gel, the current was turned off and the gel was removed from the tray. DNA fragments were observed under UV light.

RFLP (Restriction fragment length polymorphism) analysis (Fujimoto *et al*., 1994)

A 10ul sample of the PCR product was digested with 10 U of restriction enzyme MspI and AluI for 3 h at 37°C in the buffer recommended by the supplier. The digest was analyzed by agarose gel electrophoresis containing 1.6-1.7% agarose. The gel was then examined by UV-transilluminator and the photograph was taken.

3.4 Application of gelatinase positive microbes on carp scales

Ten gelatinase positive bacterial cultures were inoculated in sterilized carp scales to check whether they are capable of hydrolyzing protein present in carp scales. Selected cultures were inoculated into the fresh broth and incubated at 37^{o}C for 24 hours. Pellet was prepared by centrifuging the broth. The pellet was suspended in 10 ml of 0.85% saline. Then the saline suspension was added into 10g surface-sterilized carp scales. The incubation was done at room temperature. Sampling was done at 7 days interval to check the % of protein removed from the scales.

Three extremely halophilic microbial cultures were also selected to check their ability to hydrolyze fish scale protein. Selected species were-*Halobacterium salinarum Halococcus dombrowskii, Halococcus morrhuae.* In this case, 20% saline was used to make pellet and sampling was done at 15 days interval.

3.5 Utilization of fish scale by using microbes

Fish scales are very difficult to utilize because of their hardy nature. With a view to valorizing these under-utilized bio-wastes, the compositional analysis was done to know which is the most abundant component of the carp scale that can be utilized for the production of some high valued products. From the compositional analysis, it was found that protein is the most abundant component of carp scale. The carp scale protein was characterized as type I collagen. Collagen gets denatured and

converted to gelatin when it is heated. Therefore microorganisms having gelatinolytic activity were used to hydrolyze the gelatin protein of the scale. The hydrolysis of protein will give a mixture of oligopeptides, peptides and free amino acids which may have bio-active properties.

3.5.1 Preparation of fish scale protein hydrolysate

Fresh scales of Indian major carps were collected from the Four Bungalow fish market, Andheri West. They were washed thoroughly to remove dirt, mucus, blood etc. Washed scales were dried in a hot air oven and powdered by using food grinder. Powdered scales were used for the preparation of protein hydrolysate. Two bacterial cultures were selected based on their high gelatinolytic activity. Cultures were grown in Luria Bertani broth for overnight at 37^{o}C. Overnight cultures were centrifuged to get a pellet. Pellet was suspended in 0.85% saline solution. In a 2 liter beaker scale powder and water was mixed at a ratio of 4:1 (water: scale). The mixture was heated at 72^{o}C for 15 minutes in a hot water bath to kill all the microorganisms present in the mixture. It was cooled down to room temperature and pH was maintained at 7.2±2. The bacterial suspension was used at a concentration of 10^{8} CFU/g. The mixture was incubated at room temperature for 7 days. After 7days mixture was heated at 90-100^{o}C to kill the microbes. Then filtration was done using filter paper (Whatman 41, 125 mm Ø). Centrifugation was also done to get a clear solution. The supernatant was collected and dried using spray drier. Hydrolysate powder was stored at 4^{o}C.

3.5.2 Determination of Degree of hydrolysis of fish scale protein hydrolysate

The degree of hydrolysis was determined using the method described by Seniman *et al*. (2014). 20 ml of fish scale protein hydrolysate was added to 20 ml of 20% (w/v) TCA solution to produce 10% TCA soluble material. The mixture was allowed to stand for 30 min to allow precipitation, followed by centrifugation (7800 × g for 15 min). The supernatant was collected and protein content was determined by

using the Kjeldahl method. The degree of hydrolysis (DH) was calculated by the formula given below:

$$\text{DH (\%)} = \frac{10\%\ \text{TCA soluble nitrogen in the sample}}{\text{Total nitrogen in the sample}} \text{ X } 100$$

3.5.3 DPPH (1,1-diphenyl-2-picryl-hidrazyl) Radical Scavenging Activity

DPPH radical-scavenging activity hydrolysates were determined by DPPH assay, as described by Daud *et al.,* 2013. Different concentrations of hydrolysate made viz. 1mg/ml, 2mg/m, 3mg/ml, 4mg/ml, 5mg/ml. To the sample, 1.5 ml of 0.15 mM DPPH in 95% ethanol was added. Then the mixture was mixed vigorously and incubated at room temperature in the dark for 30 min. The absorbance was measured using a UV-1800 spectrophotometer (Shimadzu) at 517 nm. The activity was expressed as DPPH radical scavenging activity % radical scavenging activity. The percentage of scavenging activity was calculated using the following formula-

$$\text{\% scavenging activity} = \frac{Absorbance\ of\ control - absorbance\ of\ sample}{Absorbance\ of\ control} \text{ X } 100$$

3.5.4 ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) Radical Scavenging Activity

The ABTS radical-scavenging activity of hydrolysates was determined by the ABTS assay, as described by Daud *et al.*, 2013. The chemicals required are - 7.4mM ABTS solution and 2.6mM potassium persulphate solution. Then these two solutions were mixed in equal quantities and allowed them to react at room temperature in the dark for 12 hours. Sample (150µl) was mixed with 2850µl of ABTS solution and the mixture was incubated at room temperature for 2 hours in the dark. The absorbance was then measured using a UV-Vis spectrophotometer at 734 nm.

The activity was expressed as % ABTS radical scavenging activity. The percentage of scavenging activity was calculated using the following formula-

$$\% \text{ scavenging activity} = \frac{\text{Absorbance of control} - \text{absorbance of sample}}{\text{Absorbance of control}} \times 100$$

3.5.5 FRAP (Ferric reducing antioxidant power)

FRAP was assayed according to Da Rocha *et al.,* 2018. Chemicals required for FRAP assay are-stock solution of 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solution. Everytime analysis was done by using a fresh working solution prepared by mixing 25 ml of acetate buffer, 2.5 ml of TPTZ solution and 2.5 ml of $FeCl_3.6H_2O$ solution. After mixing the solution was incubated at 37 °C for 30 min. Then 150µl sample was mixed with 2850µl of FRAP solution and kept for 30 min in dark. The absorbance was measured at 593 nm. All determinations were performed in triplicate.

3.5.6 Antimicrobial test of fish protein hydrolysate

The antibacterial activity of the fish protein hydrolysate prepared from carp scales was determined by agar well diffusion method described by Kuppusamy and Ulagesan (2016) on Mueller Hinton agar medium. The bacterial cultures used for the test were *Bacillus subtilis* (MTCC2960), *Staphylococcus aureus* (ATCC25923), *Escherichia coli* (KAM32), *Pseudomonas aeruginosa* (repository of QC Lab, CIFE, Mumbai). The Mueller Hinton agar (MHA) Medium was sterilized and poured into the Petri plates. Plates were allowed to solidify. Then the inoculum was spread on the solid plates with a sterile cotton swab moistened with the bacterial suspension. Wells were made in MHA plates by using sterile micropipette tips whose ends were cut at 3mm size and 20µl of protein hydrolysate of different concentrations: 5%, 10%, 15%, 20% and 25% were added to the wells. Distilled water used as the negative control. The plates were incubated at 37°C for 24 hours. The diameters of the zone of inhibition were measured in millimeters.

3.6 Preparation of value-added products from fish scale

Carp scales were washed properly to remove dirt, blood, mucus, soluble proteins etc. They were divided into two groups. One group was treated with gelatinase positive bacteria for 7 days to make the texture soft and thereby easy to process. Another group was preserved at -20^{o}C for further use. Two different products were prepared using scales and their consumer acceptability was checked by sensory evaluation. For each product there were two groups- in one group fish scales without any treatment were used (A) and in second one microbe treated scales (B) were used.

3.6.1 Preparation of fish scale wafers

Table 2. Ingredients for fish scale wafers

Ingredients	Quantity
Fish scale	30g
Tapioca sago	100g
Salt	7g
Chili flakes	2g
Water	700ml

Procedure

Carp scales were washed properly to remove all the mucus, blood, dirt etc. Scales were dried and crushed into small pieces. Then all the ingredients mentioned above were mixed together. Water of required quantity was added and mixed properly to avoid any clump formation. The mixture was cooked at 100^{o}C until the mixture becomes thick. Spreading of the mixture was done on the trays. Then kept for drying at 50-60^{o}C for 12-16 hours. After drying the product was packed in polyethylene bags.

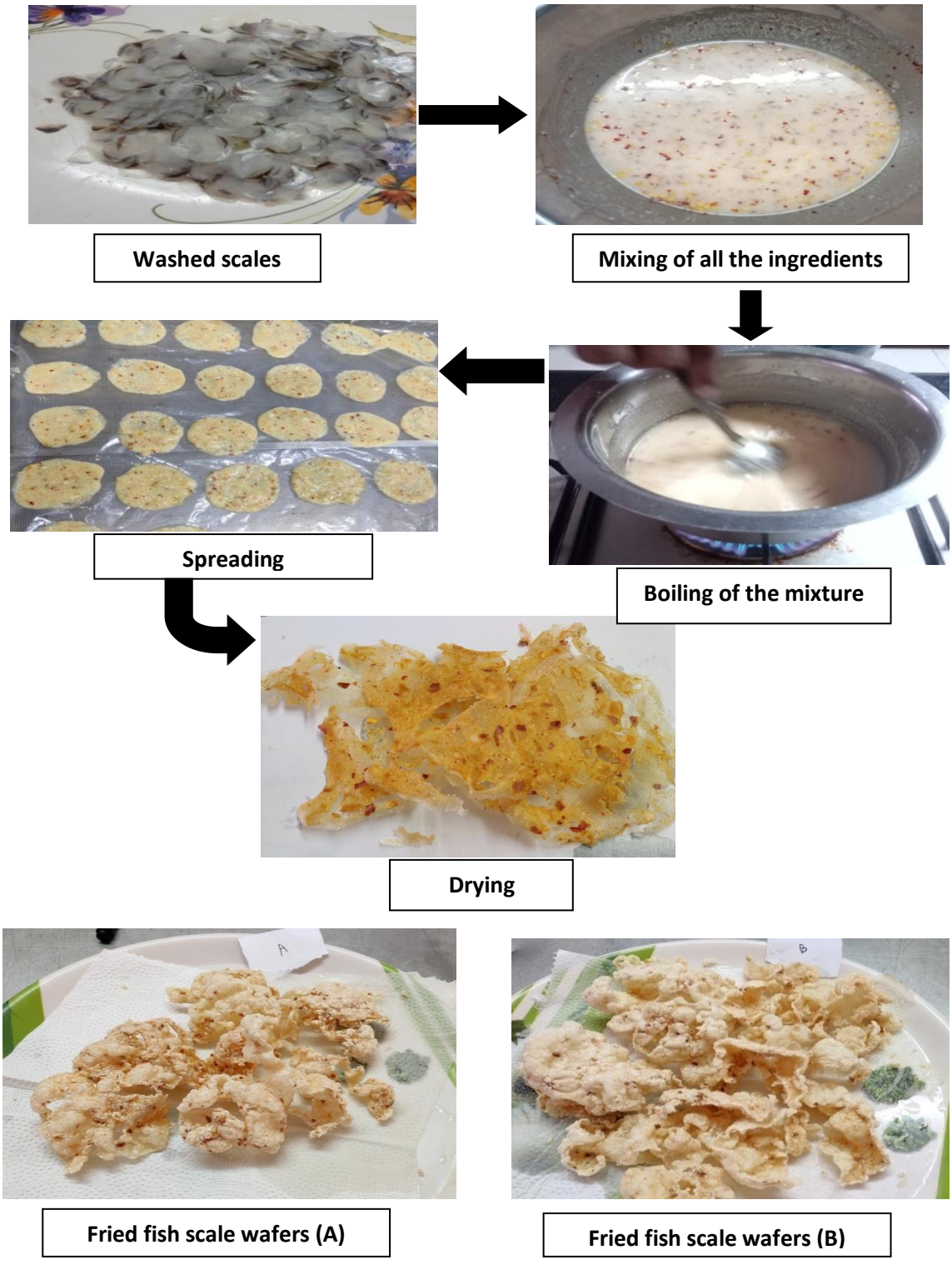


Plate 1. Procedure for the preparation of fish scale wafers

3.6.2 Preparation of coated fish scales

Table 3. Ingredients for coated fish scales

INGREDIENTS	QUANTITY
Carp scales	100g
Wheat flour	60g
Gram flour	30g
Salt	6g
Turmeric	2g
Spices	5g

Procedure

Carp scales were washed properly to remove all the mucus, blood, dirt etc. The batter was prepared by mixing all the ingredients mentioned above except scales. Scales were coated with batter. Coated scales were fried in oil.

