Study of Purification Profile of Soluble and Immobilized Protease from Visceral Organ Waste of *Sphyraena jello* Fish

Mohanapriya. K

(12PBC008)

Thesis submitted to

Avinashilingam Institute for Home Science and Higher Education for Women,

Coimbatore – 641 043

In Partial Fulfillment of the Requirements for the

Degree of Master of Science in Biochemistry

March, 2014

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Signature of Head of the Department

Signature of Supervisor

Acknowledgement

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Introduction

1.0 INTRODUCTION

Pollution causes damage to health and growth of human beings, animals and trees and all natural patterns of air flow, water flow, temperature and rain get disturbed. Pollution affects physically and physiologically all living objects (Bansal and Bansal, 2012). There are various causes for pollution and among those, animal waste is a major one. Animal wastes are let into the environment by improper disposal. Globally, fisheries and aquaculture industries produce 130 million tonnes of fish waste per annum (Sharpa and Mariojouls, 2012).

The growing consumer demand for healthy fish products has led to a thriving fish processing industry worldwide. Processing of fish includes: scaling, cutting, filleting, cooking, salting and canning. When fish fillets or cans are produced, a large fraction of fish (flesh, heads, bones, fins, skin, tails and viscera) is left as waste. Fish waste is usually disposed off in landfills or in the sea resulting in environmental problems which underscore the need for proper utilization of fish wastes for the recovery of valuable products (Zhao *et al.*, 2011). Marine organisms are rich potential sources of several enzymes that may have some unique properties of interest for both basic research and industrial applications (Salamone *et al.*, 2012).

Fish processing waste can be used to produce commercially valuable by-products (Zhou *et al.,* 2011). New sources of useful enzymes concentrate on the marine environment because of the potential to make use of processing wastes. About 35% of the mass of the fish caught is waste that is disposed off into sea or in landfills. The extraction of enzymes from fish processing waste can reduce environmental problems and improve the economics of the fish industry (Daboor *et al.,* 2010).

Enzymes are proteins that catalyze chemical reactions (Malmiri *et al.,* 2012). Enzyme, which is produced from active cells, is a highly efficient catalyst. Compared with chemical catalyst, it has many advantages such as a high specificity, a high catalytic efficiency, and an adjustable activity, which greatly promote enzyme to be used in pharmaceutical, chemical, and food industries (Zhang *et al.,* 2013). Enzymes can be used in industrial processes with many advantages over chemical techniques. They allow for a better control of the production processes, are environmentally friendly (biodegradable), safe, easy to use and effective in low concentrations (Daboor *et al.,* 2012).

Proteases are enzymes that catalyse hydrolytic reactions in which the protein molecules are degraded to peptides and amino acids. Proteases are one of the most important industrially useful enzymes. These proteolytic biocatalysts have been in use for many centuries (Sumantha *et al.*, 2006). Based upon the protease structures or properties of the active site, there are several kinds of proteases such as serine, metallo, carboxyl, acidic, neutral and alkaline proteases (Raj *et al.*, 2012).

Proteases have diverse applications in a wide variety of industries such as detergent, food, pharmaceutical, leather, peptide synthesis, and in the recovery of silver from used X-ray films. Proteases are mainly derived from animal, plant, and microbial sources. Today, there is an increasing demand for fish proteolytic enzymes in food processing. Fish viscera, one of the most important by-products of the fishing industry is known to be a rich source of digestive enzymes, especially proteases that have high activity over a wide range of pH and temperature conditions and exhibit high catalytic activity at relatively low concentrations. These characteristics of fish proteases have made them suitable for some interesting new applications in food-processing operations. In addition, fish enzymes could be utilized to produce bioactive peptides from fish proteins (Nasri *et al.*, 2011).

Proteinases from cold-water fish are of interest owing to their greater proteolytic activity towards native protein substrates and lower activation energy for catalysis compared to proteinases from mammalian or microbial sources (Fuchise *et al.*, 2011). Most of the enzymes are relatively unstable, their costs of isolation are still high, and it is technically very difficult to recover the active enzyme (Brena *et al.*, 2006) but the immobilization technique can be used to increase the stability of enzyme.

Immobilised enzymes are preferred over native ones owing to their multiple and repetitive use. In addition, the reaction product is not contaminated with the enzyme. Furthermore, immobilised enzymes have longer half-life and predictable decay rate (Sanjay and Sugunan, 2005). Immobilized enzymes are widely used for variety of applications. Based on the type of application, method of immobilization and support material can be selected (Nisha *et al.*, 2012).

Calcium alginate beads are widely used in enzyme immobilization because the gel formation occurs at mild conditions and poses no risk of harm to humans. Process yield per unit of enzyme can be improved by immobilization. In this study, enzyme is immobilized by surface binding or by physical entrapment using calcium alginate gel beads (Meyer, 2007).

Immobilization, leads to minimal inactivation of the enzyme. The immobilized enzyme membrane should also be stable for multiple use as well as storage. A variety of synthetic as well as natural polymeric materials have been used for immobilization of enzyme. One of the natural materials is onion membrane (D'Souza *et al.,* 2012). Immobilization of enzyme on membrane, involves adsorption followed by cross-linking with glutaraldehyde.

With this in view the present work was taken up with the following objectives :

To

- ✓ Isolate protease from fish visceral waste
- ✓ Precipitate protease with 40-60% ammonium sulphate
- ✓ Study the purification profile of the protease
- ✓ Immobilize the protease in calcium alginate and onion membrane and study the purification profile, stability and reusability of the immobilized enzyme.

Review of literature

2.0 REVIEW OF LITERATURE

The review of literature pertaining to the research work "Study of Purification Profile of Soluble and Immobilized Protease from Visceral Organ Waste of *Sphyraena jello* Fish" is explained under the following headings.

2.1 Pollution

2.1.1 Solid waste pollution

2.1.2 Fish waste pollution

2.2 Fish

2.2.1 Types of fish

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2.3.1 Classification of enzymes

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2.3.4 Immobilization of protease

2.3.4.1 Types of immobilization

2.3.4.2 Entrapment of protease

2.3.4.3 Cross linkage method

2.1 Pollution

Environmental pollution is a wide-reaching problem and it influences the health of human populations a lot (Khan and Ghouri, 2011).

2.1.1 Solid waste pollution

Solid waste becomes dangerous if we are not handled properly. The solid waste has adverse effects on the life of human beings and environment. Cities in India with growing population, changing life styles, migration of people from rural areas to urban areas and rapid industrialization end up generating an enormous quantity of urban waste every day. By and large, the Municipal Bodies / Urban Local Bodies in various cities/towns collect municipal solid waste, transport it to the dump yards and dispose it off in open ground dumping or non-sanitary landfill. These landfill sites are an environmental hazard – emanating methane causing greenhouse effect, smell & dirt causing health problems, and leachate contaminating the ground water, etc. (Bajaj and Kumar, 2013).

More than 90% of the Municipal Solid Waste generated in India is directly dumped on land in an unsatisfactory manner. The solid waste placed in landfills or open dumps are subjected to either groundwater underflow or infiltration from precipitation or any other possibility of infiltration of water. During rainfall, the dumped solid wastes receives water and by-products of its decomposition move into the water through the waste deposition. Areas near landfills have a greater possibility of groundwater contamination because of the potential pollution source of leachate originating from the nearby dumping site. Such contamination of groundwater results in a substantial risk to local groundwater resource user and to the natural environment (Nagarajan *et al.,* 2012).

2.1.2 Fish waste pollution

Every year, a huge quantity of fishery wastes and by-products are generated by fish processing industries (Sheriff *et al.*, 2014). The disposal of these wastes represents an increasing environmental and health problems (Rebah and Miled, 2013).

Now-a-days, fish processing industry, wet market and fish loading are looked upon as producers of worthless garbage by discarding a huge number of wastes which are parts of fish body. The fish waste is discarded without attempt for recovery. Without proper utilization, these wastes may cause environmental problems and for now, most of the fish waste is dumped as garbage or directly used as feedstuff. Fish waste consists of fish head that constitutes approximately 20% of the fresh water fish biomass, and are a rich source of protein and polyunsaturated lipids. This solid waste has approximately the same protein content as fish flesh. Although some amount of fish waste is being utilized today as feedstuff, a huge amount is still being discarded (Nurdiyana *et al.*, 2008). The discards from the processing plants amount to 20 million tonnes which is equivalent to 25% of the world's total production from marine capture fisheries (Ghaly *et al.*, 2013).

2.2 Fish

2.2.1 Types of fish

Fishes are aquatic vertebrates that are members of the largest and most diverse vertebrate taxon (25,000 species) that dates back over 500 million years. They have evolved into three major lineages: Agnatha (Hagfish and Lampreys), Chondrichthyes (sharks, skates, and rays usually referred to as Elasmobranchs), and Actinopterygii (bony fishes) (Evans *et al.*,2005).

1. Agnatha (Hagfish and Lampreys)

Fish of the class Agnatha are the most primitive of the fishes. They lack a jaw and a bony skeleton. The Hagfish and the Lamprey are the only living representatives of this class. As they lack true bones, these fish are very flexible. The Hagfish has no eyes, while the Lamprey has well-developed eyes

2. Chondrichthyes (Sharks, Skates, Rays)

Members of this class Chondrichthyes include the sharks, skates, rays, and ratfish. These fish have a cartilaginous skeleton, but their ancestors were bony animals. These were the first fish to exhibit paired fins. Chondrichthyes lack swim bladders, have spiral valve intestines, exhibit internal fertilization, and possess 5-7 gill arches.

3. Actinopterygii (bony fishes)

The bony fish comprise the largest section of the vertebrates, with over 20,000 species worldwide. They are called bony fish because their skeletons are calcified, making them much harder than the cartilage bones of the chondrichthyes.

Sphyraena jello

Sphyraena jello (Pickhandle Barracuda) are migratory pelagic predators that are distributed over continental shelves, estuaries and also in open oceans of tropical, subtropical and warm temperate seas. The *Sphyraena jello* is a long, slender schooling fish. This species is of

importance to fisheries in many countries and is considered a good fish due to its excellent flesh qualities. *S. jello* is a commercially important pelagic fish and is caught with different types of fishing gears such as of trawls, long line (Hosseini *et al*, 2009).

