Application Studies of Immobilized Protease from the Waste of Fish in Detergent Industry

P.Sophia

(15PBC010)

Thesis submitted to

DEPARTMENT OF BIOCHEMISTRY, BIOTECHNOLOGY AND BIOINFORMATICS

Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore – 641043

In Partial Fulfilment of the Requirements for the Degree of

MASTER OF SCIENCE IN BIOCHEMISTRY

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10/04/2017

Signature of the Head of the Department

10/04/17

Signature of the Supervisor

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1.0 INTRODUCTION

Fish wastes resulting from industrial fish processing operations often consists of flesh, skins, bones, entrails, shells etc. Generally fish industries that want to be effectively clean must use fish processing wastes in the manufacture of new products, contributing not only to environmental preservation, but also in increasing their own revenues. Fish waste represents half of the raw material volume of the industry and is a source of low–cost nutrients (Arruda*et al.*, 2007).

In India, on October 2014 there was1107 exporters of fish, out of which 489 are manufacture exporters and 504 are merchant exporters. Exports aggregated 928215 tones valued at Rs. 18856.26 crores and USD 3511.67 million in 2012-13. There are 470 processing plants, 573 storages, 610 peeling sheds and 78 ice plant total number of register (Ancyand Raju, 2014).

In many Asian countries, fishing is an activity that contributes directly to food security, and indirectly to the generation of income that can be used to buy food. The contribution of fish to nutrition is particularly high, as inland capture fish and other aquatic animals contribute between 47% and 80% of animal protein consumption depending on different countries (Vilain*et al.*, 2016).

Alkaline proteases are present in microbial sources like bacteria, fungi and yeasts. Among microbial sources, bacterial proteases are of more interest with various applications in industries such as in detergent, textile, leather, food and feed industry (Vishnupriya*et al.*, 2016*).*

Solid fish wastes are heads, tails, fins, frames, offal (guts, kidney and liver) and skin. Fish wastes are disposed to prevent the production of toxic by-products during decomposition process. (Zhou *et al*., 2012).

The constant development of large quantity of wastes from fish industry (which contains large quantity of protein) are discarded per annually but increases environment pollution (63.6% million tons of waste are generated) (Wandzel and Medrzycka, 2013).

Use of fish silage wastes were used for recycling process for the production of fish oil, particularly to its EPA and DHA fatty acids (Soccol and Oetterer, 2003**).**Fish oil normally contains 30% of omega-3 fatty acids, with 10% EPA (eicosapentaenoic acid) and 12% DHA(docosahexaenoic acid). They are used to prevent and treat several health problems, functional foods, and parenteral nutrition, pharmaceutical and nutritional supplements (Arruda*et al.,* 2007).

The enzymatic treatment of proteins produces peptides and amino acids, which can widely develop the biological and functional characteristics of the proteins and improve their quality and great opportunities for food application (Taheri*et al.,* 2012) .

Extracted biomolecules (Polyunsaturated fatty acids, Collagen and Gelatin) were collected using extraction method of substrate and extraction method distillation, low temperature crystallization, enzymatic methods, urea complexation, alkaline hydrolysis,extraction, microwave assisted extraction, acid treatment of the by-products from fish wastes. The principle in the process of immobilized enzyme system is the enzyme, the matrix and mode of attachment. The moving forces for enzyme immobilization are the improvement of enzyme stability downstream processing simplification of biocatalyst recycling (Liese and Hilterhaus, 2013).

When compared to enzyme immobilization, cell immobilization was recorded as the convenient method because of its low cost and highest stability. These immobilized cells are mainly used in the areas of industrial production and biotechnology. The cost of isolation and purification is very high in enzyme immobilization. Whole cell immobilized has proven application in the waste water treatment and biodiesel production further immobilization biological material in eco-friendly method as used to other applications of solution to human resource management(Elakkiya*et al.*, 2016).

Immobilized enzymes are also used in biosensors and ELISA (Enzyme Linked Immune Sorbent Assay) for the detection of various bioactive substances in the diagnosis of disease states. Biosensors are also used for the removal of waste metabolites.The most widely used biosensors are glucose dehydrogenase based on electrodes that have been developed to monitor glucose concentration (Aggarwal and Sahni, 2012).