Coated scales (A)

Coated scales (B)

Plate 2. Coated fish scales

3.6.3 Sensory evaluation

The sensory characteristics of the scale products were evaluated by a taste panel member on a nine-point scale. Scores were assigned with 1 being the least and 9 being the greatest for each attribute. Scores for different sensory characteristics are given in Appendix-II. The characteristics covered under the taste panel were appearance, color, crispness, odor, flavor, taste and overall acceptability.

3.7 Statistical Analysis

The SPSS 16.1 (SPSS, 2000) statistical package was used for the analysis of the experimental results. A sufficient number of samples were used for each analysis. The results were expressed as mean± standard deviation and Duncan's test was used for Post hoc multiple comparisons to asses statistical significance ($p< 0.05$) between samples. One way ANOVA and independent sample t-test was used to compare two hydrolysates and also to compare two groups of each product,

4. RESULTS

4.1 Composition of Carp Scales

Fish scales, which are generally considered as waste can be effectively used as a rich source of protein and minerals. There are so many chemical and enzymatic methods available for the extraction of valuable components from the fish scales. The important drawback of these methods is the use of hazardous chemicals and their harmful effect on the environment on the release of waste. This study aims to utilize the fish scales for the extraction of commercially important products by using microbial cultures has the ability to hydrolyze fish scale protein. It is very important to analyze the composition of the fish scales before using them for the production of any valuable product.

4.1.1 Nutrient Profiling of Carp Scales

The moisture, protein, ash and calcium content of the scales from two carps, *Catla catla* and *Labeo rohita* were analyzed. Three scale samples for each *sp*ecies were collected from fishes of different weight groups. Each sample was analyzed in triplicate and results are shown in Tab.4.

Table 4. Composition of carp scales (*C.catla* and *L. rohita)* expressed as percentage of wet weight

Fish	Sample	Weight of Fish	Moisture content (%)	Protein content (%)	Ash content (%)	Calcium (gm %)
Catla catla	C_1	872g	69.71 ± 1.54^{b}	22.83 ± 0.79^{a}	4.49 ± 1.14^{a}	3.16 ± 0.56^{a}
	C_2	1053g	67.82 ± 1.56^{b}	23.63 ± 1.16^{a}	5.35 ± 1.07^{a}	3.99 ± 0.59^{ab}
	C_3	1520g	64.29 ± 1.23^{a}	24.10 ± 0.86^{a}	6.72 ± 1.07^{a}	4.70 ± 0.43^{b}
Labeo rohita	R_1	717 g	70.65 ± 1.72^{a}	21.27 ± 0.74^{a}	4.32 ± 1.04^{a}	3.11 ± 0.35^{a}
	R_2	850g	68.92 ± 2.92^{a}	21.64 ± 1.08^{a}	5.27 ± 0.54^{a}	3.66 ± 0.47^{a}
	R_3	1030 g	66.63 ± 2.68^{a}	22.72 ± 1.02^{a}	5.94 ± 1.22^{a}	4.20 ± 0.90^{a}

Each value is represented by the mean± SD on wet weight basis of n=3

Different letters in the superscript indicate significant difference within the column for individual *sp*ecies according to Duncan's test ($P<0.05$)

For the analysis of the proximate composition of fish scales, three samples were collected from three different catla fishes of varying weight. C_1 was the sample collected from fish weighed less than 1kg, C_2 was from approximately 1kg, while C_3 was taken from the fish greater than 1kg (Table 1). The moisture contents in the scales of *C. catla* were 69.71±1.54%, 67.82±1.56% and 64.29±1.23 % for the C_1, C_2, and C_3 samples re*sp*ectively. The moisture content of scale sample C_3 was significantly different ($P<0.05$) from the C_1 and C_2. The moisture content was observed to be decreasing with the increasing weight of the fishes. The protein contents of the *C. catla* scales were 22.83 ±0.79, 23.63 ±1.16, and 24.10± 0.86 % in the three samples of increasing weight re*sp*ectively. The ash content was 4.49±1.14, 5.35±1.07 and 6.72±1.07% in the three samples of increasing weight re*sp*ectively. There was no significant difference ($P<0.05$) in the protein and ash contents between three different samples. The calcium contents in the *C. catla* scales were 3.16±0.56, 3.99±0.59 and 4.70±0.43% in the three samples of increasing re*sp*ectively. A significant difference ($P<0.05$) in the calcium contents between the C_1 and C_3 samples was observed. The calcium content was observed to be increasing with increasing weight of the fishes.

The weight of the two rohu fishes from where the sample R_1 and R_2 were collected was less than 1kg, while the sample R_3collected from a fish weighed approximately 1kg. The moisture content in *L. rohita* scales were 70.65±1.72 %, 68.92±2.92%, 66.63±2.68% for R_1, R_2 and R_3 samples re*sp*ectively (Tab.1). The protein content of the *L. rohita* scales were 21.27±0.74, 21.64±1.08 and 22.72±1.02% in the three samples of increasing weight re*sp*ectively. The ash content was 4.32±1.04, 5.27±0.54 and 5.94±1.22% in the three samples of increasing weight re*sp*ectively. The calcium content was 3.11 ±0.35, 3.66±0.47 and 4.20±0.90% in the three samples of increasing weight re*sp*ectively. There was no significant difference ($P<0.05$) in the moisture, protein contents, ash and calcium contents among three different samples of *L. rohita* scales.

4.1.2The Molecular Weight Distribution of Carp Scale Protein

Protein characterization of *C. catla* and *L. rohita* was done by using SDS-PAGE. SDS-PAGE gives a clear idea about the molecular weight distribution of the protein.

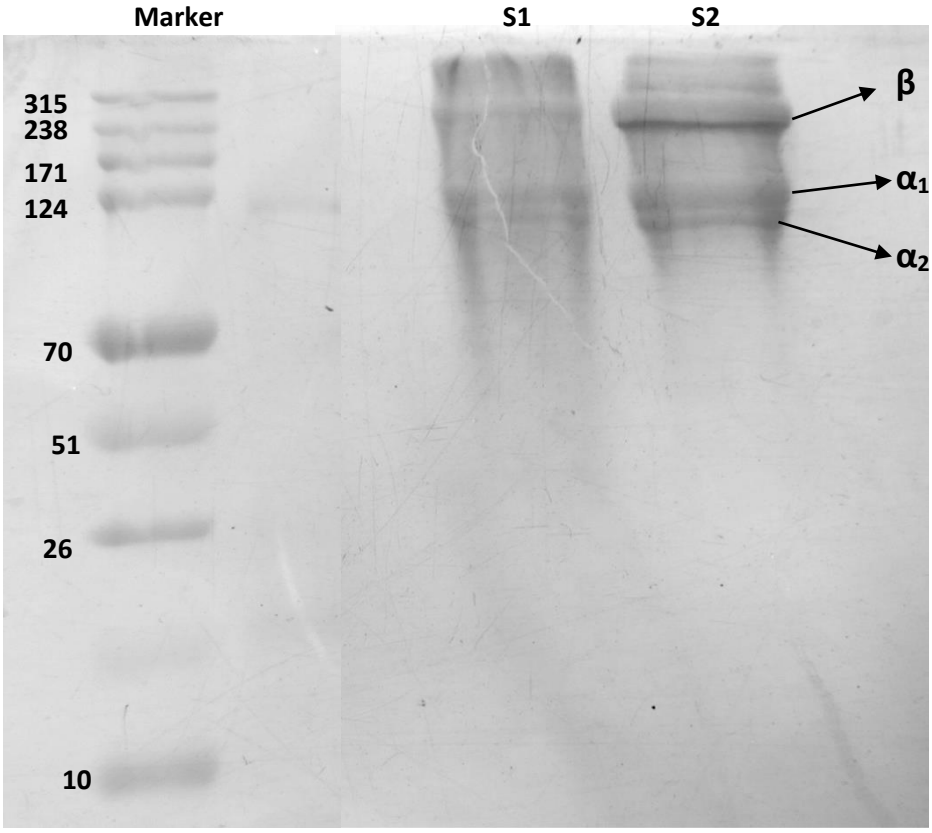


Figure 1. SDS- PAGE of protein extracted from carp scales, S_1- *Catla catla* scale protein, S_2- *Labeo rohita* scale protein

Fig.1 shows the electrophoretic patterns of carp scale proteins. From the figure it is clear that carp scale protein is mainly composed of two different α-chains viz. α_1 and α_2. The molecular weight of the α-chains of the scale proteins was between 70 to 124 KDa. The high molecular weight component of β chainswas also observed. Its molecular weight was between 238 to 315 KDa. The marker used in the analysis had molecular weight ranged from 10 to 315KDa.

4.2 Isolation and Identification of Microbes Targeting *Sp*ecific Component of Fish Scales

Scales are generally coated with mucus having an antimicrobial activity effect. The bacteria surviving on scales therefore must be capable of using the fish scale as a nutrient. In view of this, scale samples and scale deposited soil samples from different locations were taken for the isolation of gelatinolytic bacteria.