Plate – 1

***Sphyraena jello* fish**

Scientific classification

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Perciformes

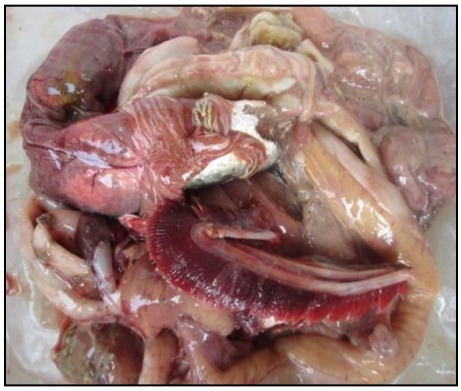
Family: Sphyraenidae

Genus: *Sphyraena*

Species: *S. jello*

2.2.2 Fish waste

Current global fish production stands at about 141 million metric tonnes. At present,



more than 76% of fish production is utilized globally for human consumption with the remaining going for miscellaneous purpose. Both from marine and freshwater fish processing, the wastes generated include scales, skins, visceral mass (viscera, air bladder, gonads and other organs), head and fins different types of waste generated, fish viscera alone contributes 15–25% of the total body weight (Jini *et al.,* 2011).

Plate -2

Fish waste of *sphyraena jello*

To avoid wasting these by-products, various disposal methods have been applied including, ensilation, fermentation, hydrolysate and fish oil production. Interestingly, fish

byproducts provide an excellent nutrient source for microbial growth useful in enzyme production process, which is largely governed by the cost related to the growth media. Fish wastes were prepared and tested as growth substrates for microbial enzyme production such as protease, lipase, chitinolytic and ligninolytic enzymes (Faouzi *et al.*, 2013).

2.3 Enzymes

More than 1700 enzymes have been reported in living cells, each with efficiency unique in nature (Nadeemullah and Mukhtar, 2013)

Enzymes are natural catalysts. They are produced by living organisms to increase the rate of an immense and diverse set of chemical reactions required for life (Li *et al.,*2012). In general biocatalysts are applied in the transformation of macro structures to new materials and energy, besides growth, maintenance and repair of cells (Eslahi *et al.*, 2014)

2.3.1 Classification of enzymes

The internal organs of the fish are a rich source of enzymes, many of which exhibit high catalytic activities at relatively low concentrations. The enzymes which are available in fish include pepsin, trysin, chymotrypsin and collagenase. These enzymes are commercially extracted from the fish viscera in a large scale (Ghaly *et al.*, 2013).

2.3.2 Applications of enzymes

Enzymes are used in many environmental-friendly industrial purposes, as they are efficient, selective, accelerate and speed up reactions by forming transition state complexes with their substrate which reduces the activation energy of the reaction. Enzymes are applied in detergents, for pulp and paper applications, in textiles manufacturing, leather industry, for fuel production and for the production of pharmaceuticals and chiral substances in the chemical industry (Binod *et al.,* 2013).

2.3.3 Protease

Proteases are proteolytic enzymes that catalyze the breakdown of proteins by hydrolysis of peptide bonds. Proteolytic enzymes are ubiquitous in occurrence, being
found in all living organisms and are essential for cell growth and differentiation. (Vadlamani and Parcha, 2011).

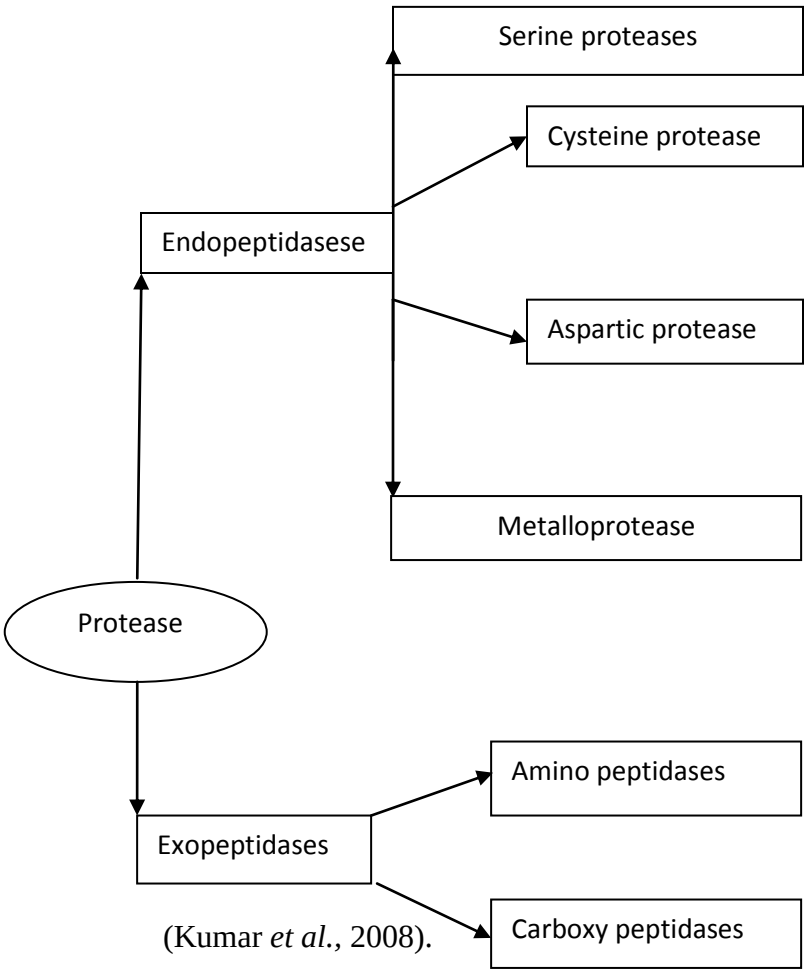
Fish waste can be used to produce various value-added by-products such as proteins, oil, omega-3 fatty acids, biodiesel, amino acids, peptides, collagen, gelatin and silage, each of which has various applications in the food industry, renewable energy and medicinal purposes. Fish protein contains amino acids and many bioactive peptides (Ramakrishnan and Vegneshwaran, 2013).

Proteases, a group of enzymes hydrolyzing proteins into peptides and amino acids, are indispensable tools of all life forms including, viruses, bacteria, fungi, plants and animals. They have been associated with diverse functionality such as pathogenecity in viruses, hydrolysis of reserved protein during germination of plant seeds, assimilation of proteins from environment, digestion of food in animals, removal of unwanted proteins from a developing structures, turning on of other proteins during a complicated physiological cascade, etc. among several (Khan, 2013).

Proteases are the class of enzymes, which holds an important place in both physiological and commercial fields. Proteases are one of the largest and most diverse families which are primarily known as enzymes of digestion (Prasad *et al.*, 2014).

2.3.3.1 Types of proteases

Proteases are broadly classified as endo or exoenzymes on the basis of their site of action on protein substrates. They are further categorized as serine protease, aspartic protease, cysteine protease or metallo protease depending on their catalytic mechanism (Padmapriya *et al.*, 2012).



(Kumar *et al.*, 2008).

The most important digestive proteolytic enzymes from fish and aquatic invertebrate viscera are the aspartic protease pepsin secreted from gastric mucosa, and the serine proteases, trypsin, and chymotrypsin secreted from the pancreas, pyloric caeca, and intestine, acidic proteases from fish stomach (Nasri *et al.*, 2011).

2.3.3.2 Applications of protease

Generation of immense quantity of underutilized marine processing by-products has long been recognized as wastes and use these materials in various applications (Kim and Mendis, 2006).

Proteases account for nearly 60% of the total industrial market and have wide applications in many industries viz., textiles, detergents, food processing especially for cheese

ripening, meat tenderizing, animal nutrition, pharmaceuticals, paper industry and food industry (Prasad *et al.*, 2014)

Proteases are present in different brands of detergents for use in home and commercial establishments and improving the stability and performance of detergent enzymes (Choudhary, 2012).

Proteases represent one of the major groups of industrial enzymes and a number of detergent stable proteases have been isolated and characterized because of their widespread use in detergents (Kumar *et al.*,2008). They are used in the soaking, dehairing and bating stages of preparing skins and hides. Alkaline protease play a crucial role in the bioprocessing of used x-ray or photographic films for silver recovery (Ray, 2012).

2.3.4 Immobilization of protease

In addition, purified enzymes are rather costly and discarding them after each use is not economical. Enzyme immobilization has been studied and utilized to overcome these problems. The enzyme immobilization is to entrap the enzymes in a semi-permeable support, which prevents the enzyme from leaving while allowing substrates and products to pass through. Immobilized enzymes typically exhibit greater stability and enzymatic activity over a broad range of pH and temperature. In addition, immobilization of enzymes also contributes to the development of continuous processes, and immobilized enzyme is adaptable to a variety of configurations and specific processes carried out in reactors. In immobilization of enzyme, the enzyme is attached to a carrier; the properties of the carrier (chemical and mechanical) influence the entrapment efficiency and catalytic activity of the enzyme as well, making it even more important to find a proper immobilization method (Shuang *et al.*,2013).

Enzyme immobilization can be defined as the attachment of free or soluble enzymes to different types of supports resulting in reduction or loss of mobility of the enzyme. In immobilization, enzymes retain biological activity. Enzymes should be attached onto surfaces without affecting their conformational and functional properties (Khan and Alzohairy, 2010).

The detergent industry also employs immobilized enzymes for removal of stains. The enzymes used in detergent industry are proteases which can remove stains of blood, egg, grass and human sweat (Nisha *et al.*,2012).

Immobilized enzymes are of great value in the food industry for processing of food samples and its analysis. Immobilized enzymes such as glucoamylase, protease, lactase have some attention recently for food processing in food industries (Sujoy and Aparna, 2013).

2.3.4.1 Types of immobilization

Methods for immobilization of enzymes include adsorption, cross-linking, covalent bonding, entrapment and encapsulation. The most common methods are adsorption and covalent bonding. Some biomaterials, for instances, silk, collagen and eggshell membrane have been employed as platforms for the immobilization of enzymes (Wu *et al.*,2004).

2.3.4.2 Entrapment of protease

Immobilized enzymes are widely used in different industries especially in food and pharmaceutical and offer several advantages over bulk or free enzymes. Advantages include high productivity, automation, continuous processing, precise control of the extent of reaction, easy product recovery and no contamination of the final product by enzyme. Entrapment of enzyme in sodium alginate is one of the important methods of immobilization (Anwar *et al.,* 2009). Immobilization by entrapment is fast, cheap and requiring mild conditions (Nisha *et al.,* 2012).

2.3.4.3 Cross linkage method

The immobilization method here employs covalent cross-linking of enzymes to inert supports. This can facilitate product separation and sometimes helps to improve the stability of biocatalysts (Chen *et al.*,2013).