However, the main use of common material for enzymes immobilization namely, silica-based carriers, synthetic polymers, active membranes, acrylic resins and exchange resins faces such as high cost of materials and technology necessary to apply fixation which greatly increase the cost of biocatalyst. Various immobilization methods have been improving enzyme activity. In time, these techniques combine with chemistry and molecular biology can help to improve enzyme immobilization strategies and develop application in various fields (Mohmoud and Helmy,2009). Proteases are the type of hydrolase enzymes that chemical reaction of break the polypeptide chain that connects the amino acids in the proteins (Singh *et al*., 2015).

The application of Protease enzymes constitutes about 60% of the total market in detergent, tannery, food industries, metal recovery and waste treatment. Industries increasingly used in treatment of inflammation, cancer, cardiovascular disorder, preparation of medicines in pharmaceutical industry such as ointments, mainly in the detergent, food and leather industries (Mahmoud and Helmy, 2009). At present application of pectinase, amylase, lipase, protease, catalase and xylanases are used in textile industry (Mojsov, 2011).

The immobilized protease has 15-20% increased stability and the entrapped enzyme retained 83% of its initial activity after six cycles. With respect to properties of the enzyme and its able for degradation of different protein sources, this protease finds out application for waste treatment, used with detergents for removal of blood stains from cotton cloth and in leather industry for dehairing from bating skins (Najafi*et al*., 2005).

The main advantage for enzyme immobilization is the easy separation of the enzyme from the reaction mixture and its reusability for ten times. Remove the enzyme from the reaction solution (or vice versa) have the ability to stop the reaction rapidly. Product is not contaminated with the enzyme that can be easy separated of enzyme from the product (in food and pharmaceutical industries).Enhancement of enzyme stability against pH, temperature, solvents, contaminants and impurities. Immobilization of enzymes is one of the methods used to stability free enzymes (Elnashar, 2010 and Danial*et al*., 2010).

The enzymes are the potential catalyst that works in low temperature, pressure, pH, substrate specificity under suitable reaction conditions to desired products that enzyme are used in variety of application such as cosmetics, paper industry, textile industry, food industry, pharmaceutical industry, biodiesel product, laundry, biomedical application and detergents. In food industry useimmobilized beta-galactosidase for lactose hydrolysis for production of baker'syeast. The enzymes such as cellulose, amylase, laccase, cutinase, pectinase etc and used for various textile applications such as scouring, biopolishing, desizing, denim finishing, treatment woods (Hemalatha*et al*., 2016)

The present study entitled**"Application Studies of Immobilized Protease from the Waste of Fish in Detergent Industry"** was carried out with the following objectives

- To Immobilize protease isolated from fish waste and determine the immobilized activity and optimization of condition
- To partially characterize immobilized protease
- To qualitatively check the activity of the immobilized protease

2.0 REVIEW OF LITERATURE

Fish processing industries generate different variety of by-products after process disposal of these wastes cause environmental and health problems.In recent years, worldwide fish production contributes 75% then remaining 25% (34.8 million tonnes)are considered as waste. Fish processing industry generates large quantities of solid waste and waste water Fish processing waste can be converted into proteins for human consumption. Fish proteins are found in the flesh, head, frames,fin,tail,skin and guts. Fish processing waste can be used to produce fish protein which contains amino acids and many bioactive peptides(Ramakrishnan*et al.,* 2013).

Fish waste can also be used for production of various value added products such as proteins, oil, amino acids, minerals, enzymes, bioactive peptides, collagen and gelatin. There are three types of proteins in fish such as structural proteins, sacroplasmic proteins and connective tissue proteins. The fish proteins can be extracted by chemical and enzymatic process (Ghaly*et al.,* 2013).

