

# **REVIEW OF LITERATURE**

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## **2.0 REVIEW OF LITERATURE**

Fishing and the fish processing industries are important economic sectors in the world. According to the Food and Agriculture Organisation (FAO), the global fishery production (catch and aquaculture) in 2008 was estimated to be around 110 million tonnes, with a global catch exceeding 76 million tonnes (FAO, 2010). The fish industry generates a significant amount of waste.

Annual discard of the fish waste industry is estimated to be approximately 20 million tones (or 25% of the total production). The major fish waste from fish processing industry include bone frame, bones, viscera, skin and scales and they contribute as high as 70% of the original raw materials. Fish waste is a good source of protein (Arnesen and Gildberg, 2007), but a huge amount of the waste is still being discarded without much effort to recover its protein. Besides that, the discarding of fish waste creates disposal problems (Amiza *et al.*, 2011).

The review of literature pertaining to the present study “Comparison of various Precipitation Techniques and Partial Purification of Protease isolated from Visceral organ and Head and Tail Wastes of Indian Oil Sardine (*Sardinella longiceps*) Fish are discussed under the following headings:

### **2.1 INTRODUCTION**

### **2.2 TYPES OF FISH**

### **2.3 FISH PRODUCTS AND BY-PRODUCTS**

#### **2.3.1 BENEFICIAL COMPONENTS FROM FISH WASTE**

#### **2.3.2 BY-PRODUCTS FROM FISH WASTE**

### **2.4 MANAGEMENT OF FISH WASTE**

### **2.5 INDIAN OIL SARDINE (*Sardinella longiceps*)**

## **2.6 PROTEASES**

### **2.6.1 CLASSIFICATION OF PROTEASE**

### **2.6.2 PROTEASES FROM FISH WASTE**

### **2.6.3 APPLICATIONS OF PROTEASES**

## **2.1 INTRODUCTION**

Fish are a class of aquatic vertebrates. The combination of gills, fins and the fact that they live only in the water make fish different from all other animals. Fish are cold- blooded with the exception of Tuna family and the Mackerel shark family. Scientists believe that there are more than 24,000 different species of fish in the world. They range from the largest whale shark, which is 16 mm (51ft) long, to the smallest 8 mm (1/4 in.) stout infant fish.

(<http://www.kidzone.ws.animals/fish1.htm>)

The classification of fishes has undergone a lot of change over the last few decades, and further changes are expected, partly because so many groups are poorly known. There are many conflicting hypotheses of relationships, some based on conflicting evidence between morphological studies and molecular studies; however, progress is being made and there are many areas of basic agreement. There are about 28,000 living species of fishes recognized as valid (formally described and recognized). This is slightly over half the number of recognized tetrapods. About 11,950 of these species are confined to fresh water. Many new species are described every year, and if we use the same species concept as the above figure reflects, then there may be an estimated 32,500 species alive today (some estimated 4500 are yet to be found and described).

## **2.2 TYPES OF FISH**

The 24,000 known species of fish are divided into three main groups.

They are

- a) Jawless fish
- b) Cartilaginous fish
- c) Bony fish

### **a) Jawless fish**

Jawless fish are the last survivors of the world's first vertebrate animals which means, "back-boned". They lack both scales and jaws. Dating from over 5000 million years ago, only the hagfish and lampreys remain.

### **b) Cartilaginous fish**

Cartilaginous fish developed about 100 million years later as ancestors of today's sharks. The skeleton of these fish is made of cartilage, which is not as hard as bone. These fish have jaws, as well as teeth, which are usually hard and sharp. Their bodies are covered with hard scales.

### **c) Bony fish**

Fish with bony skeletons appeared at the same time as cartilaginous fish. They are the largest group, with about 20,000 species. The fish have an organ called a swim bladder, which gives the animal buoyancy, the ability to float ([http:// www.iwrc-online.org/kids/ facts/ fish/fish.htm](http://www.iwrc-online.org/kids/facts/fish/fish.htm)).