In the light of the background information, the study was designed as given in the following chapter.

Methodology

3.0 METHODOLOGY

Pollution is the greatest upcoming problem in the present environment. There are varieties of waste which cause pollution, one among them being generated from fish processing industries, which cause increasing environmental and health problems. To avoid these hazards, fish waste could be utilized for any means.

Enzymes are protein molecules functioning as specialized catalysts for chemical reactions. They have contributed greatly to the traditional and modern chemical industries by improving existing processes. Enzymes are applied in various fields, including technical, food manufacturing, animal nutrition, cosmetics, medication and as tools for research and development (Li *et al.*, 2012).

Thus this project was aimed at using fish visceral waste to isolate the enzyme protease, which has industrial applications and further immobilizing it with sodium alginate and onion membrane and studying its purification profile.

The design of the experiment is as follows:

3.1 Collection of fish visceral organ waste

3.2 Isolation of protease

3.2.1 Preparation of crude enzyme extract

3.2.2 Precipitation with ammonium sulphate

3.3 Study of purification profile of ammonium sulphate precipitated protease

3.3.1 Protein estimation by Lowry's method

3.3.2 Assay of protease

3.3.3 Calculation of specific activity

3.3.4 Calculation of recovery percentage

3.3.5 Calculation of purification fold

3.4 Immobilization of protease

3.4.1 In sodium alginate

3.4.2 On onion membrane

3.5 Study of purification profile of immobilized protease

3.6 Determination of storage stability of immobilized protease

3.7 Determination of reusability of immobilized enzyme

3.1 Collection of fish visceral organ waste

The fresh visceral waste of the fish *Sphyraena jello (oooli)* was collected from a fish market in Coimbatore, maintained in ice and brought to the place of work. The waste was washed with tap water, rinsed with distilled water and was stored in a deep freezer for further use.

3.2 Isolation of protease

3.2.1 Preparation of crude enzyme extract

(Sadasivam and Manickam, 2008)

A crude extract of the enzyme was prepared with phosphate buffer as given in Appendix I. The supernatant thus obtained was used for precipitation of protease.

3.2.2 Precipitation with ammonium sulphate

The studies of Geethanjali and Subash (2013), have shown that 40-60% precipitated protease sample registered higher specific activity than the other precipitated protease samples of the same group. Hence 40-60% ammonium sulphate was used for the precipitation of protease.

The enzyme supernatants were saturated with solid 40-60% ammonium sulphate. The procedure for this is given in Appendix II

3.3 Study of purification profile of ammonium sulphate precipitated fish visceral protease

3.3.1 Protein estimation by Lowry's method

Protein content the 40-60% ammonium sulphate precipitated and crude enzyme samples were estimated by Lowry's method (Sadasivam and Manickam, 2008) as detailed in Appendix III.

3.3.2 Assay of protease

The enzyme activities of the 40-60% ammonium sulphate precipitated and crude enzyme samples were determined by following the protocol of Nigam and Ayyagari, 2007 as detailed in Appendix IV.

3.3.3 Calculation of specific activity

The specific activities of the 40-60% ammonium sulphate precipitated and crude enzyme samples were calculated by dividing the total protease activity with the protein content

$$\text{Specific activity} = \frac{\text{Total enzyme activity (units)}}{\text{Total protein content (mg)}}$$

3.3.4 Calculation of recovery percentage

Recovery percentage of the 40-60% ammonium precipitated sample and crude enzyme were calculated by dividing the total protease activity of the sample with the total protease activity of the crude extract

$$\text{Recovery percentage} = \frac{\text{Total activity of the sample}}{\text{Total activity of crude extract}}$$

3.3.5 Calculation of purification fold

Purification fold was calculated by dividing the specific activity of the

respective fraction of enzyme with that of the crude extract

$$\text{Purification fold} = \frac{\text{Specific activity of the sample}}{\text{Specific activity of crude extract}}$$

3.4 Immobilization of protease

Immobilized enzymes have been widely used in processing of a variety of products. New strategies are continuously emerging for the formation of diverse immobilized enzymes which have superior efficiency and usage. Immobilized enzymes have biomedical and industrial applications and for this reason this area continues to develop into an ever-expanding and multidisciplinary field during the last couple of decades (Khan and Alzohairy, 2010).

3.4.1 In Sodium alginate

The ammonium sulphate precipitated protease sample was immobilized in sodium alginate beads (Sadasivam and Manickam 2008).The details are given in the Appendix V.

3.4.2 On Onion membrane

The affixed onion membrane was immobilized with sample ,using 2.5% glutaraldehyde solution as cross linking agent as given in Appendix VI.

3.5 Study of purification profile of immobilized protease

The protein content of the immobilized enzyme was determined by Lowry's method as given in Appendix III, the protease assay as given in Appendix IV, the specific activity by way of calculation and the recovery percentage.

3.6 Storage stability of immobilized protease

The sodium alginate immobilized protease was checked for its activity for period of 10 days by following the protocol given in Appendix III.

3.7 Reusability of immobilized protease

The initial activity of the immobilized enzyme protease was tested by subjecting it to four cycles of use and after each cycle monitoring the amount of tyrosine liberated from the enzyme as given in Appendix III.

Result and discussion

4.0 RESULTS AND DISCUSSION

India is bestowed with vast livestock wealth and it is growing at the rate of 6% per annum. The contribution of livestock industry including poultry and fish is increasing. Efficient utilization of by-products has a direct impact on the economy and environmental pollution of the country. Non-utilization or under utilization of by-products not only lead to loss of potential revenues but also to increasing cost of disposal of these products. Non-utilization of animal by-products in a proper way may create major aesthetic and catastrophic health problems. Besides pollution and hazard aspects, in many cases meat, poultry and fish processing wastes have a potential for recycling raw materials or for conversion into useful products of higher value (Jayathilakan *et al.,* 2012). Therefore, the objective of the present work entitled **"Study of Purification Profile of Soluble and Immobilized Protease from Visceral Organ Waste of *Sphyraena jello* Fish"** was to isolate, purify and immobilize protease enzyme from visceral waste of the fish *Sphyraena jello* and analyze the purification profile of the same.

Waste of *Sphyraena jello* fish were collected, cleaned, homogenized, precipitated with 40-60% ammonium sulphate and then immobilized in sodium alginate and in onion membrane. The purification profile of the isolated and purified enzyme, namely, protease activity, protein content, specific activity, recovery percentage and purification fold were studied and the results discussed under the following headings

4.1 Purification profile of crude, 40-60% ammonium sulphate precipitated, sodium alginate immobilized and onion membrane immobilized protease from visceral waste of *Sphyraena jello* fish

4.1.1 Total protease activity

4.1.2 Total protein content

4.1.3 Specific activity

4.1.4 Recovery percentage

4.1.5 Purifiction fold

4.2 Storage stability of Immobilized Protease

4.2.1 Sodium alginate immobilized protease

4.2.2 Onion membrane immobilized protease

4.3 Reusability of Immobilized Protease

4.3.1 Sodium alginate immobilized protease

4.3.2 Onion membrane Immobilized protease

4.1 Purification profile of crude, 40-60% ammonium sulphate precipitated, sodium alginate immobilized and onion membrane immobilized protease from visceral waste of *Sphyraena jello* fish

4.1.1 Total protease activity

Table 1 and Figure 1 represent the total protease activity of crude, 60% ammonium sulphate precipitated, sodium alginate immobilized and onion membrane immobilized protease from visceral waste of the fish *Sphyraena jello.*

Table -1

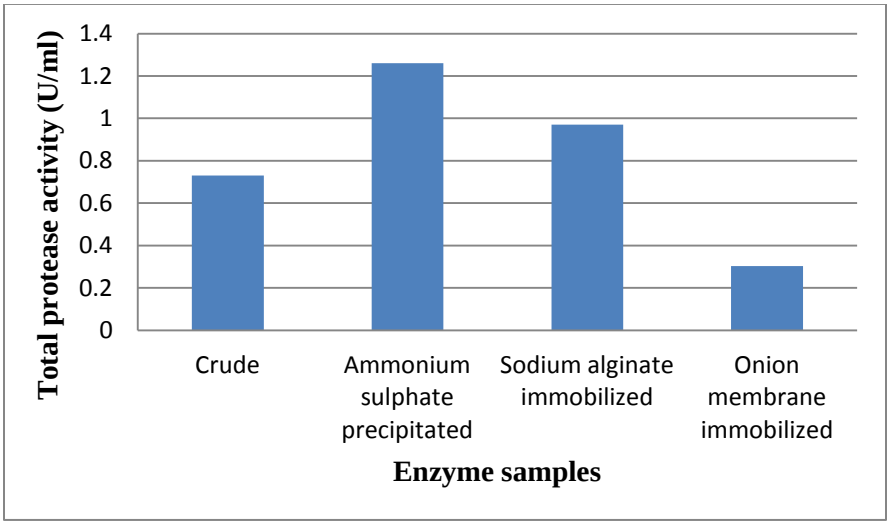
Activity of different protease samples from visceral waste of *Sphyraena jello* fish

S.No	Enzyme samples	Total protease activity U/ml
1	Crude	0.730 ± 0.028
2	40-60% ammonium sulphate precipitated	1.260 ± 0.000
3	Sodium alginate immobilized	0.970 ± 0.014
4	Onion membrane immobilized	0.302 ± 0.017

It can be observed from Table 1 and Figure 1 that the 40-60% ammonium sulphate precipitated protease sample exhibited the highest value (1.260 ± 0.00 U/ml) for protease activity followed by the sodium alginate immobilized sample (0.970 ± 0.014 U/ml) when compared to the crude and the onion membrane immobilized sample. Sodium alginate immobilized sample recorded a higher protease activity (0.970±0.014 U/ml) than the onion membrane immobilization (0.302 ± 0.017 U/ml) sample.

Figure-1

Activity of different protease samples from visceral waste of *Sphyraena jello* fish



A similar finding that ammonium sulphate precipitated sample showed protease activity higher than crude sample has been reported by Linnaeus, (2011) who stated that the crude sample of protease from the viscera of skipjack tuna fish showed lesser activity than the precipitated sample.

This finding is also in agreement with the results of El-Safey and Raouf, (2004) who reported that in *Bacillus subtilis,* ammonium precipitated protease activity was more than the activity in the crude sample.

4.1.2 Total protein content

The protein content of the crude, 40-60% ammonium sulphate precipitated, sodium alginate immobilized and onion membrane immobilized protease are shown in Table 2 and Figure 2.