Table 5. Isolation and screening of bacteria for gelatinolytic activity

Sl. No.	Type of sample	Place of sampling	Total number of samples	Total number of isolates	Number of gelatinase positive isolates
1	Scale	Four Bungalow fish market	2	18	9
2	Scale	Versova landing center	2	16	10
3	Soil	Versova landing center	1	10	7
4	Scale	Star bazaar, Andheri	1	7	2
5	Scale	Versova fish market	1	8	4
6	Soil	Four Bungalow fish market	1	12	7
7	Scale	Andheri fish market	2	18	11
8	Scale in 25% salt	Four bungalow market	1	8	0
9	Scale in 25% salt	Versova fish market	1	8	0
			Total	105	50

Tab.5 depicts that a total of 12 samplings have been done from different markets of Mumbai such as Versova fish market, Four bungalow market, Andheri fish market and Versova landing center at different time. Scale and scale deposited soil samples were collected for the isolation of bacteria having the ability to hydrolyze scale protein. Out of 12 samples, 2 samples were kept in 25% salt so that only extremely halophilic microbes can be enriched and isolated. A total of 105 isolates were taken based on unique colony morphology. Out of the total 105 cultures, 50 showed gelatinase activity. From the samples kept in 25% salt, no isolates were found to have gelatinolytic activity. Therefore, previously isolated extremely halophilic microbes having proteolytic activity were taken for the experiment.

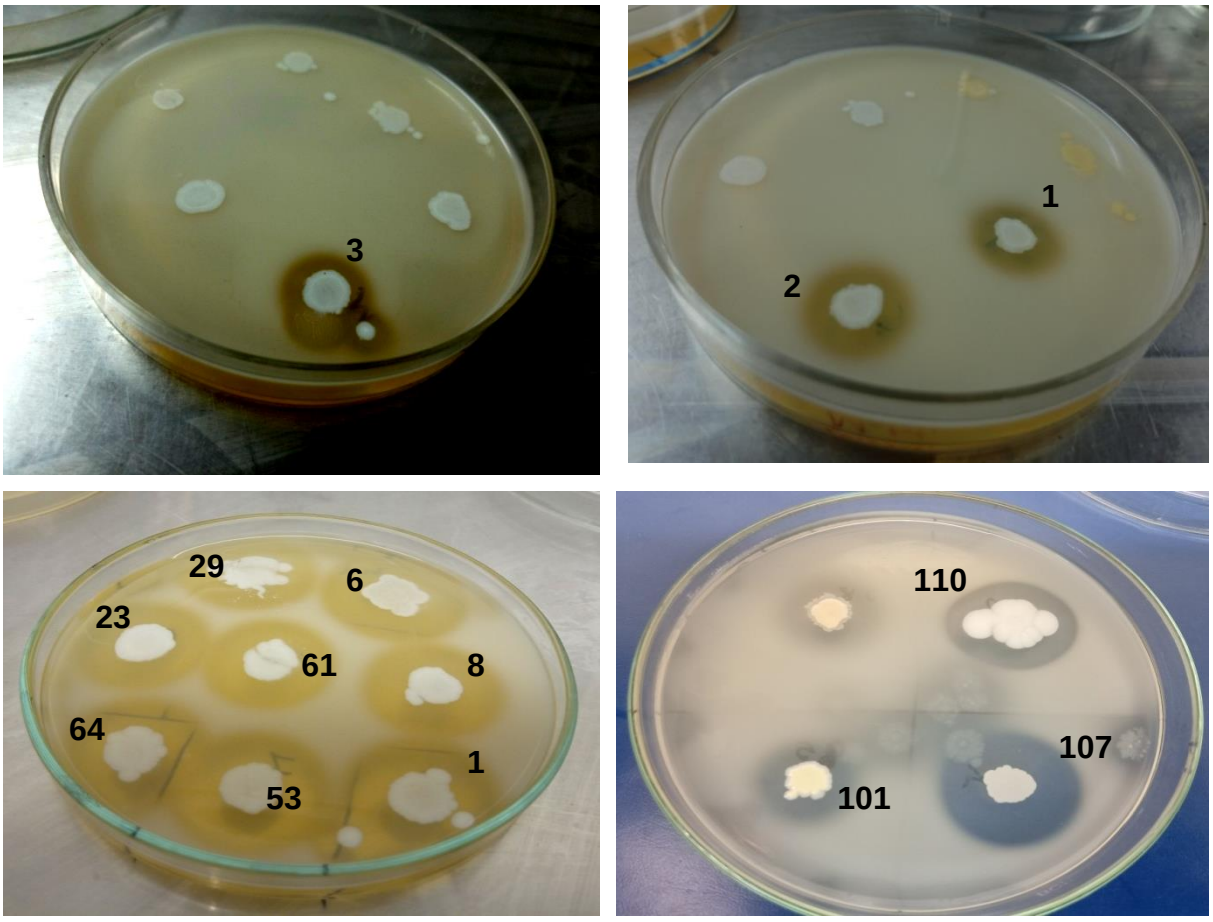


Plate 3. Gelatinase positive cultures showing clear zone on gelatin agar plate

Table 6.Sequencing results of PCR products

Isolate code	Query length	Max. score	Total score	Query cover	E value	Identity	Organism
1	862	191	1328	13%	4e-44	96.52%	*Proteus vulgaris*
10	951	1570	1570	99%	0.0	96.63%	*Staphylococcus sciuri*
53	588	538	538	55%	9e-149	96.34%	*Acinetobacter towneri*
61	1274	1406	1406	63%	0.0	97.79%	*Myroides sp.*
64	1266	2001	19868	99%	0.0	96.21%	*Lysinibacillus sphaericus*
101	1154	1520	1520	76%	0.0	97.64%	*Staphylococcus sciuri*
110	1176	721	721	35%	0.0	97.84%	*Aeromonas sp*.
56	1017	1736	1136	99	0.0	97.54%	*Pseudomonas alcaligens*
8	967	1592	1592	98	0.0	96.86	*Enterobacter sp*.
23	1294	1181	1181	51%	0.0	98.65%	*Macrococcus caseolyticus*
29	884	1528	1528	99%	0.0	98.07%	*Proteus mirabilis*
43	990	1655	1655	99%	0.0	96.97%	*Aeromonas veronii*
82	1035	1712	1712	97%	0.0	97.24%	*Aeromonas hydrophila*
84	831	484	2380	96%	2e-132	77.98%	*Psychrobacter sp.*
107	1154	1520	1520	76%	0.0	97.64%	*Staphylococcus sciuri*

The PCR was done to amplify the 16s rRNA gene of the bacteria (Fig. 2). After that RFLP of the PCR products was done using 2 restriction enzymes namely M*sp*I and AluI. RFLP results revealed that out of 50 gelatinase positive cultures only 28 cultures showed different digestion patterns for the 2 restriction enzymes (Fig. 3). The PCR products of total of 15 gelatinase positive culture were sequenced and analyzed in the NCBI nucleotide blast software. The isolates were identified as *Proteus vulgaris,Acinetobacter towneri*, *Myroides sp*. *Lysinibacillus sphaericus, Aeromonas sp*., *Pseudomonas alcaligens,Enterobacter sp., Macrococcus caseolyticus, Proteus mirabilis, Aeromonas veronii, Aeromanas hydrophilla, Psychrobacter sp*. and three isolates were *Staphylococcus sciuri.*

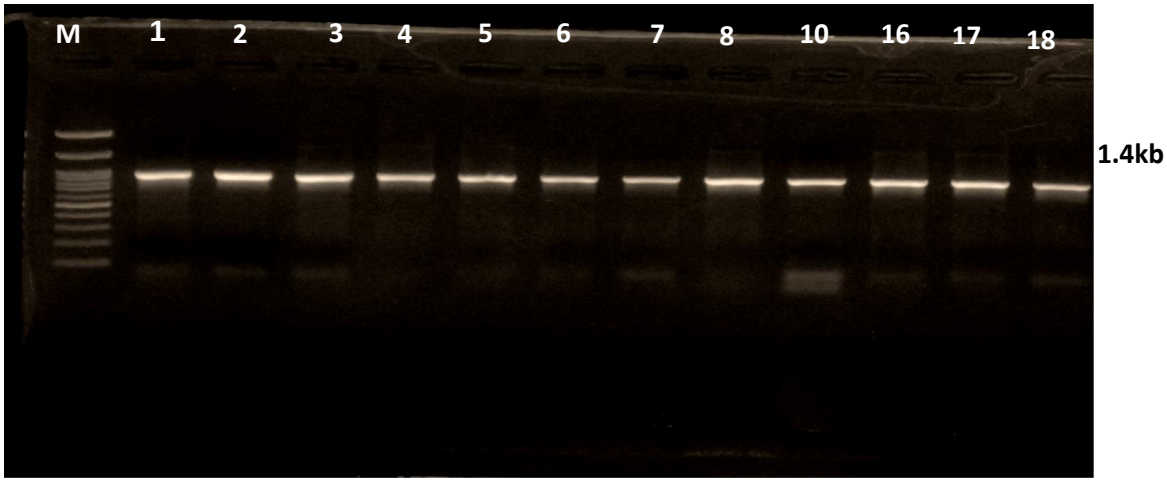


Figure 2. PCR assay for 16s rRNA gene

Lane M	1kb DNA ladder
Lane 1-10	isolates from scale samples of Four Bungalow fish market
Lane 16-18	isolates from soil samples of Versova fish market

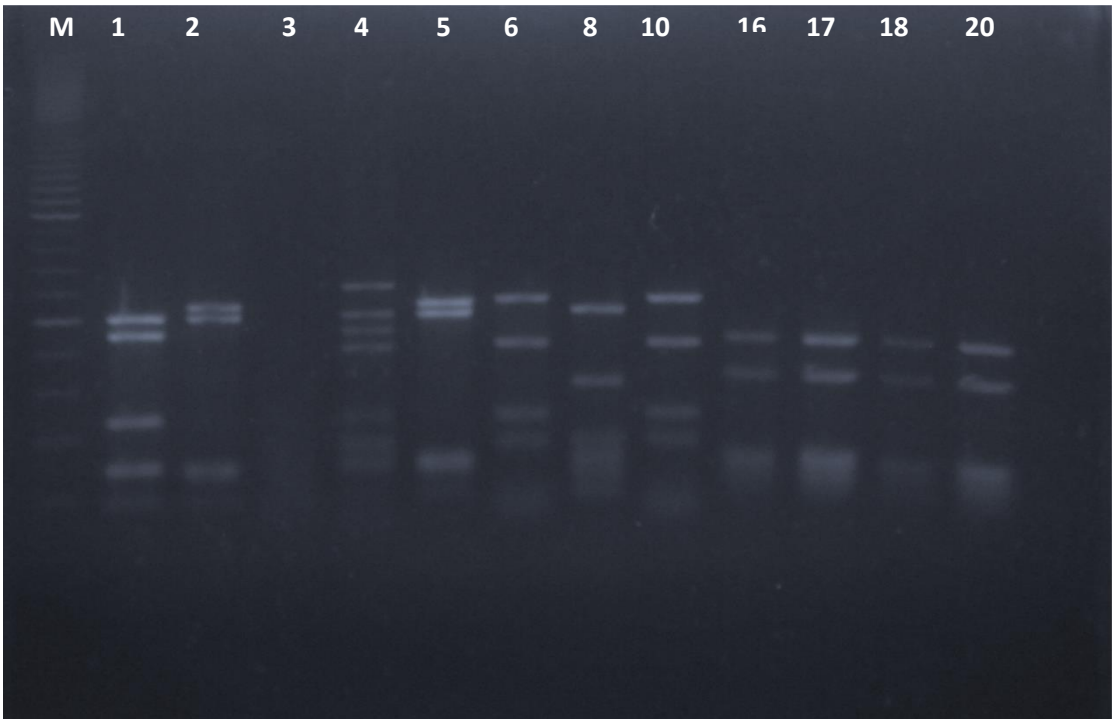


Figure 3. RFLP(Restriction fragment length polymorphism) assay

Lane M	1kb ladder
Lane 1-10	Isolates from scale samples of Four Bungalow fish market
Lane 16-20	Isolates from scale deposited soil samples of Versova fish market

Table 7.Screening of extremely halophilic microorganisms for gelatinase activity

Sl. No.	*Sp*ecies	Type of microorganism	Gelatinase test
1	*Halococcus sp.*	Archaea	-
2	*Hlc. qingdaonensis*	Archaea	-
3	*Halobacterium trapanicum*	Archaea	-
4	*Hlc. thailandensis*	Archaea	-
5	*Hbr. salinarum*	Archaea	**+**
6	*Hlc. dombrowskii*	Archaea	**+**
7	*Halobacterium sp.*	Archaea	**+**
8	*Salimicrobium jeotgali*	Bacteria	-
9	*Hlc. morrhuae*	Archaea	**+**
1o	*Lentibacillus juripiscarius*	Bacteria	-

Ten extremely halophilic bacterial and archaeal cultures were collected from the repository of QC Laboratory, CIFE, Mumbai. Out of 10 cultures, only 4 were positive for the gelatinase test on gelatin agar plate (Tab. 7). The positive cultures were *Halobacterium salinarum, Halococcus dombrowskii, Halococcus morrhuae and Halobacterium sp*.The other 6 isolates were found to be gelatinase negative.