## **2.3 FISH PRODUCTS AND BY-PRODUCTS**

The amount of by-products from fish varies with fish species, size, season and fishing ground . The by-products are generated when the fish is gutted, headed and further processed into fillets. Depending on the efficiency of the production process, only 50-60% of the total fish catch goes for human consumption. The by-products include heads, viscera, skin, trimmings and fish rejects. They are often dumped, used as animal feed or as fertilizer. Due to the worldwide decline of fish stocks, a better use of by-catch and by-products is important. These biomasses have great potential as a source of high valued products due to their high protein content, high levels of essential nutrients such as vitamins, minerals, and in particular, fish oils (Souza *et al.*, 2007).

### **2.3.1 BENEFICIAL COMPONENTS FROM FISH WASTE**

Seafood and fish waste may be source of proteins of high biological value, unsaturated essential fatty acids, vitamins and antioxidants, minerals or trace metals and physiological beneficial amino acids and peptides.

Fish waste can be value added by converting it into fish protein hydrolysate (FPH) by utilizing proteolytic enzymes to hydrolyze the fish protein (Venugopal, 2006). Enzymes used to produce FPH should be of food grade, and if they are of microbial origin, the producing microorganisms have to be non-pathogenic. The enzymatic protein hydrolysis of fish waste produces soluble and insoluble fractions. The insoluble fraction may be used as animal feed and is normally dried to produce a stable concentrated protein called fish protein hydrolysate. Alcalase®, a serine bacterial endopeptidase prepared from a strain of *Bacillus licheniformis* has been proven to be one of the best enzymes by many researchers which has been used in the preparation of fish protein hydrolysates (Bhaskar *et al.*, 2006). Several studies has been reported on the

optimization of enzymatic fish protein hydrolysis such as in Catla viscera (Bhaskar *et al.*, 2008), pacific whiting solid waste (Nilsang *et al.*, 2005), threadfin bream (Normah *et al.*, 2005) and grass carp skin (Wasswa *et al.*, 2008).

**2.3.1.1 Fish body oil** - It is used as an artificial rubber-filling compound, as a lubricating agent and in the manufacture of detergents

**2.3.1.2 Chitosan:** - Used as food additive, sizing material in leather industry, chelating agent and artificial skin

**2.3.1.3 Shark fin Rays** - It is used in soup preparation

**2.3.1.4. Fish Silage** - It is used as cattle and poultry feed

**2.3.1.5. Fish Fertilizer**

**2.3.1.6. Shrimp Extract** - Large quantities of prawn waste are thrown away as a waste. The high percentage of protein present in the waste can be isolated for human conception or as animal feed.

## **2.3.2 BY-PRODUCTS FROM FISH WASTE**

Fish waste can be turned into a commercially valuable by product.

Fish by-products contain valuable protein and lipid fractions as well as vitamins and minerals, but are also an important source of environmental contamination. Environmental regulations are becoming stricter, requiring new methods for managing these fish by-products. There are many options for seafood waste management that could help to resolve these problems. Among them, the production of fish meal and fish oil is the most used worldwide. However, enzymatic hydrolysis is one of the most efficient methods to recover protein and thus to increase the commercial value of such biomass. Indeed, a lot

of fish by-products have been hydrolysed such as salmon head, salmon frame, sole frame, sardine viscera and head. Preferred enzymes are large spectra proteases such as Alcalase, Neutrase, Protamex and Kojizyme.

Majority of fishery by-products are presently employed to produce fish oil, fishmeal, fertilizer, pet food and fish silage. However, most of these recycled products possess low economic value. Recent studies have identified a number of bioactive compounds from remaining fish muscle proteins, collagen and gelatin, fish oil, fish bone, internal organs and shellfish and crustacean shells (Jung & Kim, 2005). Generally, a far better profitability is obtained by producing human consumables and the highest profitability is currently expected from bioactive compounds. These bioactive compounds can be extracted and purified with technologies varying from simple to complex and such compounds may include preparation and isolation of bioactive peptides, oligosaccharides, fatty acids, enzymes, water-soluble minerals and biopolymers for biotechnological and pharmaceutical applications (Kim and Mendis, 2006).