Table -2

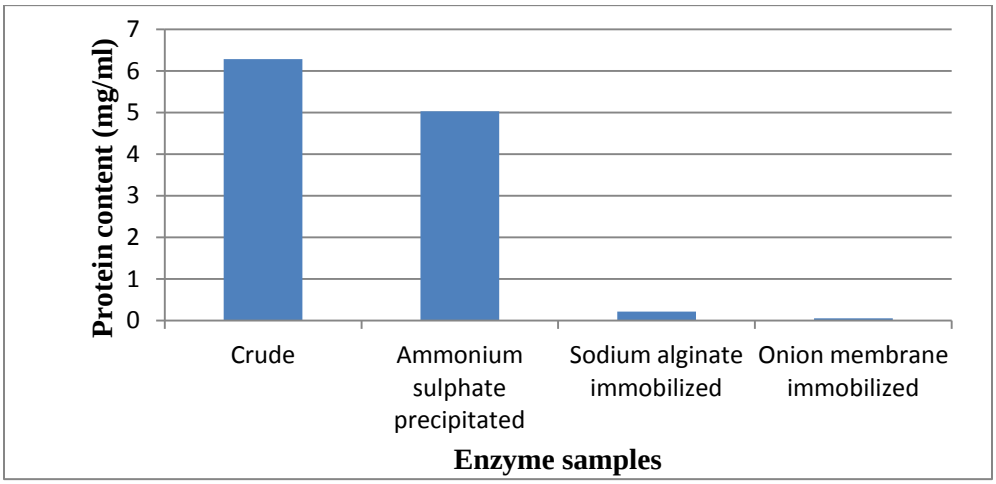
Protein content of different protease samples from visceral waste of *Sphyraena jello* fish

S.No	Enzyme samples	Total protein content mg/ml
1	Crude	6.284±0.040
2	Precipitated 40-60% ammonium sulphate	5.028 ±0.016
3	Sodium alginate immobilized	0.209 ±0.003
4	Onion membrane immobilized	0.054 ±0.001

It can be seen from the table and figure that precipitation with 40–60% ammonium sulphate and immobilization did not increase the protein content of the sample. Hence, the crude sample showed the highest value (6.284±0.040 mg/ml) for protein content followed by the ammonium sulphate precipitated sample (5.028 ±0.016 mg/ml) when compared to the sodium alginate and the onion membrane immobilized samples. Here again sodium alginate was found to be better than onion membrane for immobilization, since it recorded a higher protein content (0.209 ±0.003 mg/ml) than the onion membrane (0.054 ±0.001 mg/ml).

Figure -2

Protein content of different protease samples from visceral waste of *Sphyraena jello* fish



The present finding that the protein content of the ammonium sulphate precipitated protease sample was less than that of the crude sample agrees with the results of Shobana and Subash, (2013) who stated that purified protease sample from viscera of fish *Rachycentron canadum* had a protein content less than the crude enzyme.

4.1.3 Specific activity

The specific activity of the different samples of crude, 40-60% ammonium sulphate precipitated, sodium alginate immobilized and onion membrane immobilized protease is given in Table 3 and Figure 3.

Table – 3

Specific activity of different protease samples from visceral waste of *Sphyraena jello* fish

S.No	Enzyme samples	Specific activity U/mg
1	Crude	0.116
2	40-60% ammonium sulphate precipitated	0.250
3	Sodium alginate immobilized	4.626
4	Onion membrane immobilized	5.570

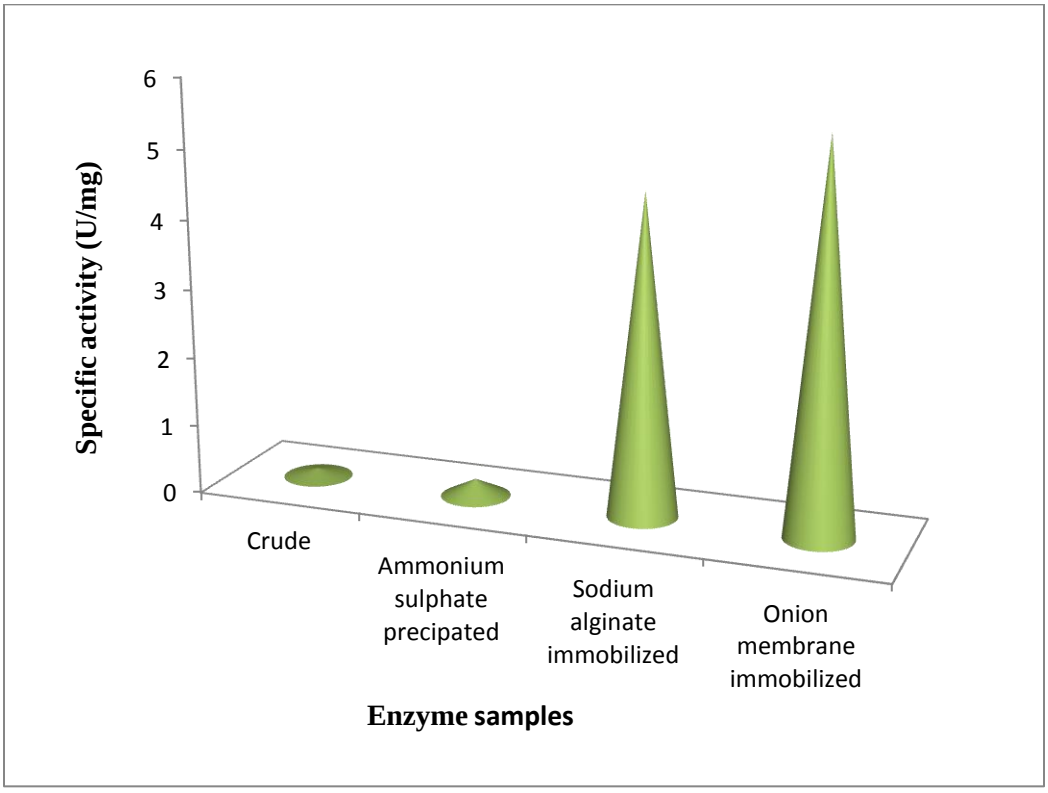
The specific activity of the onion membrane immobilized sample, as shown in Table 3 and Figure 3 marked the highest value (5.570 U/mg) followed by sodium alginate immobilized protease (4.626 U/ml) when compared to the 40-60% ammonium sulphate precipitated sample (0.250 U/mg) and crude enzyme (0.116 U/ml). The sodium alginate sample marked a lesser activity than the onion membrane immobilized sample.

The above finding that the specific activity of the ammonium precipitated sample (0.250 U/mg) is more than that of the crude sample (0.116 U/ml) on parr with the findings of

Nadeemullah and Mukhtar, (2013) who stated that specific activity of the ammonium sulphate precipitated protease sample was higher than the specific activity of the *Bacillus subtilis* of the protease sample.

Figure - 3

Specific activity of different protease samples from visceral waste of *Sphyraena jello* fish



4.1.4 Recovery percentage

Table 4 and Figure 4 depict the Recovery Percentage of crude 40-60% ammonium precipitated sample, sodium alginate immobilized and onion membrane immobilized protease samples.

Table – 4

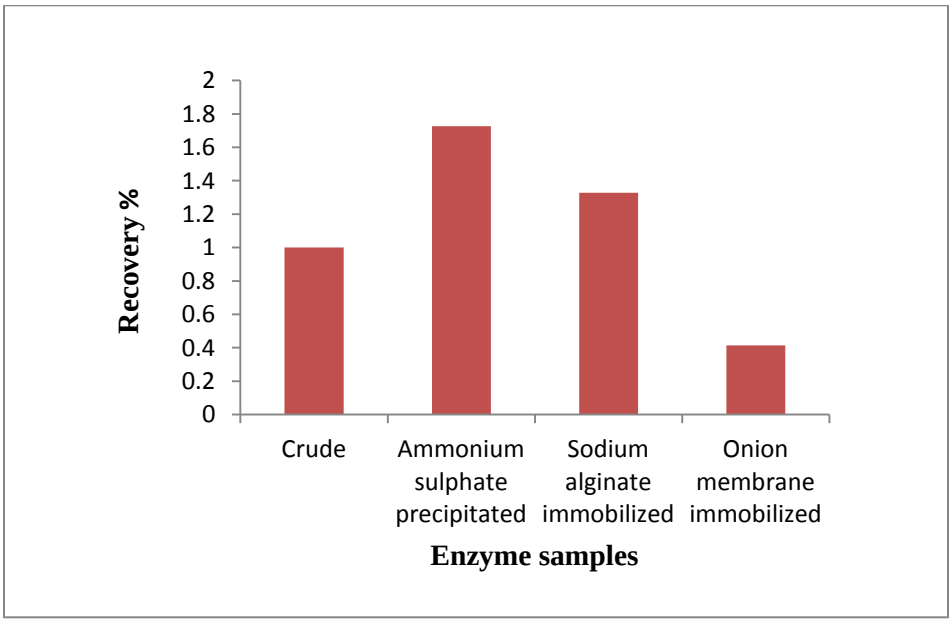
Recovery percentage of different protease samples from visceral waste of *Sphyraena jello* fish

S.No	Enzyme samples	Recovery percentage
1	Crude	1.000
2	40 - 60% ammonium sulphate Precipitated	1.726
3	Sodium alginate Immobilized	1.328
4	Onion membrane Immobilized	0.414

It is understood from table and figure that the recovery percentage of 40-60% ammonium sulphate precipitated sample exhibited the highest value (1.726 %) compared to the other samples of immobilized protease and the crude enzyme (1.000%). Since the recovery percentage was more for the sodium alginate immobilized sample (1.328%) than the onion membrane immobilized sample (0.414%), it can be deduced that sodium alginate is better than onion membrane for immobilization of protease. Thus precipitation with ammonium sulphate and immobilization with sodium alginate increased the recovery percentage of protease.

Figure – 4

Recovery percentage of different protease samples from visceral waste of *Sphyraena jello* fish



4.1.5 Purifiction fold

The purification fold of the crude, ammonium sulphate precipitated, sodium alginate immobilized and onion membrane immobilized protease samples are recorded in Table 5 and Figure 5.