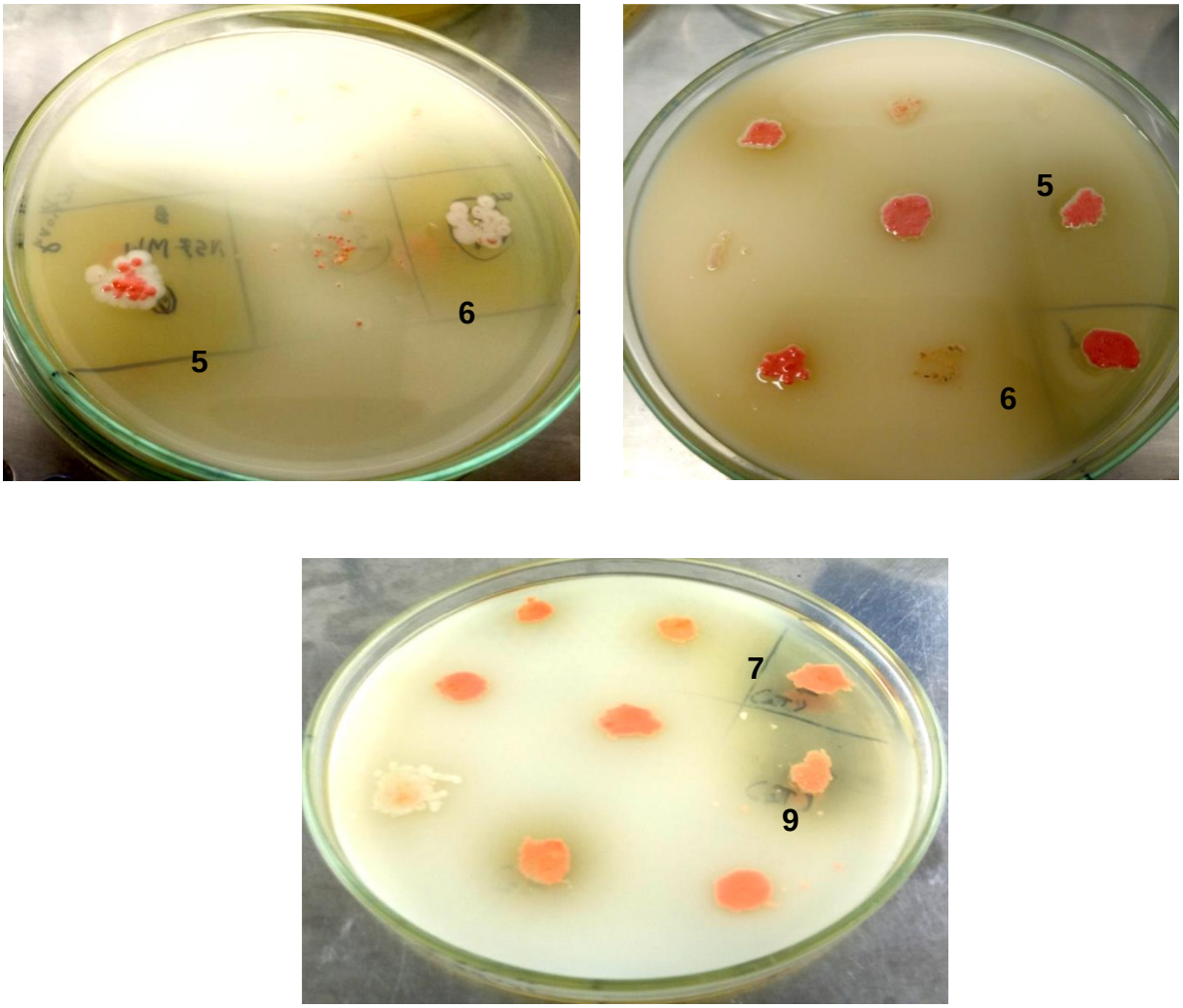


Plate 4. Gelatinase test of extremely halophilic cultures

4.3 Application of Selected Microbes to Utilize Carp Scale Protein

Ten bacterial cultures and 3 halophilic archaeal cultures were selected based on their gelatinase activity and inoculated into the surface-sterilized carp scales to check whether they are capable of hydrolyzing carp scale protein. The protein removal efficiency of these microbes was estimated.

Table 8. Percentage protein removal from carp scales by gelatinase positive bacterial cultures at 7 days interval

Isolate No.	*Species*	Protein removal (%)			
		7th day	14th day	21th day	28th day
1	*Proteus vulgaris*	25.43±2.69^{a}	44.49±2.17^{a}	57.82±3.61^{a}	72.49±2.30^{a}
3	NI	32.87±3.24^{bc}	54.62±1.72^{cd}	70.48±1.31^{bc}	78.12±1.99^{b}
8	*Enterobacter sp*.	30.77±2.70^{b}	49.19±2.50^{b}	69.54±2.04^{b}	80.92±3.03^{c}
10	NI	35.34±2.18^{cd}	55.74±3.16^{cd}	73.92±3.12^{bcd}	85.36±1.68^{d}
53	*Acinetobacter towneri*	47.23±2.00^{g}	65.21±2.30^{f}	83.79±2.43^{fg}	97.73±1.50^{g}
61	*Myroides sp*.	39.61±2.29^{def}	60.24±2.39^{e}	80.16±2.06^{ef}	94.32±2.46^{efg}
64	*Lysinibacillus sphaericus*	41.52±1.60^{f}	61.90±3.38^{ef}	86.51±3.02^{g}	96.60±1.93^{fg}
91	NI	38.45±2.32d^{ef}	60.57±1.29^{e}	75.91±2.66^{de}	90.48±2.39^{de}
101	*Staphylococcus sciuri*	37.01±1.70^{cde}	51.57±1.78^{bc}	74.65±2.23^{cd}	87.75±1.34^{cd}
107	*Staphylococcus sciuri*	40.32±2.20^{ef}	57.83±2.55^{de}	78.48±2.36^{de}	92.81±3.29^{ef}

Each value is represented by the mean± SD on wet weight basis of n=3

Different letters in the superscript indicate significant difference by Duncan's test (P0<.05) within the column

NI – Not identified

The protein removal efficiency of ten selected gelatinase positive bacterial isolates was estimated. Tab. 8is representing the % protein removal from carp scales by those bacteria at every 7 days interval up to the 28th day. All the microbes showed a significant difference ($p<0.05$) in all the samplings viz. 7th, 14th, 21thand 28th day. Out of these 10 microbes, *Acinetobacter towneri* and *Lysinibacillus sphaericus* showed the highest protein removal efficiency from fish scales. *Acinetobacter towneri removed* 47.23±2.00%, 65.21±2.30%, 83.79±2.43% and 97.73±1.50% protein from carp scales on the 7th, 14th, 21thand 28th day of sampling res*p*ectively. The protein removal efficiency of *Lysinibacillus sphaericus* was 41.52±1.60%, 61.90±3.38%, 86.51±3.02% and 96.60±1.93% on the 7th, 14th, 21thand 28th day of sampling res*p*ectively.

Table 9. Percentage protein removal from carp scales by gelatinase positive extremely halophilic microbial cultures at 15 days interval

***Sp*ecies**	**% Protein removal**				
	15th day	**30th day**	**45th day**	**60th day**	**75th day**
Halococcus morrhuae	9.45±1.53[a]	25.72±2.4[a]	53.35±1.9[a]	69.27±2.2[a]	87.46±2.3[a]
Halococcus dombrowskii	20.33±2.9[c]	40.26±1.94[c]	72.18±1.81[c]	88.32±2.53[c]	96.54±2.27[c]
Halobacterium salinarum	14.68±2.3[b]	33.82±2.36[b]	64.95±2.98[b]	82.45±2.55[b]	92.10±1.76[b]

Each value is represented by the mean± SD on wet weight basis of n=3

Different letters in the superscript indicate significant difference by Duncan's test (P0<.05) within the column

The protein removal efficiency of three extremely halophillic microbes were determined. Tab.9 is representing the % protein removal from carp scales by haloarchaeal isolates at every 15 days interval up to the 75th day. All the three microbes showed a significant difference in all the samplings viz. 15th, 30th, 45th, 60th and 75th day. *Halococcus dombrowskii* showed highest protein removal efficiency in all the samplings- 20.33±2.9%, 40.26±1.94%, 72.18±1.81%, 88.32±2.53% and 96.54±2.27%; followed by *Halobacterium salinarum* 14.68±2.3%, 33.82±2.36%,

64.95±2.98%, 82.45±2.55% and 92.10±1.76% on 15th, 30th, 45th, 60th and 75th day sampling respectively.

4.4 Fish Scale Protein Hydrolysate

The microbes showed higher efficiency in the hydrolysis of carp scale protein viz. *Acinetobacter towneri* and *Lysinibacillus sphaericus* were selected for the production of fish scale protein hydrolysate by the microbial fermentation process. Two fish scale protein hydrolysates FPHa and FPHb prepared by using two different bacteria viz. *Acinetobacter towneri* and *Lysinibacillus sphaericus* respectively.The hydrolysates produced by using these two bacterial cultures were analyzed for % yield, protein content and degree of hydrolysis.