Fish wastes have a huge unexploited potential for value adding. Every year 18 – 30 million tons of waste is dumped around the world. The goal is to increase the use in foods, functional foods and biochemical products for human consumption. By-products from Norwegian fisheries, included fish farming consist of viscera (liver, roe, stomachs, etc.), heads, backbones, cuts and rejected fish from processing (<http://www.rubin.no/eng/>).

### **2.3.2.1 FISH SILAGE**

Fish silage in its liquid form (i.e. not clogging nozzles) is an organic fertilizer that can be mechanically sprayed onto (organic) food crops. The fish waste alluded here is principally composed of: heads, guts, skin and cartilage. In this regard most kinds of fish can be made into silage. But the parts

used for processing should be fresh. Thawed and previously frozen fish can also be used. Sharks and rays are difficult to liquify and so should be mixed with other fish species. The inclusion of the guts, in this mixture, is very important. These viscera contain enzymes that aid in liquefying the fish. That is why it is very important to encourage fishermen not to dump viscera into the sea. Other than that, a suitable supplement has to be found to help the fish enzymes in achieving a better liquefaction, should the fish continue to be handled without the gut enzymes. The other usage for the fish waste, since it contains minerals, trace elements, complex nutrients and amino acids can be used as a supplement to animal food.

#### **2.3.2.2 FISH MUSCLE PROTEIN**

Fish frames and cut offs result from mechanically deboned fish containing considerable amounts of muscle proteins. These muscle proteins are nutritionally valuable and easily digestible with well-balanced amino acid composition. Therefore, fish proteins derived from processing by-products can be hydrolysed enzymatically to recover protein biomass otherwise discarded as processing waste. Protein hydrolysates from by-products of several marine species have been analysed for their nutritional and functional properties and researches have mainly explored the possibility of obtaining biologically active peptides. Moreover, with the increasing knowledge on functional properties of fish protein hydrolysates, there are many researches performing on the developments and applications into functional foods and nutraceuticals (Kim and Mendis, 2006).

#### **2.3.2.3 BIOACTIVITIES OF FISH MUSCLE DERIVED PEPTIDES**

Fish skin waste could be used as a potential source to isolate collagen and gelatin. Collagen and gelatin are currently used in diverse fields including food,

cosmetic, and biomedical industries. Collagen is structurally formed as a triple helix by three extended protein chains that wrap around one another. Collagen and gelatin are different forms of the same macromolecule and gelatin is the partially hydrolyzed form of collagen. Heat denaturation easily converts collagen into gelatin. In addition to fish skin, collagen and gelatin could be isolated from bone and fins of fish processing by-products. Collagen and gelatin are unique proteins compared to fish muscle proteins and this uniqueness of fish lies in the amino acid content and they are rich in non-polar amino acids (above 80%) such as Glycine , Alanine, Valine and Proline. Even though main industrial sources of collagen and gelatin are bovine and porcine skin, many studies have been conducted to extract collagen and gelatin from fish skin and have been used to screen for their potential industrial applications (Gomez-Gillen *et al.*, 2002).

#### **2.3.2.4 FISH OIL**

Better utilization of marine fish processing by-products could be achieved by converting these materials into fish oil. In general, the fat content of fish is varied from 2– 30% and it basically depends on the type of species, dietary, geographic, environmental, reproductive and seasonal variations. However, with the depleting marine fishery resources, fishing for oil is not encouraged. Therefore, a large amount of offal generated from processing, would be a potential source to produce good quality fish oil for human consumption, especially from fatty fish processing by-products. Composition of fish oil is different from that of other oils and mainly composed of two types of fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These are polyunsaturated fatty acids classified as omega-3 fatty acids and predominantly found in many marine animals including cold-water fish species with a higher unsaturated fat content. Compared to saturated fats, polyunsaturated fatty acids

in fish oil are readily digested for energy production and have been reported to have various bioactivities.