Table – 5

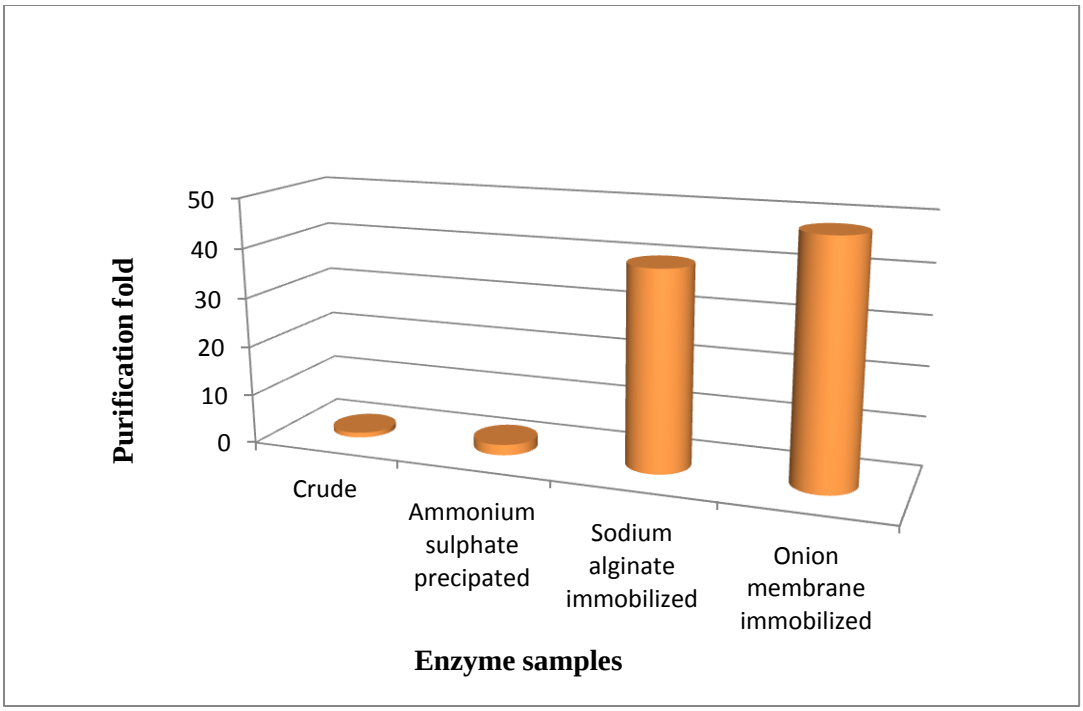
Purification fold of different protease samples from visceral waste of *Sphyraena jello* fish

S.No	Enzyme samples	Purification fold
1	Crude	1.000
2	40-60% ammonium sulphate Precipitated	2.155
3	Sodium alginate immobilized	39.879
4	Onion membrane immobilized	48.017

It can be seen from the table and figure that onion membrane immobilized protease sample registered the highest value (48.017) for purification fold when compared to the other samples. This was followed by sodium alginate immobilized sample (39.879). The increase in the purification fold for onion membrane and sodium alginate immobilized sample were much higher than the ammonium sulphate precipitated (2.155) and crude enzyme samples (1.000).

Figure -5

Purification fold of different protease samples from visceral waste of *Sphyraena jello* fish



The finding that the purification fold of the enzyme sample increased on ammonium sulphate precipitation sample as compared to the crude sample is supported by the report of El-Safey and Raouf, (2004) who stated that crude amylase enzyme sample from *Aspergillus falvus var.* showed purification fold less than the ammonium precipitated sample.

4.2 Storage Stability of Immobilized Protease

Storage stability is a prominent factor for commercialization of an enzyme. Immobilization is a form of storage and it is advisable to immobilize enzymes because free enzymes can lose their activities quickly. In general, if an enzyme is in aqueous solution, it is not stable during storage and the activity gradually reduces.

4.2.1 Sodium alginate Immobilized Protease

This is explained in terms of the residual activity of crude, ammonium sulphate precipitated, sodium alginate immobilized and onion membrane immobilized samples for 10 days as shown in Table 6 and Figure 6.

Table- 6

Storage stability of Sodium Alginate Immobilized Protease

Days	Residual activity %
1	39.280
2	35.714
3	27.380
4	25.000
5	22.222
6	19.840
7	18.650
8	16.269
9	12.698
10	7.939

It can be observed from the table and figure that the storage stability of sodium alginate immobilized protease gradually decreased from day 1 (39.280%) to day 10 (7.939%).

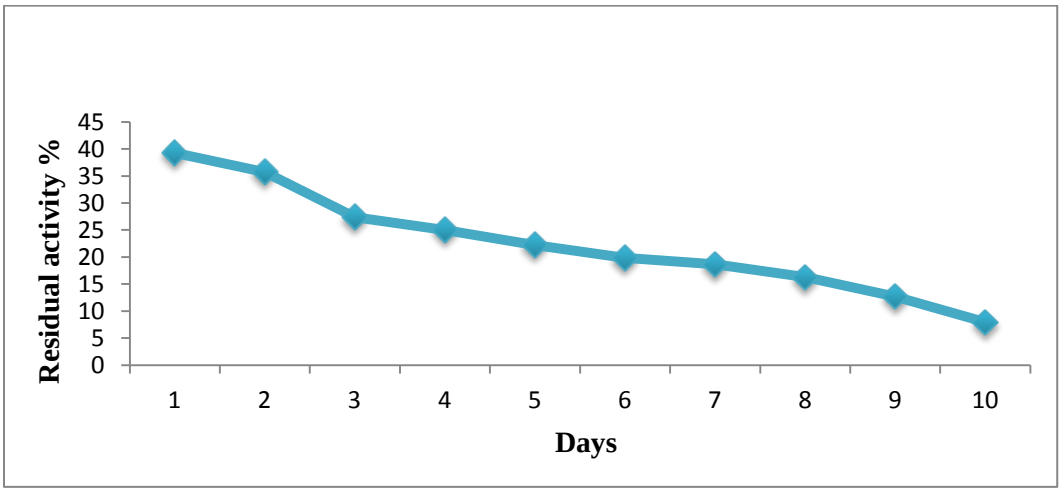
These results are similair to those of Kharkrang and Ambasht, (2013) who stated that immobilized α amylase of *pennisetum typhoides* activity is retained even after 90 days. Yin *et al.,(*2013) also stated that immobilized cellulase on modified mesoporous silica storage stability showed a gradual decrease in activity only after 4 weeks.

Plate-3

Protease immobilized with sodium alginate

Figure - 6

Storage stability of Sodium alginate Immobilized Protease



4.2.2 Onion membrane Immobilized Protease

Table 7 and Figure 7 highlight the 5 days storage stability of onion membrane immobilized protease sample.

Table – 7

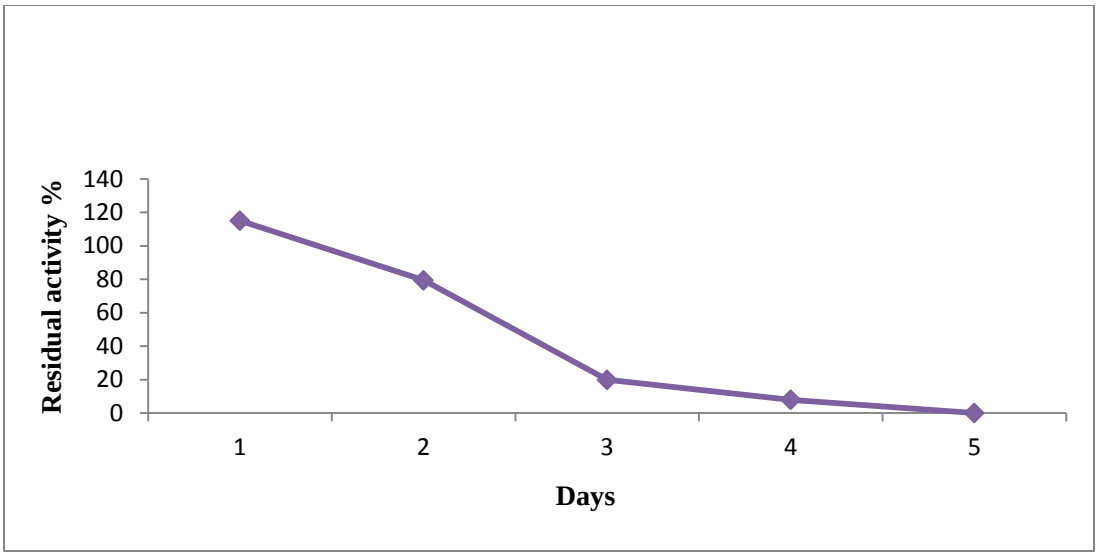
Storage Stability Onion membrane Immobilized Protease

Days	Residual activity %
1	115.07
2	79.36
3	19.84
4	7.93
5	0.00

The protease activity of the onion membrane immobilized sample was the highest (115.07%) on day 1 and this decreased gradually with 0% activity on the 5^{th} day.

Figure – 7

Storage stability Onion membrane Immobilized Protease



The above result coincides with the report of Jobanputra *et al.,* (2011) who inferred that immobilized rifamycin oxidase from *Chryseobacterium* species showed loss of activity after 8 days of storage.

Thus on comparison of Tables 6 & 7, it can be inferred that sodium alginate immobilization of protease than onion membrane, retained the enzyme activity upto the 10^{th} day unlike the onion membrane immobilized sample where the activity was retained only upto the 4^{th} day

Plate-4

Immobilization of Onion membrane

Before immobilization **After immobilization**

4.3 Reusability of Immobilized Protease

The reuse of the immobilized enzyme is very important from the point of view of reducing the cost of the enzyme.

4.3.1 Sodium Alginate immobilized protease

The reusability of sodium alginate immobilized protease is depicted in Table 8 and Figure 8.