The review of literature pertaining to the study entitled **"Application Studies of Immobilized Protease from the Waste of Fish in Detergent Industry"** is discussed under the following headings,

2.1 Growth and development of Indian fishing industry

2.2 Statistical analysis of fish production in India

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2.8.1 Detergent industry

2.1 Growth and development of India fishing industry

From 1990 through 2010, Indian fish industry growth has accelerated, reaching a total marine and fresh water fish production to about 8 million metric tons. India's fish industries efforts led to a more than fourfold increase in coastal fish production from 5,20,000 tons in 1950 to 3.35 million tons in 2013.Between 1990 to 2007 fish production in India has grown at a higher rate than food grains, milk,eggs and other food items. In India fisheries and aquaculture are important sector of food production.Fish and fish product have presently exports of India, with 5.2 lakh tonnes of quantity and Rs 7,200 crores in value.50 different types of fish and shell fish products are exported 75% countries around the world. Approximately 67 per cent of the total fish produced in the country,23 percent as consumed in processed 16 per cent used for drying and 7 per cent for freezing(Nedumaran., 2014)

2.2 Statistical analysis of fish production in India

India is producer of fish through aquaculture and ranks second in the world after China.in recent years 2013-2014 India is the second largest producer of fish in the world contributing to 5.68% of global fish production(Goswami and Zade, 2015)

Table-1

State wise production of fish (In tonnes)

S.No	States	2010-11	2011-12	2012-13
		Total	Total	Total
1	Andhra Pradesh	1368202	1603168	1808077
2	Arunachal Pradesh	3150	3300	3710
3	Assam	227242	228621	254270
4.	Goa	93270	89956	77879
5.	Gujarat	774902	783719	786086
6.	Haryana	96197	106000	96704

7.	Himachal Pradesh	7381	8045	8561
8.	Karnataka	526579	546436	575383
9.	Kerala	681613	693208	633490
10.	Tamil Nadu	614809	611488	620397

Source: (Goswami and Zade, 2015)

Aquaculture supplied the world about 148 million tonnes of fish in 2010, of which about 128 million tonnes was utilized as food and preliminary data for 2011, increase there was an production of 154 million tonnes, which 131 million tonnes was utilized as for food**.**
(FAO 2012).

The fish production had a significant growth in all states, except Rajasthan. In other states like Andra Pradesh, Gujarat, Karnataka, Kerala, Maharashtra Orissa West Bengal and Tamil Nadu there are growth inland fisheries but in, marine fisheries growth.(Mruthyunjaya, 2004)

2.3 Special products from fish waste:

2.3.1. PUFAs, EDA and DHA

Marine fish accounts for 90% of fish resource.Fish product of Fillets,chips, canned food, protein product, fish and fish oil. 75% of fish stock are used for human consumption and 25% is used for fish oil production (Bin *et al*., 2013). The fish industry generates high volume of waste from fish oil. Production of 30.78% polyunsaturated fatty acids (PUFA) with 9,12 of dicosahexaenoic (DHA) and 10,36% of eicosapentaenoic (EPA) was found while the fish waste is processed (Nascimentd *et al.,* 2015)**.**

In fish and poultry processing, viscera are generally considered as a waste product and are discarded. Chicken and fish (hilsailisa) viscera were used for the production of PUFA (84%) and in fish viscera were containing 78.2% EPA-DHA(Patil and Nag, 2011).

Long chain polyunsaturated fatty acids (*LC-PUFA*)**,** DHA and arachidonic acid (AA, 20:4n-6) are important constituents of the phospholipids of all cell membranes. That can be able to play roles assuring the correct environment for membrane protein function, maintaining membrane fluidity, regulating cell signaling, gene expression and cellular function and serving as substrates for the synthesis of lipids mediators (Giuseppe *et al.,* 2014).

2.3.2 Chitin/Chitosan

Chitin is a natural polysaccharide synthesized by many number of different number of crustacean exoskeleton, insects and fungi and function as a structural polysaccharide. Chitosan is modified from N-acetyl glucosamine derivative and carbohydrate polymer of glucosamine. Chitosan degradation used in ozone treatment combined with ultraviolet radiation is promising technique (Guirguis*et al*., 2016).