#### **2.3.2.5 FISH BONE**

Fish bone, which is separated after removal of muscle proteins on the frame, is another valuable source in identifying health-promoting components. The organic component of fish bone, which accounts for 30% of the material, is made out of collagen (Nagai *et al.*, 2004). Therefore, fish bone is considered as a source of collagen and gelatin in addition to fish skin (Nagai & Suzuki, 2000). In contrast, fish bone consists of 60–70% of inorganic substances and is mainly composed of calcium phosphate and hydroxyapatite.

#### **2.3.2.6 FISH INTERNAL ORGANS**

Fish internal organs represent rich sources of enzymes, and many of them exhibit high catalytic activity at relatively low concentrations. Considering the specific characteristics of these enzymes, fish processing by-products are currently used for enzyme extraction. A range of proteolytic enzymes including pepsin, trypsin, chymotrypsin and collagenases (Park *et al.*, 2002) are commercially extracted from marine fish viscera in large scale.

Processing of fish fillets for certain markets require methods to remove scales to obtain scale free superior fish products. Direct bioactivities or functional properties of fish scales have not been studied but it is a potential source to extract collagen (Nagai *et al.*, 2004). The composition, structure, and properties of fish scales have been analysed to search for more potential applications for their optimum utilization (Ikoma *et al.*, 2003). Fish eggs resulted from fish processing can be easily processed in to caviar or fish bait. An unrevealed nutraceutical potential exists in fish eggs because they are rich sources of lectins. Lectins are naturally occurring glycoproteins that can bind

with carbohydrates to form stable complexes. Reproductive cells, eggs and sperms are thought to be rich sources of lectins because of its role in fertilization. Due to the ability of lectins to bind to carbohydrates they may have a potential to be used as a better alternative for antibiotics to make pathogens incapable of causing diseases by making lectin-pathogen complexes. Some recent researches have identified lectins from fish eggs and a great potential exists to development of methods to explore their nutraceutical effects (Park *et al.*, 2002).

## **2.4 TYPES OF FISH WASTE**

The fishing industry also creates large amounts of scales and bones as waste by-products. Assuming that 10% of the animal weight is wasted, the total amount of waste generated from these sources can be as high as 900,000 tonnes per year (Morimura *et al.*, 2002).

During the processing of canning of fish, vast amounts of liquid and solid wastes are generated. These include the pre-cooking water, viscera, head, bone, blood and dark meat. The viscera may account for 7-8% of the whole body weight (Prasertsan and Prachumratana, 2008).

## **2.5 INDIAN OIL SARDINE (*Sardinella longiceps*)**

**Domain** : Eukaryota

**Kingdom** : Animalia

**Phylum** : Chordata

**Subphylum** : Vertebrata

**Superclass** : Gnathostomata

**Grade** : Teleostomi

**Class** : Actinopterygii

**Subclass** : Neopterygii

**Division** : Teleostei

**Subdivision** : Clupeomorpha

**Order** : Clupeiformes

**Family** : Clupeidae

**Genus** : *Sardinella*

**Species** : *longiceps*

([http://en.wikipedia.org/wiki/Indian\\_oil\\_sardine](http://en.wikipedia.org/wiki/Indian_oil_sardine))

### 2.5.1 Diagnostic Features

Body elongate, subcylindrical, its depth less than 30% of standard length, belly rounded. The pelvic finray count of 8 distinguishes *S. longiceps* from all other clupeids in the northern Indian Ocean. Very closely resembles *Sardinella neglecta* of East African coasts, but head longer (29 to 35% of standard length; cf. 26 to 29% in *S. neglecta*) and more lower gillrakers (150 to 253 in fishes of 8 to 15.5 cm standard length, usually more than 180; cf. 143 to 188 in *S. neglecta* of 9.5 to 13 cm standard length, usually less than 185). Distinguished in the same way from *Sardinella lemuru* (whose range it may overlap in the Andaman Sea), but the latter has even fewer gillrakers (77 to 188 in *S. lemuru* of 6.5 to 22 cm standard length). A faint golden spot behind gill opening, followed by a faint golden midlateral line; a distinct black spot at hind border of gill cover (absence of pigment).