Table – 8

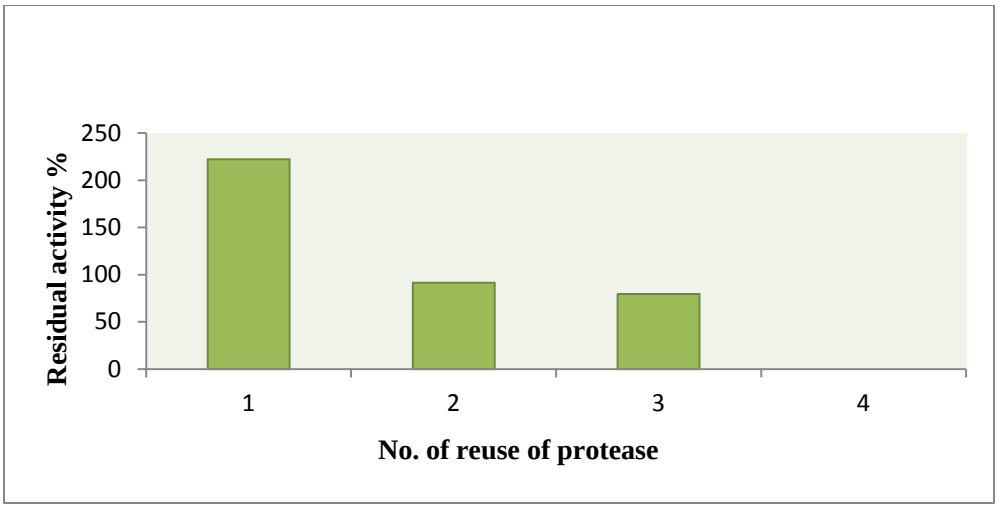
Reusability of Sodium alginate Immobilized protease

Cycle	Protease activity %
1	222.22
2	91.26
3	79.36
4	0

It can be observed from the table that the activity of the sodium alginate immobilized protease was at the maximum during the 1st cycle of enzyme use (222.22%). This value decreased to 91.26% during 2nd cycle, 79.36% during 3rd cycle and 0% during the 4th cycle. Hence this form of enzyme can be used 3 times.

Figure– 8

Reusability of Sodium alginate Immobilized protease



These findings are in agreement with the results of Pithawala *et al.*(2010) who observed that the enzyme jack bean meal urease entrapped in alginate could be reused upto five times. Anwar *et al*. (2009) also stated that entrapped protease in calcium alginate beads could be reused upto three times and that, the decrease in activity occurred on further reuse which was due to the leakage of enzyme from the beads, during washing at the end of each cycle.

4.3.2 Onion membrane Immobilized protease

Table 9 and Figure 9 show the reusability of onion membrane immobilized protease

Table – 9

Reusability of Onion membrane Immobilized protease

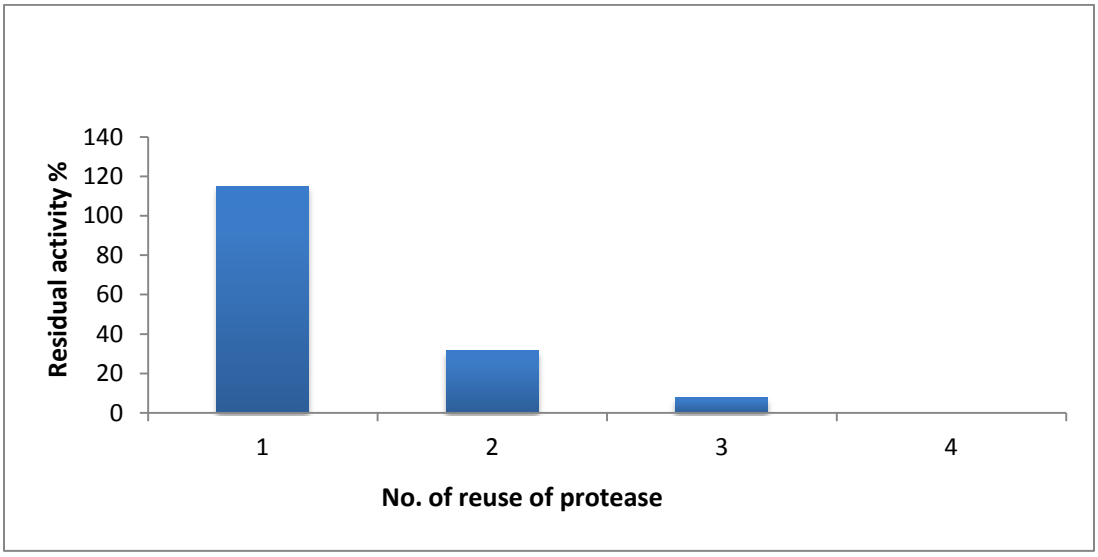
Cycle	Protease activity %
1	115.07
2	31.74
3	7.93
4	0

It is understood from the Table 9 and Figure 9 that the reusability of onion membrane immobilized protease decreased from 115.07% for the 1st cycle, followed by 31.74% for the 2nd time and 7.93% for the third reuse and on 4th reuse there was complete loss in activity.

Similar findings by Chen *et al.,* (2013) stated that immobilization of *Bacillus licheniformis* γ-glutamyl transpeptidase enzyme retained initial activity even after 10 cycles of usage.

Figure – 9

Reusability of Onion membrane Immobilized protease



Highlights of the study

- Protease activity was found to be higher in 40-60% ammonium sulphate precipitated sample.
- Storage stability of sodium alginate immobilization protease was better than onion membrane immobilized protease.
- Sodium alginate immobilized protease had better reusability than onion membrane immobilized protease because it had higher protease activity.

The findings of the present study are summarized and concluded in the next chapter.

Summary and Conclusion

5.0 SUMMARY AND CONCLUSION

Fish industries create large amount of fish wastes increasing the environmental pollution. Fish waste utilization is an alternative method for controlling pollution. Fish waste are rich in oil, fatty acids, proteins, enzymes and amino acids. These products are useful in various applications in industries like leather, textiles and pharmaceuticals. Fish wastes consists of heads, tails, bones, scales and visceral organs. The visceral organs have digestive enzymes like protease, trypsin, collagen, pepsin.

The fish *Sphyraena jello's* visceral organ waste was collected and a homogenate prepared. This was precipitated with 40-60% ammonium sulphate. Ammonium sulphate precipitated protease sample was then immobilized in sodium alginate and onion membrane.

Purification profile was determined in the samples like crude, 40-60% ammonium precipitated, sodium alginate immobilized and onion membrane immobilized protease. From the purification profile, of was clear that the protease activity showed higher value in 40-60% ammonium precipitated sample followed by onion membrane immobilized protease when compared with crude and sodium alginate immobilized samples, which represented less activity.

The crude enzyme sample recorded the a highest protein content than the precipitated, sodium alginate immobilized and onion membrane immobilized protease. Immobilization of onion membrane protease samples registered the highest specific activity when compared to crude, 40-60% ammonium sulphate precipitated and sodium alginate immobilized protease samples.

Recovery percentage was found to be high in ammonium sulphate precipitated sample than the crude, sodium alginate immobilized and onion membrane immobilized protease. Onion membrane immobilized protease sample showed an increased purification fold when compared to the other samples.

Storage stability of sodium alginate and onion membrane immobilized protease gradually decreased. Sodium alginate beads showed higher activity (79.36%) and protease immobilized on onion membrane had only 7.93% in the activity third cycle of reuse.

Thus from the present study it can be concluded that pollution caused by fish waste can be managed effectively for isolating industrially important enzymes like protease.

Suggestions for future study

- Various types of enzymes can be isolated from fish waste
- Enzyme can also be isolated from head and tail wastes of *Sphyraena jello* fish
- Other immobilization methods can also be carried out
- Various types of industrial applications of enzymes can be studied.

Bibliography

BIBLIOGRAPHY

- Anwar, A., Qader, S.A.U., Raiz, A., Iqbal, S. and Azhar, A. (2009). Calcium Alginate: A Support Material for Immobilization of Proteases from Newly Isolated Strain of *Bacillus subtilis* KIBGE-HAS, *World Appl. Sci. J.,* 7: 1281-1286 .
- Bajaj, A. and Kumar, V. (2013). Solid Waste Management; Clean India by Reduce Solid Waste, *IJGHC*, 2(3): 610-617.
- Bansal, J. and Bansal, P. (2012). Changed Human Values in India & Pollution: Analysis of Some Contemporary Issues, *Global Journals Inc.,* 12 (9): 1-6.
- Binod, P., Palkhiwala, P., Gaikaiwari,R., Nampoothiri, Duggal, A., Dey, K. and Pandey, A.(2013). Industrial Enzymes-Present Status And Future Perspectives For India, *Journal of Scientific and industrial research,*72: 271-286.
- Brena, B.M, and Batista-Viera, F. (2006). Methods in Biotechnology, Immobilization of Enzymes and Cells, Second Edition. Guisan, J.M.(Ed.), Humana Press Inc., Totowa, NJ.
- Chen,Y., Tsai. M., Chi, M., Wang, T. and Lin, L. (2013). Covalent Immobilization of Bacillus licheniformis γ-Glutamyl Transpeptidase on Aldehyde-Functionalized Magnetic Nanoparticles, *Int. J. Mol. Sci., 14*: 4613-4628.
- Choudhary, V. (2012). Compatibility with commercial detergents and stain removal capability of Aspergillus versicolor protease, *J. Acad. Indus. Res*., 1(6): 301-305.
- Daboor, S.M., Budge, S.M., Ghaly, A.E., Brooks, M.S. and Dave, D. (2010). Extraction and Purification of Collagenase Enzymes: A Critical Review, *American Journal of Biochemistry and Biotechnology,* 6 (4): 239-263.
- Daboor, S.M., Budge, S.M., Ghaly, A.E., Brooks, M.S. and Dave, D. (2012). Isolation and activation of collagenase from fish processing waste, *Advances in Bioscience and Biotechnology*, 3(4): 349-353.
- D'Souza, S.F., Kumar, J. Jha, S.K., Kubal, B.S. (2012). Immobilization of the urease on eggshell membrane and its application in biosensor, *Materials Science and Engineering*, 33: 850–854.
- El-Safey, E.M. and Ammar, M.S. (2004). Purification and Characteraization of -α-amylase isolated from *aspergillus falvus var. Columnaris, Ass. Univ. Bull. Environ. Res.*, 7(1): 93-100.