Table 10. The yield, degree of hydrolysis and protein content of Fish Protein Hydrolysates

Fish protein hydrolysate	Yield of hydrolysate (%)	Protein content (%)	Degree of hydrolysis (%)
FPHa	3.51%	76.56±1.17	16.64±1.51
FPHb	2.74%	82.62±1.14	22.19±1.15
p-value		**0.03**	**0.02**

Means in the same column are significantly different according to t-test for independent samples at 95% confidence interval (P<0.05)

Tab.10 representing the yield, protein content and degree of hydrolysis of two protein hydrolysates FPHa and FPHb prepared by using two different bacteria viz. *Acinetobacter towneri* and *Lysinibacillus sphaericus* respectively. The yield of FPHa was 3.51% and 2.74% for FPHb. There was a significant difference (p<0.05) in the protein content and degree of hydrolysis between the two hydrolysates. The protein content of FPHa and FPHb was 76.56±1.17% and 82.62±1.14% respectively. The degree of hydrolysis was 16.64±0.51% and 22.19±1.15% for FPHa and FPHb respectively.

4.4.1 Amino Acid Composition

Table 11. Amino acid profiling of fish scale protein hydrolysate

Amino Acids	%	
	FPHa	FPHb
Aspartic acid	3.68	5.95
Glutamic Acid	8.47	7.47
Asparagine	ND	ND
Serine	15.53	1.97
Glutamine	7.57	3.15
Histidine	ND	ND
Glycine	22.45	23.68
Threonine	ND	ND
Arginine	ND	ND
Alanine	15.63	19.04
Tyrosine	4.30	ND
Valine	3.60	5.88
Methionine	0.45	ND
Tryptophan	ND	5.72
phenylalanine	1.98	ND
Isoleucine	4.31	4.32
leucine	4.51	6.80
Lysine	1.37	5.57
Hydroxproline	6.11	10.41

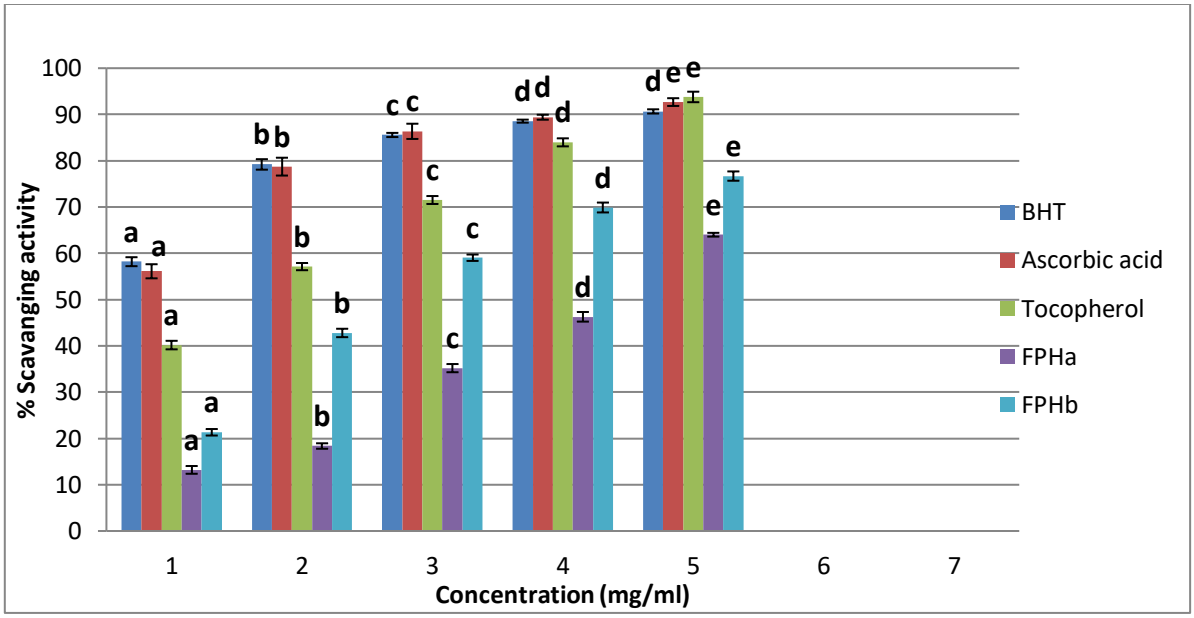
ND- Not detected

Amino acid profiling was done for both the hydrolysates –FPHa and FPHb. Glycine was the most abundant amino acid identified in both the hydrolysates, followed by alanine. Fish protein hydrolysates have been reported to exhibit variation in their amino acid composition. Non-essential amino acids found to be more abundant in both the hydrolysates. A*sp*aragine, histidine, threonine, arginine and tryptophan were not detected in FPHa. In case of FPHb, A*sp*aragine, histidine, threonine, arginine, tyrosine, methionine and phenylalanine were absent (Tab.11).

4.4.2 Antioxidant Activity of Fish Scale Protein Hydrolysate

The fish scale protein hydrolysates prepared using *Acinetobacter towneri* (FPHa) and *Lysinibacillus sphaericus* (FPHb). Their DPPH radical scavenging activity was compared with standard antioxidants viz. α- Tocopherol, ascorbic acid and BHT. The ABTS radical scavenging and ferric reducing activity of the hydrolysates were compared with standard antioxidant ascorbic acid.

4.4.2.1 DPPH Radical Scavenging Activity of Fish Scale Protein Hydrolysates

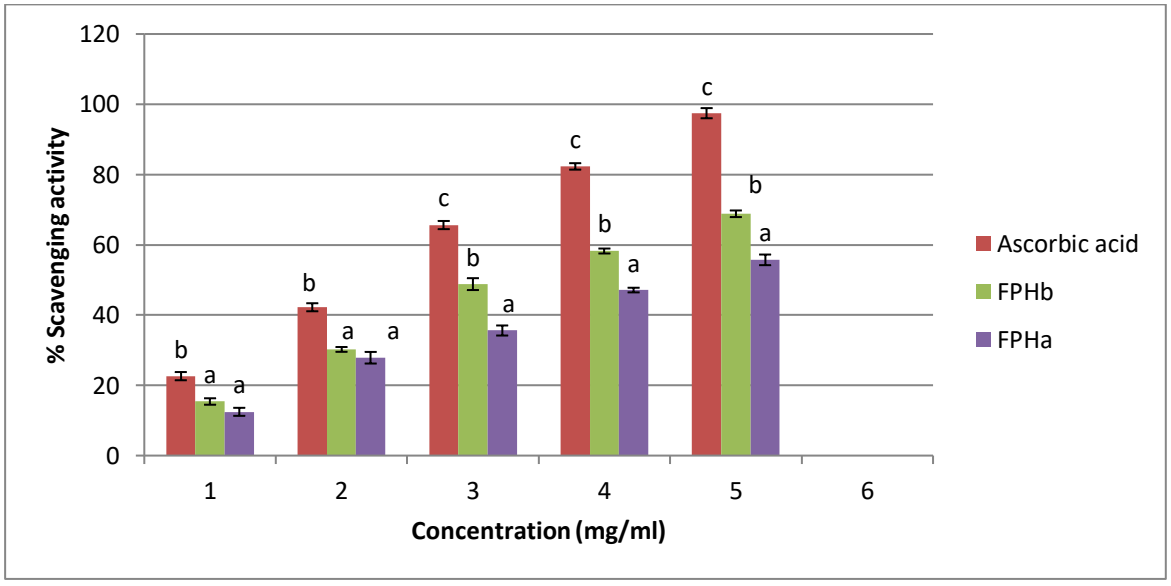


Different letters in the superscript indicate significant difference for the same concentration according to Duncan's test (P0<.05)

Figure 4: Comparison of DPPH radical scavenging activity of fish protein hydrolysates prepared by using microbes

The DPPH radical scavenging activity of both the hydrolysates was found to be dose-dependent. Therefore, the highest DPPH radical scavenging activity was found at the concentration of 5mg/ml. At this concentration, the % scavenging activity of the hydrolysate FPHa and FPHb were 64.02±0.70 and 76.69±1.00 res*p*ectively (Fig. 4).FPHb showed higher DPPH radical activity as compared to FPHa. The scavengingactivity of the hydrolysates were compared with standard antioxidants viz. α-tocopherol, ascorbic acid andBHT. It was found that these antioxidants have higher scavenging activity than these hydrolysates. α- Tocopherol showed the highest DPPH radical scavenging activity in this experiment.

4.4.2.2 ABTS Radical Scavenging Activity Of Fish scale Protein Hydrolysates

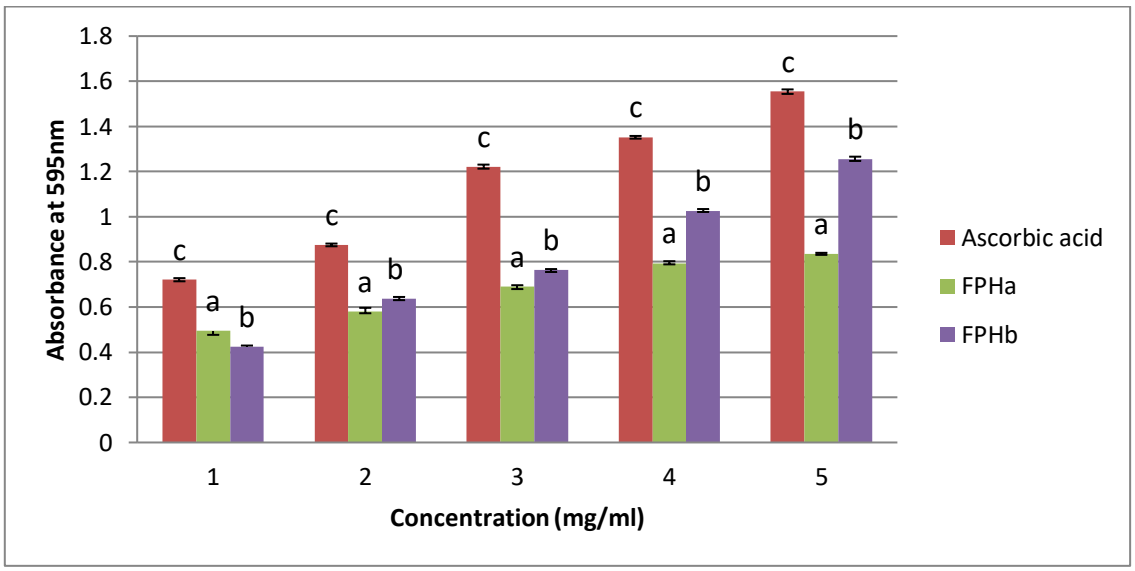


Different letters in the superscript indicate significant difference for the same concentration according to Duncan's test (P0<.05)

Figure 5.Comparison of ABTS radical scavenging activity of fish protein hydrolysates prepared by using microbes

The highest ABTS radical scavenging activity of both the hydrolysates was observed at the concentration of 5mg/ml. At this concentration, the % scavenging activity of the hydrolysate FPHa and FPHb was 55.71 and 68.83% re*sp*ectively (Figure 5). There was a significant difference between the hydrolysates ($p<0.05$) for the same concentrations. FPHb showed higher ABTS radical activity as compared to FPHa.The scavenging activity of the hydrolysates was compared with standard antioxidant ascorbic acid and it was found that ascorbic acid showed higher activity (97.46%).