### 2.5.2 Geographical Distribution

Indian Ocean (northern and western parts only, Gulf of Aden, Gulf of Oman, but apparently not Red Sea or the "Gulf", eastward to southern part of India, on eastern coast to Andhra; possibly to Andamans). Coastal, pelagic, strongly migratory. Feeds mainly on phytoplankton (especially diatoms), both as juveniles and adults, but also on zooplankton. Breeds once a year, the spawners arriving off western coasts of India in June-July when temperatures and salinity are

low during the southwest monsoon months; extended spawning season, but most intense in August-September; exact spawning grounds not located. ([http://en.wikipedia.org/wiki/Indian\\_oil\\_sardine](http://en.wikipedia.org/wiki/Indian_oil_sardine))

**INDIA** : Mathi (Malayalam and Tamil), Boothai (Kannada), Taralai , Haid (Marathi).

## **2.6 MANAGEMENT OF FISH WASTE**

Fish waste management has been one of the problems having the greatest impact on the environment. Fish farming has detrimental effects on the marine environment in particular and has become an issue of public concern. To regulate the water disposal problems, fish waste management programmes are established. The measures promoted for fish waste management through a combination of fish-cleaning restrictions, public education and proper disposal of fish waste, which are to be implemented by various states where fish waste was determined to be a source of water pollution. The programme development and the approval guidance were published by the Environmental Protection Agency (EPA) and the National Oceanic and Atmospheric Administration (NOAA) of the U.S. Development of Commerce (Onokpise *et al.*, 2008).

## **2.7 PROTEASES**

Proteases, a large group of enzymes in most of the organisms, are one of the most important groups of industrial enzymes (Ozturk *et al.*, 2011). These enzymes play an important role in blood-clotting and metabolic control, and also can either break specific peptide bonds, depending on the amino acid sequence of a protein, or breakdown a complete peptide to amino acids. Proteases are widely applied in various fields of industry, such as detergent

formulations, leather preparation, protein recovery or solubilisation, food processing industries and organic synthesis and so on (Jellouli *et al.*, 2009).

### 2.7.1 CLASSIFICATION OF PROTEASES

Proteases are present in all living forms as they are involved in various metabolic processes. They are mainly involved in hydrolysis of peptide bonds. Proteases are classified into six types based on the functional groups in their active sites. They are aspartic, cysteine, glutamic, metallo, serine, and threonine proteases. They are also classified as exo-peptidases and endo-peptidases, based on the position of the peptide bond cleavage. Proteases find wide range of applications in food, pharmaceutical, leather and textile, detergent, diagnostics industries and also in waste management. Thus, they contribute to almost 40% of enzyme sales in the industrial market. Though proteases are found in plants and animals, microbial proteases account for two-third of share in the commercially available proteases.

Depending upon the pH range proteases are generally divided into three broad categories:

- i) Acidic proteases, which show maximum activity between pH 2.0 – 5.0.
- ii) Neutral proteases with a maximal activity at pH 7.0 or around 7.0 and are inhibited by metal chelating agents and
- iii) Alkaline proteases having maximum activity at pH 9.0 – 11.0 and are unaffected by metal chelating agents and cleave a wide range of peptide bonds (Haq *et al.*, 2006).

The largest share of the enzyme market is occupied by detergent proteases, which are mostly alkaline serine protease and active at neutral to alkaline pH range. Alkaline serine proteases have aspartate and histidine

residues with serine in their active site forming a catalytic triad. Serine proteases contribute to one third of the share in the enzyme market and are readily inactivated by Phenyl Methane Sulfonyl Fluoride (PMSF) (Page and Di Cera 2008). Based on the sequence and structural similarities, all the known proteases are classified into clans and families and are available in the MEROPS database (Paul Lavanya *et al.*, 2011).