Chitin is the second most natural polysaccharide that is present in many organism of crustacean exoskeleton.The shell fish industry discard huge amount waste per processing which utilized as an source of chitin and its derivative chitosan. Chitin and chitosan are promising biomaterials. The deacetylated chitin derivative is useful as bioactive polymer

2.4. Fish waste utilization

The present utilization of important product that used from fish wastes and discarded of fish processing industrial wastes is given below

Table-2

Fish waste utilization

S. No	Source	Products	Processes	Uses
1.	Fish head & viscera	PUFA Fish meal	Capsule, solvent extraction	Pharmaceutical uses
2.	Skin	Collagen & gelatin	Acid and alkali wash, Edible coatings	Medicine and food
3.	Scales & Bones	Calcium	Enzyme treatment, Dry powdering	Nutraceutical products

Source: Zynudheen, 2005

2.4.1 Fish oil and biodiesel production

Generally fish oils are potentially have advantage as petroleum and virgin vegetable oil based due to waste utilization (Jayasinghe*et al*., 2013).

Fish oil can be extracted from the whole fish, skin or liver of fish. Fish oil is 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 with higher activity of unsaturated fat content. Compared to saturated fats, poly unsaturated fatty acids in fish oil are readily digested for energy production finally that reported bioactivities (Geethanjali and Subash, 2012).

Wide range of fatty acid present in fish oils that is found with 18 carbon atoms of their carbon chain and even some contain an odd number of carbons. Commonly fatty waste materials that can be improve quality of biodiesel production from utilization of fatty materials, in particular waste frying oils and animal fat produce from mainly from meat and fish processing industries (Mata *et al*., 2015).

2.4.2 Biogas production

The world growing populations lead to increase in the generation of waste. To solve that problem using production of energy from different wastes (Waste-to energy) come from agricultural, industrial and domestic wastes. Biogas contains by methane (CH_4) and carbon dioxide(CO_2) that come from fish canning industry. Which resulted in 67% methane (CH) used for biogas produced from the sludge finally biogas ranges between 65 to 70% (Carvalho.,*et al*2012)

Fish processing wastes have great potential energy production. The fish wastes process renewable energy market finally plays in the future of bio-fuels. The biogas production fish waste was determined and anaerobic digestion process was using kinetic models(Kafle*et al.,* 2012).

2.4.3 Meal and fodder

They may still be disposed as waste such as fish skin, bones, and fins are present in nutritional value that is used in fish meal manufacturing. The nutritional value depends on amino acid composition and protein rich products, such as fish meal and silages (Mahboob*et al.*, 2014)

Mullet have been identified as species for pond culture in China, Egypt, Hawai, Italy, Japan, Philippine and Taiwan. Mainly grown in polyculture with milkfish, tilapia and carps. Mullet fry at 21% was best growth performance and feed utilization(El-Dahhar*et al.*, 2014).

2.4.4 Fertilizer:

Fish remains are also used as fertilizer, given their more nutritive elements are present such as nitrogen and potassium and quickly take the decomposition. Composting initiatives using fish from aquaculture have been carried out in alternative and viable techniques for transforming fish wastes that is useful for agricultural products(Mosquera*et al.,* 2011).

Production of fish fertilizer involves hydrolysis of by-products, lawns, row crops and turf are used by fish fertilizer. Some recent present in the nitrogen from fish fertilizer remain soil longer than nitrogen from inorganic fertilizer (Abdelmajeed*et al*., 2011).

2.4.5 Medical application:

Iron concentration is present in fish waste of head and viscera. Iron mainly functions to oxygen transportin immunity, healing and energy level. Vitamin A important develops a strong immune system and proper eyesight. Fish liver oil highest concentration in preformed vitamin A. Zinc is present in small fully consumed fish. Mineral of iodine is found in largely in seafood that mainly functions of form thyroid hormones. (Vilain*et al*., 2016).

Cardiovascular diseases(CVD) are treated by use of streptokinase and tissue-type plasminogen activator (t-PA) among fibrinolytic agents. However fibrinolytic agents can cause allergic reactions, bleeding complications and short half- lives (Biji*et al*., 2016).