- Eslahi, H., Moghaddam, M.G., Khajeh, M., Omay, D., Zakipourrahimabadi, E. and Motalleb, G. (2014). General Biography, Structure And Classification Of Enzymes, *Research and Reviews in Materials Science and Chemistry*, 3(1): 1-83.
- Evans, D., Piermarini, P.M. and Choe, K.P. (2005). The Multifunctional Fish Gill: Dominant Site of Gas Exchange, Osmoregulation, Acid-Base Regulation, and Excretion of Nitrogenous Waste, *Physiol Rev.,* 85: 97–177.
- Faouzi, B.R., Nabil, M. (2013). Fish processing wastes for microbial enzyme production:a review, *Biotech.3*,255–265.
- Fuchise, T., Sekizaki, H., Kishimura, H., Klomklao, S., Nalinanon, S., Benjakul, S. and Chun, B. (2011). Simple Preparation of Pacific Cod Trypsin for Enzymatic Peptide Synthesis.2011: 1-8
- Gaur, R., Pant, H., Jain, R. and Khare, S.K.(2006).Galacto-oligo saccharidsynthesis by immobilized *Aspergillus oryzae* β-galactosidase, *Food Chemistry*, 97: 426-430.
- Geethanjali.S and Subash, A. (2013). Optimization and Immobilization of Purified Labeo rohita Visceral Protease by Entrapment Method, Hindawi Publishing Corporation Enzyme Research, 2013: 1-7.
- Ghaly, A.E., Ramakrishnan, V.V., Brooks, M.S., Budge, S.M. and Dave, D. (2013). Fish Processing Wastes as a Potential Source of Proteins, Amino Acids and Oils: A Critical Review, *J. Microb Biochem Technol* ,5: 107-129.
- Hosseini, A., Kochanian, P., Marammazi, J., Yavari, V., Savari, A. and Salari-Aliabadi, M.A. (2009). Length-Weight Relationship and Spawning Season of *Sphyraena jello* C., from Persian Gulf. *Pakistan Journal of Biological Sciences, 12: 296-300.*
- http://www.marinebiology.org/fish.htm
- Jayathilakan, K., Sultana, K., Radhakrishna, K. and Bawa, A. S. (2012). Utilization of byproducts and waste materials from meat poultry and fish processing industries: a review, *J Food Sci Technol*., 49(3): 278–293.
- Jini, R., Swapna, H.C. Rai, A.K., Vrinda, R., Halami, P.M., Sachindra, N.M. and Bhaskar, N. (2011). Isolation and characterization of potential lactic acid bacteria (LAB) from freshwater fish processing wastes for application in fermentative utilization of fish processing waste, *Braz J Microbiol.,* 42(4): 1516–1525.

- Jobanputra, A.H., Karode, B.A. and Chincholkar S.B. (2011). Calcium alginate as supporting material for the immobilization of rifamycin oxidase from *Chryseobacterium* species, Research Article, *Biotechnol. Bioinf. Bioeng*., 1(4): 529-535.
- Khan, A.A. and Alozhairy ,M.A. (2010). Recent Advances And Applications Of Immobilized Enzyme Technologies:Review,*Res.J.Biol.Sci.*,5:565-575
- Khan, F. (2013). New microbial proteases in leather and detergent industries *Innov. Res. Chem*. 1(1): 1-6.
- Khan, M. A. and Ghouri, A.M. (2011). Environmental pollution: its effects on life and its remedies, *Journal of Arts, Science and Commerce,* II (2): 276-285.
- Kharkrang, K. and Ambasht, P.K. (2013), Immobilization Of A-Amylase From Pennisetum Typhoides Inside Gelatin Beads And Its Characterization, *Journal Of Proteins And Proteomics*, 4(3): 183-189.
- Kim, S. and Mendis, E. (2006). bioactive compounds from marine processing byproducts A review, *Food Research International*, 39: 383-393.
- Kumar, D., Savitri, N., Thakur, R., Verma. and. Bhalla, T.C. (2008). Microbial Proteases and Application as Laundry Detergent Additive, *Research Journal of Microbiology, 3: 661-672.*
- Li, S.,Yang, X., Yang, S., Zhu, M.,Wang, X.(2012). Technology Prospecting on Enzymes: Application, Marketing and Engineering, *Computational and Structural Biotechnology Journal*,2(3): 1-34.
- Linnaeus, K.K.P. (2011). Extraction And Characterization Of Protease From The Viscera Of Skipjack Tuna Fish.
- Malmiri, H.J., Jahanian, M.A.J. and Berenjian, A.(2012). Potential Applications Of Chitosan Nanoparticles As Novel Support In Enzyme Immobilization , *American Journal of Biochemistry and Biotechnology*, 8 :203-219.
- Meyer, N.L. (2007). Effect of Immobilization Method on Activity of Alpha-Amylase.
- Morana, A., Mangione, A., Maurelli, L., Fiume, I., Paris, O., Cannio, R. and Rossi, M. (2006). Immobilization and characterization of a thermostable β-xylosidase to generate a reusable biocatalyst, *Enzyme and Microbial Technology*, 39: 1205–1213.
- Nadeemullah and Mukhtar, H. (2013). Partial purification of alkaline protease by mutant strain of *Bacillus subtilis*, *BIOLOGIA (PAKISTAN)*, 59 (1): 165-171.

- Nagarajan, R., Thirumalaisamy, S. and Lakshumanan, E. (2012). Impact of leachate on groundwater pollution due to non-engineered municipal solid waste landfill sites of erode city, Tamil Nadu, India, *Iranian Journal of Environmental Health Sciences & Engineering*, 9(35): 1-12.
- Nasri, R., Younes, I., Lassoued, I., Ghorbel, S., Ghorbel-Bellaaj, O.and Nasri, M. (2011). Digestive Alkaline Proteases from *Zosterisessor ophiocephalus*, Raja clavata, and Scorpaena scrofa: Characteristics and Application in Chitin Extraction, *J Amino acids*, 1-34.
- Nigam, A., Ayyagarai, A. (2007). Lab manual in Biochemistry, Immunology and Biotechnology, First edition, Tata Mcgraw hill publishing company,113-117.
- Nisha, S., Karthick, S.A.and Gobi, N. (2012). A Review on Methods, Application and Properties of Immobilized Enzyme, *Che Sci Rev Lett.*, 1(3): 148-155.
- Nurdiyana, H., Mazlina, M.K.S. and Fadhilah, M.S.N. (2008). Optimization Of Protein Extraction From Freeze Dried Fish Waste Using Response Surface Methodology (RSM), *International Journal of Engineering and Technology*, 5(1): 48-56
- Padmapriya,B., Rajeswari, T., Nandita, R. and Raj, F. (2012). Production and Purification of Alkaline Serine Protease from Marine *Bacillus* Species and Its Application in Detergent Industry, *European Journal of Applied Sciences*, 4 (1): 21-26.
- Pithawala, K., Mishra, N. and Bahadur1, A.(2010). Immobilization of urease in alginate, paraffin and lac, *J. Serb. Chem. Soc. 75* (2): 175–183.
- Prasad, M.P., Sethi, R. and Arthirajan J. (2014). Optimization studies of Protease enzyme in in-vitro conditions from *Bacillus Licheniformis, , Int. Res. J. Biological Sci.*, Vol. 3(2): 34-39.
- Raj, A., Khess, N., Pujari, N., Bhattacharya, S., Das, A. and Rajan, S.S. (2012). Enhancement of protease production by Pseudomonas aeruginosa isolated from dairy effluent sludge and determination of its fibrinolytic potential, *Asian Pacific Journal of Tropical Biomedicine*, pp1845-1851.
- Ramakrishnan, V. and Vegneshwaran (2013). Enzymatic Extraction of Proteins and Amino Acids From Whole Fish and Fish Waste.
- Ray, A. (2012). protease enzyme- potential industrial scope, *Int.J.Tech.*, 2(1): 01-04.

- Rebah, F.B. and Miled, N. (2013). Fish processing wastes for microbial enzyme production: a review, *3 Biotech*, 3: 255–265.
- Sadasivam,S. and Manickam, A. (2008). Biochemical methods, Third edition, New age international publishers, 54-60.
- Salamone, M., Cuttittab, A., Seiditac, J., Mazzolad, S., Bertuzzie, F., Ricordi, C. and Ghersi, G. (2012). Characterization of Collagenolytic/Proteolytic Marine Enzymes, *CEt*, 27: 1-6.
- Sanjay, G. and Sugunan, S. (2005). Invertase immobilised on montmorillonite: reusability enhancement and reduction in leaching, *Catalytic Communications*, 6: 81–86.
- Sharpa, M. and Mariojoulsb, C. (2012). Better utilisation of fish waste in the Pacific, *SPC* Fisheries Newsletter.
- Sheriff, S.A., Balasubramanian, S., Baranitharan, R. and Ponmurugan, P. (2014). Synthesis and in vitro antioxidant functions of protein hydrolysate from backbones of Rastrelliger, kanagurta by proteolytic enzymes, *Saudi Journal of Biological Sciences*, 21: 19–26
- Shobana, A. and Subash, A. (2013). Partial Characterization of Protease from the Visceral Organ Waste of Cobia (*Rachycentron canadum*), *Journal of Biology, Agriculture and Healthcare*, 3(14): 5-9.
- Shuang, Z., Wenting, S., Xiaoxi, Y., Shujuan, Z., Xiaogang, Z. and Jiawei, C. (2013). Immobilization of Lipase using Alginate Hydrogel Beads and Enzymatic Evaluation in Hydrolysis of p-Nitrophenol Butyrate, *Bull. Korean Chem. Soc.*, 34.
- Sujoy, B. and Aparna, A. (2013). Enzymology, Immobilization and Applications of Urease Enzyme, *Int. Res. J. Biological Sci.*, 2(6): 51-56.
- Sumantha, A. Larroche, C. and Pandey, A. (2006). Microbiology and Industrial Biotechnology of Food-Grade Proteases: A Perspective, *Food Technol. Biotechnol., 44* (2): 211–220.

.

- Vadlamani, S. and Parcha, S.N. (2011). studies on industrially important alkaline protease production From locally isolated superior microbial strain from soil microorganisms, *IJBA*, 3(1): 102-105.

- Vikas, Kumar, R. and Pundir, C.S. (2007). Covalent immobilization of lipase onto onion membrane affixed on plastic surface: Kinetic properties and application in milk fat hydrolysis, *Indian Journal of Biotechnology*, 6: 479-484.
- Wu, B., Zhang, G., Shuang, S. and. Choi, M.M.F. (2004). Biosensors for determination of glucose with glucose oxidase immobilized on an eggshell membrane, *Talanta* , 64: 546–553.
- Yin, H., Su, Z., Shao2, H., Cai, J., Wang, X. and Yin, H. (2013). Immobilization of cellulase on modified mesoporous silica shows improved thermal stability and reusability, *African Journal of Microbiology Research*, 7(25): 3248-3253.
- Zhang, D., Yuwen, L. and Peng, L. (2013). Parameters Affecting the Performance of Immobilized Enzyme, *J. Chem* , 2013: 1-7.
- Zhao, I., Budge, S.M., Ghaly, A.E., Brooks, M.S. and Dave, D. (2011). Extraction, Purification and Characterization of Fish Pepsin: A Critical Review, *J Food Process Technol.*,2:6.
- Zhou, L., Budge, S.M., Ghaly, A.E., Brooks, M.S. and Dave, D. (2011). Extraction, Purification and Characterization of Fish Chymotrypsin: A Review, *American Journal of Biochemistry and Biotechnology* , 7 (3): 104-123.