### **2.7.2 PROTEASE FROM FISH WASTE**

Proteases from fish and aquatic invertebrates may be classified into broader classes such as acidic, neutral and alkaline proteases (Shahidi and Kamil, 2001). Most of these enzymes find application in low cost operations in fish processing such as removing waste flesh from the machinery, preparation of fish protein hydrolysates, skinning of small fish and separation of oil from fatty tissue such as cod livers (Shahidi and Kamil, 2001). Acid ensilaging, using organic acids such as formic and propionic acid is a better method to stabilize the proteases in marine fish waste with potential for recovering active proteases from the liquified silage. Although many researchers have characterized and identified different proteases associated with freshwater fishes (El-Beltagy *et al.*, 2004), the effect of ensiling fresh water fish waste on stabilizing the proteases associated therewith is seldom reported.

### **2.7.3 APPLICATIONS OF PROTEASES**

Proteases are the most important class of industrial enzymes and comprise about 25% of commercial enzymes in the world. These enzymes are widely using in dairy industry as milk clotting agents and meat tenderizing agents in food industry. Proteases are mainly produced by submerged fermentation; the microorganisms and the substrate are present in the

submerged state in the liquid medium, where a large quantity in the form of solvent is present. Since the contents are in submerged state in the liquid medium, the transfer of heat and mass is more efficient and is amenable for modelling the process (Radha *et al.*, 2011).

Proteases represent one of the three largest groups of industrial enzymes and find application in detergents, leather industry, food industry, pharmaceutical industry and bioremediation processes. Proteolytic enzymes are very important in digestion as they breakdown the peptide bonds in the protein foods to liberate the amino acids needed by the body (Ogino *et al.*, 2008). Additionally, proteolytic enzymes have been used for a long time in various forms of therapy. Their use in medicine is notable based on several clinical studies indicating their benefits in oncology, inflammatory conditions, blood rheology control, and immune regulation. Parasites, fungal forms, and bacteria are protein. Viruses are cell parasites consisting of nucleic acids covered by a protein film. Enzymes can break down undigested protein, cellular debris, and toxins in the blood, sparing the immune system this task. The immune system can then concentrate its full action on the bacterial or parasitic invasion. Protease also has the ability to digest unwanted debris in the blood including certain bacteria and viruses. Proteases are widespread in nature. Microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications. *Bacillus* produces a wide variety of extra-cellular enzymes, including proteases. Several *Bacillus* species involved in protease production are e.g. *B. cereus*, *B. sterothermophilus*, *B. mojavensis*, *B. megaterium* and *B. subtilis* (Naidu S., 2011)

Probably the largest application of proteases is in laundry detergents, where they help remove protein based stains from clothing (Banik and Prakash,

2004). For an enzyme to be used as a detergent additive, it should be stable and active in the presence of typical detergent ingredients, such as surfactants, builders, bleaching agents, bleach activators, fillers, fabric softeners and various other formulation aids. In the textile industry, proteases may also be used to remove the stiff and dull gum layer of sericine from the raw silk fiber to achieve improved luster and softness. Protease treatments can modify the surface of wool and silk fibers to provide new and unique finishes. Proteases have been used in the hide dehairing process, where dehairing is carried out at pH values between 8 and 10. Proteases are also useful as important components in biopharmaceutical products such as contact-lens enzyme cleaners and enzymatic debriders (Anwar and Saleemuddin, 2000). The proteolytic enzymes also offer a gentle and selective debridement, supporting the natural healing process in the successful local management of skin ulcerations by the efficient removal of necrotic material (Sjodahl *et al.*, 2002).

Proteolytic enzymes are degradative enzymes which catalyze the cleavage of peptide bonds in other proteins. Alkaline proteases, which are referring to proteolytic enzymes which work optimally in alkaline pH are the main enzymes among proteases and constitute 60 to 65% of the global industrial enzyme market (Amoozegara *et al.*, 2007). Moreover, they are used in the food industry in meat tenderization processes, peptide synthesis, infant formula preparations, baking and brewing. They are also used in the detergent industry as additives, in pharmaceutical and medical diagnosis as well as in the textile industry in the process of dehairing and leather processing (Dodia *et al.*, 2008).

With this background literature the experimental design for the study was formulated in the following chapter.