2.5 Enzyme from marine animal

Rich source of enzymes are found in internal organs of the fish. The enzymes include: collagenase, pepsin, trypsin and chymotrypsin, polysaccharides, proteins and pectin used to physical, chemical and organoleptic properties of the original food in high nutritive value

2.5.1 Proteases

Proteases are a group of enzymes, whose catalytic function is to hydrolyze peptide bonds of proteins and break down into polypeptides or free amino acids. They constitute 59% of the global market of industrial enzymes.They have got wide range of commercial used in detergents, leather, food and pharmaceutical industries (Alnahdi, 2012).

Amino acids have an important role of protein synthesis as present compounds carriers of hydrogen, vitamins, carbon dioxide, enzymes and structural proteins and they also influence bioactive and functional properties (Villamil*et al*., 2017).

Wastes product of meat, poultry and fish processing industries can supply a large amount of protein rich material for bioconversion to recoverable products (Vinoth*et al*., 2014).

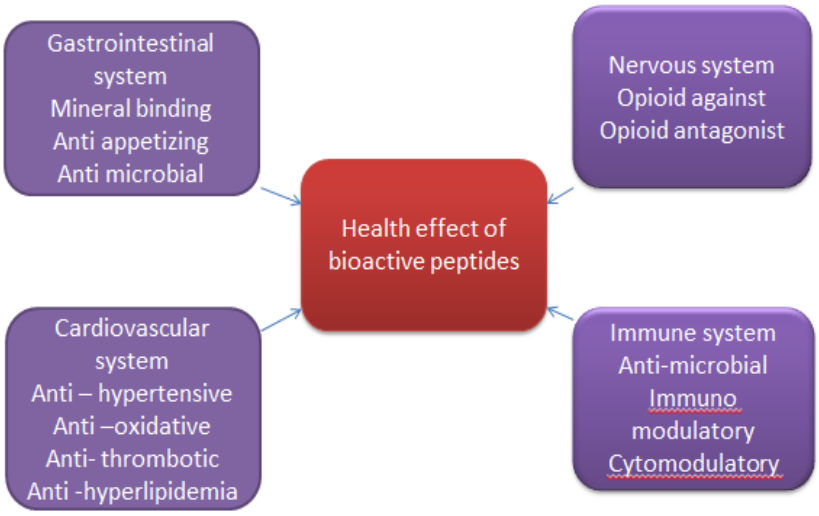
Collagen and gelatin fish skin waste to convert into fish protein hydrolysates. Fish head by product waste have converted to protein hydrolysates these are mainly present in various species of salmon and red salmo. Protein hydrolysates are produced using various fish muscle such as *Salmosalar, Decaptersmaruadsi, Selaroidesleptolepis* and *Catlacatla* (Chalamaiah*et al.,* 2012).

The protease enzyme condition in laboratory have been characterized it rate of enzyme production such as mainly pH and temperature on the activity of the enzyme has observed.in

vitro (spectrophotometrically) and in vivo assay (goat skin for removal) (Arunachalam and Saritha, 2009).

Figure-1

Physiological effects of food derived bioactive proteins on major body systems



Source: (McCarth , 2013)

2.6 Method of immobilization:

The immobilization of lipase includes several methods entrapment, encapsulation, cross linking, adsorption and covalent bonding. Adsorption method of immobilization is widely used in recent years when compared to covalent bond, entrapment and cross linking(Hemalatha*et al*., 2016).

2.6.1 Enzymes can be immobilized by different techniques

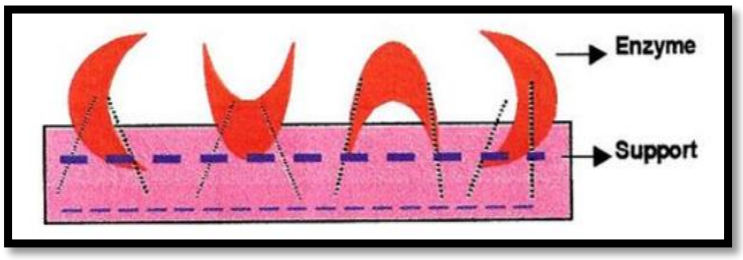
1. Adsorption onto an inert carrier.