4.4.2.3 Ferric Reducing Ability of Fish Scale Protein Hydrolysates



Different letters in the superscript indicate significant difference for the same concentration according to Duncan's test (P0<.05)

Figure 6. Comparison of Ferric reducing ability of fish protein hydrolysates prepared by using microbes

The hydrolysates prepared from fish scale showed good ferric reducing ability. There was a significant difference between the hydrolysates ($p\leq0.05$) for the same concentrations. From the absorbance data, it can be interpreted that FPHb showed higher ferric reducing activity as compared to FPHa (Fig. 6).The reducingactivity of the hydrolysates was compared with standard antioxidant i.e. ascorbic acid and it was found that ascorbic acid showed higher reducing activity than the samples.

4.4.3 Antimicrobial Activity of Fish Protein Hydrolysate

The antimicrobial activity of the fish scale protein hydrolysates (FPHa and FPHb) was tested against *Bacillus subtilis, Escherichia coli, Pseudomonas aeroginosa and Staphylococcus aureus* by using agar well diffusion method.

Table 12. Antimicrobial activity of fish protein hydrolysate prepared by using *Lysinibacillus sphaericus*(FPHb)

Microorganisms	**FPHb**				
	5%	10%	15%	20%	25%
Bacillus subtilis	0	0	7.51±0.97	15.68±1.44	18.51±0.83
Escherichia coli	0	0	6.43±1.36	13.03±1.18	15.86±0.87
Pseudomonas aeruginosa	0	0	5.38±1.14	10.22±0.85	13.05±0.97
Staphylococcus aureus	0	0	0	0	0

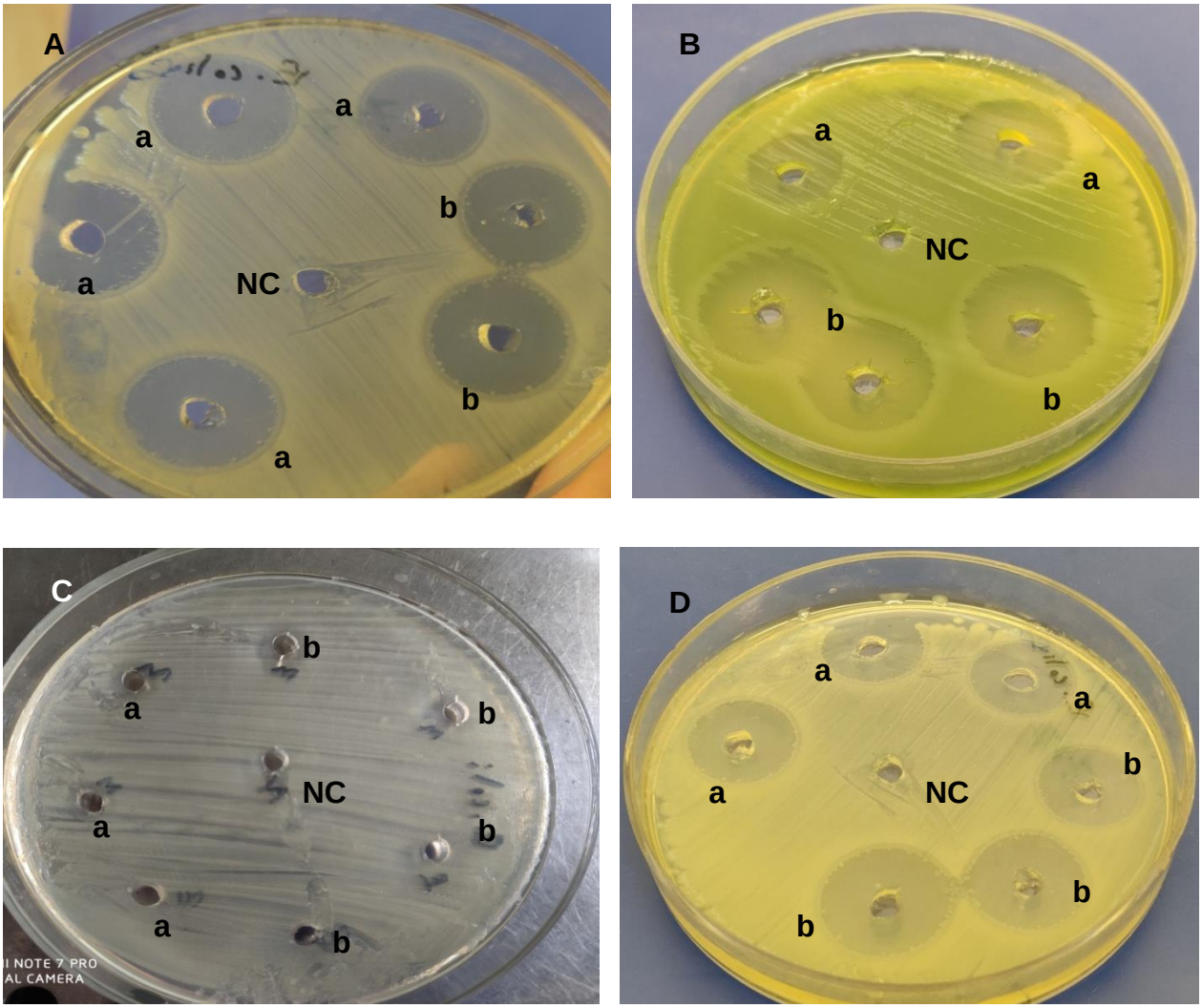
Different letters in the superscript indicate significant difference for the same concentration according to Duncan's test (P0<.05)

The antimicrobial activity of the hydrolysate FPHb was showing an increasing trend with increasing concentration (Tab.12). It showed the highest antimicrobial activity against *Bacillus subtilis,* followed by *Escherichia coli.* The hydrolysate did not exhibit an inhibitory effect against *Staphylococcus aureus.*

Table 13: Antimicrobial activity of fish protein hydrolysate by using *Acinetobacter towneri* (FPHa)

Microorganisms	**FPHa**				
	5%	10%	15%	20%	25%
Bacillus subtilis	0	0	0	5.42±0.64	13.51±0.77
Escherichia coli	0	0	0	7.24±0.91	15.39±0.67
Pseudomonas aeruginosa	0	0	0	5.38±0.95	12.68±1.12
Staphylococcus aureus	0	0	0	0	0

The antimicrobial activity of the hydrolysate FPHa was showing an increasing trend with increasing concentration. FPHa showed the highest antimicrobial activity against *Escherichia coli* (Tab.13).The hydrolysate did not show any antimicrobial activity against *Staphylococcus aureus.*



A) Escherichia coli, B) *Pseudomonas aeroginosa*, C) *Staphylococcus aureus*, D) *Bacillus subtilis*

Plate 5. Antimicrobial activity of fish scale protein hydrolysates against different microorganisms

4.5 Products Prepared for Direct Consumption of Fish Scales

Carp scales were divided into two groups. One group was treated with gelatinase positive bacteria for 7 days to make the texture soft and thereby easy to process. Another group was preserved at -20^{o}C for further use. Two different products were prepared using scales and their consumer acceptability was assessed by sensory evaluation. For each product, there were two groups- in one group scales were used without any treatment (A) and in second one microbe treated scales (B) were used. Tab.13 and 14 representing the scores of the sensory evaluation of the fish scale wafer and coated fish scales re*sp*ectively.

Table 14. Sensory scores for scale wafer product A and B

Product	Sensory attributes						
	Appearance	**Colour**	**Cris*p*ness**	**Odour**	**Flavour**	**Taste**	**Overall acceptability**
A	8.00±0.00	8.00±0.00	8.20±0.75	8.15±0.74	8.40±0.46	8.30±0.63	8.20±0.58
B	8.65±0.47	8.65±0.45	7.45±0.89	8.00±0.67	8.15±0.74	8.15±0.33	8.65±0.41
p-value	0.00	0.00	0.471	0.408	0.238	0.063	0.489

Table 15. Sensory scores for coated fish scale product A and B

Product	Sensory attributes						
	Appearance	**Colour**	**Cris*p*ness**	**Odour**	**Flavour**	**Taste**	**Overall acceptability**
A	8.40±0.70	8.00±0.67	8.10±0.87	8.20±0.78	7.90±0.73	8.30±0.48	8.00±0.81
B	8.00±0.94	7.60±0.97	7.40±0.84	7.85±0.88	7.60±1.42	7.80±1.39	7.65±1.41
p-value	1.00	0.087	0.841	0.548	0.249	0.367	0.447

There was no significant difference ($p>0.05$) between the two groups (A and B) of fish scale wafers in terms of cri*sp*ness, odour, flavour,tasteand overall acceptability. But significant difference ($p<0.05$) was found in the appearance and colour (Tab.14). These two attributes obtained the higher sensory score for group B.

There was no significant difference ($p>0.05$) between the two groups (A and B) of the coated fish scales. But based on the scores obtained by all the sensory attributes, group A obtained higher scores for all the sensory attributes as compared to group B (Tab.15).

5. DISCUSSION

Huge quantities of fish scales are generated in a number of fish processing units of India. Fish scales are a biodegradable waste but it takes a long time to degrade due to its hardy nature and constitution (Pal, 2017). Therefore, it causes hurdles in the complete utilization of mixed waste. The degradation of these wastes is also detrimental to the environment creating foul smell and pollution of water bodies. Hence, these fish processing units are facing a critical problem in the disposal of these wastes and are in search of possible solutions. Considering these wastes as a potential source of protein and minerals, the present research aims to extract valuable components from underutilized bio-wastes (scales) of rohu and catla fish which are abundant in India.

5.1 Composition of Carp Scales

Fish scales are the rich source of protein and minerals. It has been reported that fish scales and bones contain a high amount of collagen (Zhang *et al.*, 2007). They also contain a variety of calcium phosphate salts due to their intense biological response to the difference in osmolality and ionic inequality in the physiological environment (Panda *et al.,* 2014). Mahboob (2015) reported that moisture content of *Catla catla* and *Cirrhinus mrigala* scales varies from 63.20 to 70.40%.; protein content varies from 21.77 to 23.90% and ash content of carp scales varies from 4.51 to 7.16% significantly depending upon the size and weight of fish, even among the same species. The scales of Chinese carps viz. *Cyprinus carpio* and *Hypophthalmichthys molitrix* contain higher protein content as compared to Indian major carp scales which is 29.91 and 28.38% protein respectively (Mahboob *et al.*, 2014). They also reported that the protein content of carp scales vary depending upon their size and weight.

This study reports the moisture, protein, ash and calcium contents of the carp (*Catla catla* and *Labeo rohita*) scales. The moisture contents in the scales of *C. catla* were ranged between 64.29±1.23 % and 69.71±1.54% in the three samples

(Tab.4). A significant difference (P<0.05) in the scale moisture contents between the C_1 and C_3 samples was observed. The protein contents of the *C. catla* scales were varied from 22.83 ±0.79% to 24.10± 0.86%. The ash content was ranged between 4.49±1.14% and 6.72±1.07%. There was no significant difference (P>0.05) in the protein and ash contents between three different samples. The calcium contents in the *C. catla* scales were ranged between 3.16±0.56% and 4.70±0.43%. A significant difference (P<0.05) in the calcium contents between the C_1 and C_3 samples was observed.