Appendices

APPENDIX I

Preparation of Crude enzyme

(Sadasivam and Manickam, 2008)

Weigh the required amount of fish waste and cut into small pieces. Homogenize the pieces with 25 mM Phosphate buffer (pH 7.2) using a homogenizer. Centrifuge the homogenate at 15,000 rpm for 15 minutes at 4˚C and collect the supernatant. This is the crude homogenate of the enzyme.

APPENDIX II

Ammonium sulphate precipitation

(Nigam and Ayyagari, 2007)

Principle

Ammonium sulphate is a particularly useful salt for the fractional precipitation of proteins. It is available in a highly purified form, has great solubility allowing for significant changes in the ionic strength and is inexpensive. Changes in the ammonium sulphate concentration of a solution is brought about either by adding solid substance or by adding solution of known saturation, generally a fully saturated (100%) solution. Once the concentration at which the target protein precipitates is known, fractional precipitation with ammonium sulphate can be used as an early step in the purification protocol.

Reagents

1. Ammonium sulphate - Add ammonium sulphate to the protein solution as a solid.
2. Resuspension buffer - Resuspend the concentrated protein with 25 mM Phoshte buffer pH 7.2 after ammonium sulphate precipitation.
3. Protein sample - Crude extract.

Procedure

1. Use a graduated cylinder to measure the volume of the protein solution to be concentrated. Pour the solution into a beaker with a capacity approximately twice the measured volume of the protein solution. Place the beaker in a bed of ice.
2. Weigh the necessary amount of ammonium sulphate required to give the desired saturation.
3. Place the beaker containing the protein solution (and a large magnetic stir bar) on a large stir plate in the cold room.
4. Slowly add the solid ammonium sulphate to the stirred protein solution for 30 mins and allow the mixture to stand for 2 hours mins to ensure complete precipitation.
5. Transfer the mixture to screw-cap polycarbonate centrifuge tubes and carefully balance them. Ensure that the centrifuge tubes are balanced within 0.1-0.2 g across the rotar axis.
6. Centrifuge the tubes at 8000 g for 10 minutes at 4^{o}C.
7. Decant the supernatant solution, saving the protein pellet.
8. The pellet dissolved in 25 mM phosphate buffer pH7.2 and assay them for total proteins.

APPENDIX III

Determination of Protein by Lowry's method

(Sadasivam and Manickam, 2008)

Principle

The blue colour developed by the reduction of phoshphomolybdic – phosphotungstic components in the Folin Ciocalteau reagent by the amino acid tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with alkaline cupric tartarate can be measured by Lowry's method.

Materials

1. Reagent A: 2% Sodium carbonate in 0.1N Sodium hydroxide.
2. Reagent B: Copper sulphate in 1% potassium sodium tartarate.
3. Reagent C: Mix 50 ml of A and 1ml of B prior to use.
4. Reagent D: Folin Ciocalteau reagent.
5. Stock standard: weigh accurately 50 mg of bovine serum albumin and dissolve in distilled water and make up to 50ml in standard flask.
6. Working standard: Dilute 10 ml of the stock solution to 50 ml with distilled water in a standard flask. One ml of this solution contains 200 µg of protein.

Procedure

1. Pipette out 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard into a series of test tubes.
2. Pipette out 0.1ml and 0.2 ml of the sample extract in two other test tubes.
3. Make up the volume to 1ml in all the test tubes. A tube with 1ml of water serves as the blank.
4. Add 5 ml of reagent C to each tube including the blank .mix well and allow to atand for 10 mins.
5. Add 0.5 ml of reagent D , mix well and incubate at room temperature in dark 30 minutes.
6. Take the readings at 660nm.
7. Draw a standard graph and calculate the amount of protein in the sample.

APPENDIX IV

Assay of Protease

(Nigam and Ayyagari, 2007)

Principle

Protease cleaves the substrate casein and on treatment with Folin Ciocalteau reagent, the tyrosine produced reduces the phoshphomolybdic acid and phosphotungstic components of the reagent to give a blue coloured product, which is measured spectrophotometricaly at 660 nm.

Procedure:

1. Prepare a stock solution of 200 µg /ml of tyrosine. Also prepare a 1% casein solution as a substrate in the appropriate buffer.
2. Set up a reaction mixture by adding 1ml of different enzyme dilution to 1ml of substrate (1% casein).
3. Incubate the reaction mixture at 50°C for 30 min. Now add 5ml of 5% TCA to all tubes and allow them to stand further for 10 min to precipitate the undigested protein.
4. Centrifuge the contents of all the tubes at 10,000 rpm for 10 min and collect the supernatant.
5. Estimate the amount of amino acids released by means of Lowry's method using a standard curve of tyrosine. Read the absorbance at 660 nm and by using the absorbance values of test and control, obtain the amount of amino acids (µg) released in each case, with the help of standard curve values in the linear region.
6. Calculate the international units (I.U) of protease as follows

$$\text{I.U} = \frac{\text{Net amount of amino acids released (µg) x dilution factor.}}{181 \times 30}$$

where, 181 = molecular weight of tyrosine

30= incubation time in min.

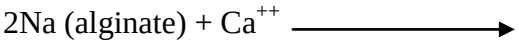
APPENDIX V

Immobilization of Protease in Alginate gel

(Sadasivam and Manickam, 2008)

Principle

The process of gelation which is simply the exchange of calcium ions for sodium ions is carried out under relatively mild conditions.



Materials required

1. Alginic acid sodium salt
2. 0.2M $CaCl_2.6H_2O$: Weigh 0.22g and dissolve in 100 ml distilled water.
3. Enzyme (sample)

Procedure

1. Dissolve 3g of sodium aliginate in 100 ml distilled water to make a 3% solution
2. Mix approximately 0.015g of enzyme with 10 ml of 3% sodium alginate solution
3. The beads are formed by dripping the polymer solution from a height of approximately 20 cm into an excess of stirred 0.2 M CaCl2 solution using a syringe and needle at room temperature.
4. Pressure and the needle gauge can control the bead size. A typical hypodermic needle produces beads of 0.5 to 2 mm diameter. Leave the beads in the calcium solution to cure for 0.5 to 3 hrs.
5. The beads can be stored by refrigeration upto 6 months period

APPENDIX VI

Immobilization of Protease on Onion Membrane

(Vikas *et al.*, 2007)

Materials required

1. Fevitite (standard epoxy adhesive)
2. Onion bulb
3. 2.5% glutaraldehyde in 0.1M sodium phoshate buffer, pH 8.0.

Procedure

1. Rub 100 ml plastic beaker on its inner bottom with a sand paper and then wash with distilled water.
2. Apply thin layer of non-reactive fixative (fevitite, standard epoxy adhesive) is uniformly on the rubbed surface .
3. Peel off white translucent membrane from water soaked onions and apply its piece ($18.1cm^2$) on the fixative layer carefully. Keep the beaker at room temperature overnight.
4. Add the affixed onion membrane inside the plastic beaker, sufficient gultaraldehyde solution so that the affixed onion membrane is fully covered.
5. Allow the onion membrane to get activated for about 2 hrs with occasional shaking.
6. Discard the excess of glutaraldehyde solution and wash affixed onion membrane with 0.1 M Sodium phosphate buffer of pH 8.0.
7. Add a measured protease solution into the beaker so as to submerge all the membrane. Allow the coupling between the enzyme and activated membrane for 48 hrs at 4° C with occasional shaking.
8. Take off the unbound enzyme solution from the beaker and test for the enzyme activity and protein content.
9. Wash the beaker in 0.1M Sodium phosphate buffer pH 8.0 several times until no activity is detected in the washing discard. Test the membrane bound enzyme activity.
10. Estimate the protein bound to membrane affixed on plastic beaker by determining the loss of protein from the enzyme solution during immobilization using the Lowry's method.

APPENDIX VII

Assay of Immobilized Enzyme

(Nigam and Ayyagari, 2007)

(Modified by Geethanjali and Subash, 2013)

Procedure

1. To 0.5 g of dry immobilized protease, add two milliliters of 1% casein solution in 25 mM phosphate buffer (pH 7.2).
2. Incubate at 50°sC for 30 minutes and add 10% TCA to precipitate out the undigested proteins.
3. Neutralize the filtrate with 500 mM sodium carbonate.
4. Estimate the amount of amino acids released by means of Lowry's method as given in Appendix III.

Calculation

Initial activity of the free enzyme = 'a'

Volume of enzyme solution = 'b'

Weight of beads formed after immobilization of enzyme solution = 'c'

Enzyme solution entrapped in 0.5 g beads = 'b/c x 0.5 = d'

Therefore activity of the enzyme entrapped must be = 'd x a /1 = e'

Activity of immobilized enzyme obtained in 0.5 g beads = ' f '

Therefore total enzyme activity after entrapment = 'x %'

APPENDIX VIII

Determination of Storage Stability of Immobilized Enzyme

(Morona *et al.*, 2006 Anwar *et al.*, 2009)

Procedure

1. Incubate aliquots of free enzyme in sealed eppendorf tubes with mineral oil overlaid to avoid evaporation. For immobilized enzyme, suspend 100 and 50 mg beads, respectively, in 25 mM phosphate buffer, pH 7.2 (1 ml).
2. Store the soluble and immobilized enzymes at two temperatures (4°C and 30°C) and note the activity for 10 days to determine the storage stability of entrapped enzymes.

3. Measure the residual activity at specific time intervals under the standard assay conditions as described in Appendix III.
4. After each activity assay, wash the immobilized enzyme with the buffer and leave aside until the next assay in buffer solution.

APPENDIX IX

Determination of Reusability of Immobilized Enzyme

(Gaur *et al.*, 2006)

Procedure

1. Measure the initial activity of the immobilized enzyme.
2. Subject the enzyme to four cycles of repeated use. Assess the reusability of the immobilized preparation at 50ºC by carrying out the hydrolysis of casein (1%, w/v) in 25 mM phosphate buffer, pH 7.2, containing 1 U (0.5 g) immobilized enzyme activity and monitor the amount of tyrosine liberated after each cycle of assay.
3. After each cycle of hydrolysis, recover the pellet containing the immobilized enzyme by centrifugation at 8000 g for 5 min and reuse for further cycles as given in Appendix III.