The moisture content in *L. rohita* scales was ranged between 66.63±2.68% and 70.65±1.72% % in the three samples (Tab.4). In the *L. rohita* scales up to 22.72±1.02% protein content was observed. The ash content was varied from 4.32±1.04% to 5.94±1.22% in the three samples. The calcium content was observed to vary between 3.11 ±0.35% and 4.20±0.90% in the three samples respectively. There was no significant difference (P>0.05) in the moisture, protein contents, ash and calcium contents between three different samples of *L. rohita* scales.

5.2 SDS-PAGE of Fish Scale Protein

The protein and minerals are the two important components of the fish scale that can be used for consumption after further processing. In order to know what kind of proteins are present SDS- PAGE was done. The first complete primary structure of a teleost type I collagen was identified in 2001 in rainbow trout (Saito *et al.,* 2001).Type I collagens consist of α- triple helix structure (Liu *et al.,* 2012). Both the *C. catla* and *L. rohita* scale protein were shown to contain at least two different α chains. From the Fig.1 it is visible that scale protein is mainly composed of two different α-chains viz. α_1 and α_2. The molecular weight of the α-chains of the scale protein ranged from 70 to 124 KDa. The high molecular weight component of β chains was also observed. Its molecular weight was between 238 to 315 KDa. A similar type of band pattern was obtained by Duan *et al. (*2009) who reported that the *Cyprinus carpio* scale and bone collagen was the type I based on its electrophoretic mobility. Mehboob *et al.* (2014) also observed similar band pattern for freshwater carp scale proteins.

5.3 Isolation and Identification of Gelatinase Positive Bacteria

The scales are hard and made up of proteins that are difficult to breakdown. Proteins are also the most valuable component to be utilized. One way to utilize the proteins is to hydrolyze them so that they can be extracted from the structure of the scale. This will help to release the digested proteins and minerals. The biological method of degrading the scales is the cheapest and most effective way. This can only be done by gelatinolytic or collagenolytic enzymes. In order to isolate collagenase or gelatinase producing bacteria, samples that are likely to contain such bacteria viz. fish scale and scale deposited soil were collected from different locations. A total of 105 isolates from 12 samples collected from different places were screened (Tab.5). Out of 105 cultures, 50 were positive for the gelatinase test.

Ten extremely halophilic microorganisms were also used in the experiment. The idea behind the use of halophiles is that they do not produce a bad smell during the fermentation process as much as bacteria. Out of 10 extremely halophilic microorganisms, only 4 were showed positive results for the gelatinase test (Tab.7). They are- *Halobacterium* spp*. Halobacterium salinarum*, *Halococcus dombrowskii*, *Halococcus morrhuae.* Similar results have been reported by Stan-Lotter *et al*. (2002) on the gelatinase activity of *Halococcus dombrowski and Halococcus morrhuae*. Mehdi and Jafar (2017) reported that *Halococcus salinarum* is able to hydrolyze casein and gelatin.

5.4 Restriction Fragment Length Polymorphism (RFLP)

RFLP is the characterization of PCR products based on sequence-specific enzymatic cleavage by restriction enzymes. It involves the generation of different size and number of DNA fragments that result in characteristic banding patterns in agarose gel electrophoresis (Rohit *et al.,* 2016). Each RFLP pattern refers to one operational taxonomic unit (OTU) thereby reducing the number of isolates to be sent for sequencing (Rathlavath *et al.,* 2017). In this study 50 gelatinase positive isolates when exposed to RFLP with MspI and AluI, 28 OTUs were generated (Fig.3). Out of 28 OTUs, 15 were sent for sequencing. After sequencing the 15 isolates were

identified as *Proteus vulgaris, Acinetobacter towneri*, *Myroides* spp. *Lysinibacillus sphaericus, Aeromonas sp., Pseudomonas alcaligens, Enterobacter sp., Macrococcus caseolyticus, Proteus mirabilis, aeromonas veronii, Aeromanas hydrophila, Psychrobacter sp.* and three isolates were *Staphylococcus sciuri* (Tab. 6).

5.5 Application of Gelatinase Positive Microbes on Carp Scales

Ghanem *et al*. (2010) used a mutant strain of *Aspergillus terreus* isolated from fishery polluted waste for the biodegradation of parrot fish scale waste. Basu and Banik (2005) studied the biodegradation of fish scale powder by using crude protease of a mutant strain of *Aspergillus niger* and found that the solubilization of proteinaceous material was maximum on the 14th day of fermentation. In the present study, all the bacterial cultures took up to 28 days for the removal of 72.49 to 96.60% protein from the carp scale. It may be due to the large size of the fish scales used in the experiment.

All 10 bacterial cultures inoculated into carp scales have the ability to hydrolyze scale protein (Tab.8). Out of them, *Acinetobacter towneri*, *Lysinibacillus sphaericus* and *Myroides* spp. showed the highest protein removal efficiency in the experiment (Table 8). *Proteus vulgaris* showed the lowest protein removal efficiency from carp scales. All the 3 extremely halophilic cultures also showed a positive result for scale protein hydrolysis, but their rate of hydrolysis was very slow. It may be due to the slow growth rate of extremophiles. It has been reported that extremophilic microbes grow at extremely slow rates and live at low densities (Rampelotto, 2010). Out of 3 cultures, *Halococcus dombrowskii* showed the highest protein removal followed by *Halococcus salinarum* (Tab. 9).

5.6 Fish Scale Protein Hydrolysate

Two hydrolysates were prepared from carp scale by using two different bacterial cultures. The hydrolysate prepared using *Acinetobacter towneri* (FPHa) and *Lysinibacillus sphaericus* (FPHb) were analyzed to determine the protein content and degree of hydrolysis. According to Nasri *et al*. (2013), the protein hydrolysates may have variation in their amino acid composition based on factors such as type of

enzyme and hydrolysis time. The percentage of peptide bond breakage was calculated by using the degree of hydrolysis.

The protein content of FPHa and FPHb was 76.56±1.17% and 82.62±1.14% respectively. The degree of hydrolysis was 16.64±0.51% and 22.19±1.15% for FPHa and FPHb respectively (Tab.10). The higher percentage of the degree of hydrolysis in fish scale protein hydrolysate prepared by using *Lysinibacillus sphaericus* (FPHb) indicates that more number of peptide was present in the hydrolysate as compared to the hydrolysate prepared using *Acinetobacter towneri* (FPHa). The peptides recovered after hydrolysis of protein is found to be more bioactive and antioxidative (Kuppusamy and Ulagesan, 2016). Therefore, the antioxidant and antimicrobial properties of the hydrolysate were evaluated.

5.7 Amino Acid Profiling of Fish Scale Protein Hydrolysates

Fish protein hydrolysates prepared by using different microbes have been reported to exhibit variation in their amino acid composition. The amino acid composition of fish protein hydrolysates influenced by several factors such as raw material, enzyme source, and hydrolysis conditions (Klompong *et al.,* 2009). Glycine was the most abundant amino acid identified in both the hydrolysates, followed by alanine. Duan *et al.* (2009) reported that glycine is a major amino acid in carp collagen. The results of the present study are also supported by the findings of Zhang *et al.* (2007) and Kim and Mendis (2006), who demonstrated that glycine is the major amino acid in each collagen type. The hydrolysates consisted of hydroxyproline, which is a unique amino acid found in collagen. The results of the present study are supported by the findings of Mahboob (2015). Glutamic acid was found in significant quantities in both the hydrolysates. Asparagine, histidine, threonine, arginine, cystine, norvaline and tryptophan were not detected in FPHa. In the case of FPHb, asparagine, histidine, threonine, arginine, cystine, norvaline, tyrosine, methionine and phenylalanine were absent (Tab.11). Amino acid methionine was significantly low in FPHa. Non-essential amino acids found to be more abundant in both the hydrolysates.

5.8 Antioxidant Properties of Fish Scale Protein Hydrolysates

5.8.1 DPPH Radical Scavenging Activity

The DPPH radical scavenging activity determines the hydrogen-donating potential of protein hydrolysate (Thiansilakul *et al*., 2007). DPPH is a free radical and it gives a maximum absorbance at 517 nm at an appropriate environment, when the DPPH facing a hydrogen-donating substance such as an antioxidant, the absorbance will be reduced and the color will be changed from purple to yellow (Nalinanon *et al*., 2011; Shimada *et al*., 1992). The antioxidant properties of the protein hydrolysate may be due to the presence of peptide sequences that have the ability to transfer H-atom/electron and thereby terminate the free radical-induced chain reaction (Elavarasan *et al.*, 2014).

In this study, the DPPH radical scavenging activity increased with the increased concentration of protein hydrolysate. Similar results were reported with *Sardinella longiceps* hydrolysate (Samanta and Khora, 2014) in which radical scavenging activity of the *Sardinella longiceps* hydrolysate was found to increase with the increased concentration of hydrolysate. The DPPH radical scavenging activity of fermented protein hydrolysates prepared from the meat of different fishes viz. sardinella, zebra blenny, goby was reported by Jemil *et al*. (2014), who found that the antioxidant activities of the hydrolysate were dose-dependent. Godinho *et al.* (2016) reported that fermentative protein hydrolysates from cod protein hydrolysate by using *Bacillus sp.* exhibited DPPH radical scavenging activity. In this study, the highest DPPH radical scavenging activity of both the hydrolysates was observed at the concentration of 5mg/ml. At this concentration, the percentage scavenging activity of the hydrolysate FPHa and FPHb was 64.02±0.70 and 76.69±1.00 respectively (Fig.4). There was a significant difference between the hydrolysates ($p<0.05$) for the same concentrations. FPHb showed higher DPPH radical activity as compared to FPHa. The higher degree of hydrolysis of FPHb may be the reason behind its higher DPPH radical activity.

5.8.2 ABTS Radical Scavenging Activity

ABTS assay is a widely used method to measure antioxidant activity. In this study, the ABTS radical scavenging activity increased with the increased concentration of protein hydrolysate. Similar results were reported on protein hydrolysates prepared from the viscera of skipjack tuna (*Katsuwonus pelmamis*) by Klomklao and Benjakul in 2018. Both the hydrolysates from the carp scale have shown good ABTS scavenging activity. The presence of peptides in the hydrolysates which have the capability to donate hydrogen atoms to the free radicals are responsible for the termination of free radical-induced oxidation chain reaction (Faithong *et al.,* 2010). The highest ABTS radical scavenging activity of both the hydrolysates was observed at the concentration of 5mg/ml. At this concentration, the % scavenging activity of the hydrolysate FPHa and FPHb was 55.71% and 68.83% respectively (Fig.5). There was a significant difference between the hydrolysates ($p<0.05$) for the same concentrations. FPHb showed higher ABTS radical activity as compared to FPHa. The higher degree of hydrolysis of FPHb may be the reason behind its higher ABTS radical activity.

5.8.3 FRAP (Ferric Reducing Antioxidant Power) Assay

FRAP (Ferric reducing antioxidant power) assay is performed to determine the ability of an antioxidant to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II) complex. It measures the potential of an antioxidant to reduce ferric tripyridyltriazine (Fe3+-TPTZ) complex to a colored compound ferrous tripyridyltriazine (Fe2+-TPTZ). The termination of free radical-induced oxidation chain reaction takes place through the donation of a hydrogen atom (Binsan *et al*., 2008). The hydrolysates prepared from the fish scale showed good ferric reducing ability which was indicated by the intensity of the color of the sample due to the formation of ferrous tripyridyltriazine (Fe2+-TPTZ). The optical density (OD) value of the hydrolysates was measured at 595 nm wavelength. The ferric reducing activity of the hydrolysates was found to be dose-dependent. Therefore, the maximum reducing activity was observed at the concentration of 5mg/mL. The OD values of the hydrolysate FPHa and FPHb were 0.836 and 1.255 for respectively at the concentration of 5mg/mL (Fig.6). There was a

significant difference between the hydrolysates ($p<0.05$) for the same concentrations. FPHb showed higher ferric reducing activity as compared to FPHa. It has been reported that the anti-oxidative activity and functional properties of protein hydrolysate are influenced by the degree of hydrolysis and type of enzyme (Vilailak *et al.,* 2007). The degree of hydrolysis was higher for FPHb. This may be the reason behind the higher antioxidant activity of fish scale hydrolysate (FPHb) prepared by using *Lysinibacillus sphaericus.*

5.9 Antimicrobial Activity of Fish Scale Protein Hydrolysate

There are so many mechanisms that are found to be responsible for the antimicrobial activity of peptides. Most antimicrobial peptides (AMPs) work directly against microbes through a mechanism involving membrane disruption and pore formation which allows reflux of essential ions and nutrients (Taha *et al.,*2013). Intracellular targeting of cytoplasmic components such as DNA, RNA etc. is another mechanism that may responsible for the antimicrobial activity of peptides (Harris *et al*., 2009; Yeaman and Yount, 2003).

In the present study, the antimicrobial activity of both the hydrolysates was assessed against gram+ve bacteria *Staphylococcus aureus and Bacillus subtilis* and gram–ve *Escherichia coli and Pseudomonas aeruginosa*. Both the fish scale protein hydrolysates showed an inhibitory effect against *Bacillus subtilis, Escherichia coli and Pseudomonas aeruginosa*. The reason for the inhibitory effect of hydrolysates against microbes could be attributed to small peptide sequences. There are reports that peptides have an inhibitory effect on the selected microbes (Kuppusamy and Ulagesan, 2016). The zone of inhibition showed a positive correlation with the concentration of hydrolysate in this study. Similar results by Da Rocha *et al.* (2018) showed that under-utilized fish Argentine croaker (*Umbrina canosai)* protein hydrolysate has antibacterial activities.

The maximum zone of inhibition was observed at a concentration of 25%. There was a significant difference in the antimicrobial activity of FPHb between different microbes ($p\leq0.05$). It showed the highest antimicrobial activity against *Bacillus*

subtilis, followed by *Escherichia coli* (Tab. 12)*.* The hydrolysates did not exhibit an inhibitory effect against *Staphylococcus aureus.* It may be due to the reason that none of the mechanisms responsible for the antimicrobial activity of peptides is effective in the case of *Staphylococcus aureus*. FPHa showed the highest antimicrobial activity against *Escherichia coli* (Tab.13)*.*

5.10 Sensory Evaluation of Value Added Products Prepared by Using Fish Scales

5.10.1 Fish Scale Wafers

As mentioned in the introduction and results section (4.1.2), the collagen is the major protein in the fish scale and the results demonstrated that collagen when heated gets converted to gelatin. Therefore, the processing of scales by heating would make the scale edible. In order to add taste to the scale before heat processing minimum ingredients of Tapioca sago, salt, chili powder were added to the fish scale wafer. Wafers prepared by using microbial treated and untreated scales referred B and A respectively. There was no significant difference ($p \geq 0.05$) between the two groups (A and B) in terms of crispness, odor, flavor, taste and overall acceptability (Tab.14). But significant difference ($p \leq 0.05$) was found in the appearance and color. These two attributes obtained a higher sensory score for group B.

5.10.2 Coated Fish Scales

Coating improved taste, flavor, color and appearance of the scale which can't be consumed as such. Because scales are made up of collagen and minerals. Collagen gets converted to gelatin when heated which is tasteless. The two groups (A and B) of the product did not show any significant difference ($p > 0.05$) according to scores obtained by the sensory attributes (Tab.15).

5.11 Overall Findings

In the present study it has been found that fish scales are the rich source of protein and calcium. To utilize these valuable components of fish scales, microbes were isolated from the scale itself which are capable of hydrolyzing fish scale protein. Ten bacterial and three haloarchaeal cultures were selected to check their protein removal efficiency from carp scales. Bacterial cultures were more efficient as compared to haloarchaeal isolates. They removed up to 97.73% protein from carp scales within 28 days. Whereas, haloarchaeal cultures took 75 days to remove 96.54% protein. But the main problem associated with the bacterial fermentation process was the production of the foul smell but the smell was very less in case of Haloarchaeal fermentation. Out of the total 28 isolates 2 isolates were selected viz. *Acinetobacter towneri* and *Lysinibacillus sphaericus* for the preparation of protein hydrolysate from the carp scales based on their ability the hydrolyze scale protein. The hydrolysates prepared using microbes showed good antioxidant and antimicrobial activity. Carp scales were also used for the preparation of two value-added products viz. fish scale wafer and coated fish scales. Both the products obtained more than 7 scores for all the attributes which indicate that products are liked moderately by the panel members. Thus the present study demonstrates that microbes can be effectively used for the production of high valued products from fish scales as well as help to overcome the problem of their disposal.

6. SUMMARY

Fish scales which are generally considered as waste can be effectively used as a rich source of protein and minerals. So the present study was carried out to develop simple, cost-effective and eco-friendly methodologies for the utilization of fish scales by using microbes in order to overcome the problem of waste disposal. It has been found that the carp scales are the rich source of protein and mineral mainly calcium. The protein and calcium content of carp scales varied from 21.27±0.74 to 24.10±0.86% and 3.11 ±0.35 to 4.70±0.43% respectively. To utilize these valuable components of fish scales, microbes were isolated from the scale itself which are capable of hydrolyzing fish scale protein. A total of 105 bacterial cultures were isolated and 50 out of the total showed gelatinolytic activity. In RFLP 28 cultures out of 50 showed different digestion patterns for two restriction enzymes viz. MspI and AluI. Those 28 cultures were sent for sequencing. Ten extremely halophillic microbes were also used in the study. Out of 10 halophiles, only 4 were positive for gelatinolytic activity. Ten bacterial and three haloarchaeal cultures were selected to check their protein removal efficiency from carp scales. Bacterial cultures were more efficient as compared to haloarchaeal isolates. They removed up to 97.73% protein from carp scales within 28 days. But Haloarchaeal cultures took 75 days to remove 96.54% protein. But the main problem associated with the bacterial fermentation process was the production of the foul smell but the smell was very less in case of Haloarchaeal fermentation.

The microbes showed higher efficiency in the hydrolysis of carp scale protein viz. *Acinetobacter towneri* and *Lysinibacillus sphaericus* were selected for the production of fish scale protein hydrolysate by the microbial fermentation process. The hydrolysates produced by using these two bacterial cultures were analyzed for protein content and degree of hydrolysis. Fish scale protein hydrolysate prepared by using *Lysinibacillus sphaericus* showed higher

protein content and degree of hydrolysis as compared to the hydrolysate prepared by using *Acinetobacter towneri.* The hydrolysates were tested for antioxidant activities viz. DPPH radical scavenging activity, ABTS radical scavenging activity and ferric reducing ability. Both the hydrolysates showed good antioxidant activity. Fish scale protein hydrolysate prepared by using *Lysinibacillus sphaericus* showed higher antioxidant activity as compared to the hydrolysate prepared by using *Acinetobacter towneri.* The antioxidant activity of both the hydrolysates was found to be increased with increased concentration.

The hydrolysates were also tested for antimicrobial activity against human pathogens viz. *Escherichia coli*, *Pseudomonas aeroginosa*, *Staphylococcus aureus* and *Bacillus subtilis. Both the hydrolysate showed antimicrobial activity against Escherichia coli*, *Pseudomonas aeroginosa* and *Bacillus subtilis.* But they did not show any antimicrobial activity against *Staphylococcus aureus*.

Carp scales were also used for the preparation of two value-added products, fish scale wafers and coated fish scales. Both the products were prepared in two groups each viz. A (without treatment) and B (microbe treated).The consumer acceptability of both the products was assessed by sensory evaluation. The sensory characteristics of the scale products were evaluated by a taste panel member on a nine-point scale. Scores were assigned with 1 being the least and 9 being the greatest for each attribute. Both the products obtained more than 7 scores for all the attributes which indicate that products are liked moderately by the panel members. This work establishes that direct consumption by making wafer and coated scales are possibilities of use of scales. However, greater utilization would be by hydrolysate production using bacteria and archaea.

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APPENDIX- I

1. Nutrient agar

1.1. Composition (Ingredients –gms /Litre)

Ingredient	Gms / Litre
Peptone	5.000
Sodium chloride	5.000
HM peptone	1.500
Yeast extract	1.500
Agar	15.000
Final pH (at 25°C)	7.4±0.2

28 grams was suspended in 1000 ml of distilled water and heated to boiling to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. Mixed well and poured into sterile Petri plates.

2. Luria bertani agar (Hi-media, Mumbai)

2.1. Composition (Ingredients –gms /Litre)

Ingredients	Gms / Litre
Casein enzymic hydrolysate	10.000
Yeast extract	5.000
Sodium chloride	10.000
Agar	15.000
Final pH (at 25°C)	7.5±0.2

40 grams media was suspended into 1000 ml of distilled water and heated to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Dispense as desired. Mixed well and poured into sterile Petri plates.

3. Luria Bertani Broth, Miller (Hi-Media, Mumbai)

3.1. Composition (Ingredients –gms /Litre)

Ingredients	Gms / Litre
Casein enzymic hydrolysate	10.000
Yeast extract	5.000
Sodium chloride	10.000
Final pH (at 25°C)	7.5±0.2

25 grams was suspended in 1000 ml of distilled water and heated to dissolve the medium completely. The medium was dispensed into the tube and sterilised by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

4.Mueller Hinton Agar

4.1 Composition (Ingredients –gms /Litre)

Ingredients	gm/ L
Beef, infusion form	300.0
Casein acid hydrolysate	17.500
Starch	1.500
Agar	17.00
Final pH (at 25^0C)	7.3 ± 0.1

Direction: Suspend 38 grams of dehydrated media were suspended in 1000 ml distilled water. The medium was dissolved completely by heating and sterilized by autoclaving at 15 lbs. pressure (121°C) for 15 minutes. Then it was cooled to 50-60^{o}C and dispensed as desired.

APPENDIX- II

SENSORY EVALUATION OF FISH SCALE PRODUCTS

Product:……..

Date:……

CHARACTERISTICS	A	B
Appearance		
Colour		
Crispness		
Odour		
Flavour		
Taste		
Overall acceptability		

SCALE	
Like extremely	9
Like very much	8
Like moderately	7
Like slightly	6
Neither like nor dislike	5
Dislike slightly	4
Dislike moderately	3
Dislike very much	2
Dislike extremely	1

Signature of the evaluator