2. Entrapment within the lattice of a polymerized gel (synthetic and non-synthetic)
3. Cross linking of the protein.
4. Covalent bonding to a reactive insoluble support.
5. Ion-exchange.
6. Copolymerization.

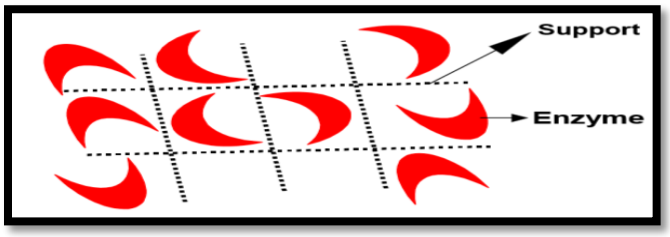
Figure-2

Enzymes can be immobilized by different techniques

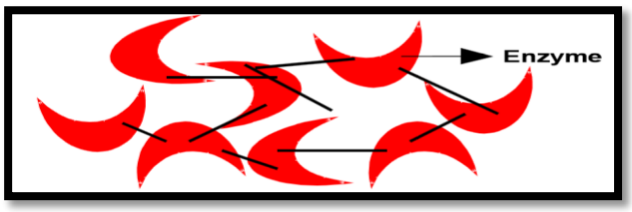
(a)Immobilization of enzymes using the adsorption technique



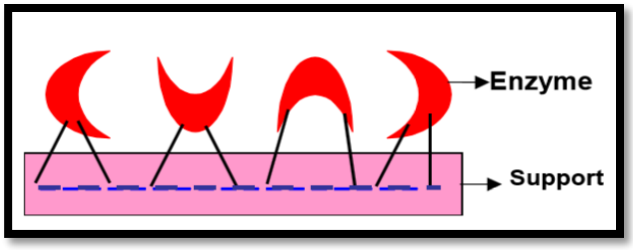
(b) Immobilization of enzyme using the entrapment technique



(c) Cross linking technique

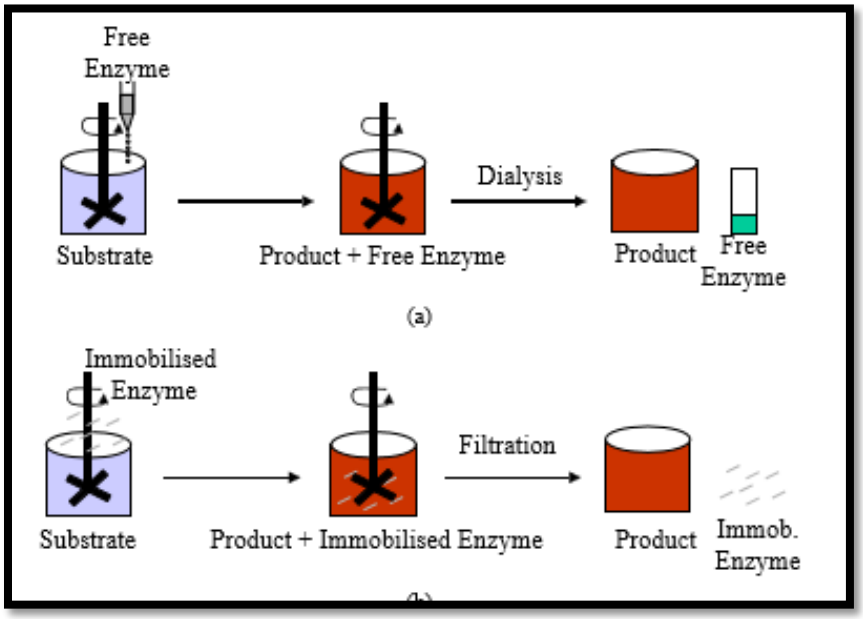


(d) Immobilization of enzymes using the covalent technique



Source: Latme*et al.*,(1998)

Figure 3.Schematic diagram of free and immobilized enzyme reactions.



Source: Elnashar, (2010)

(a) Reaction of free enzyme with substrate and formation of product, which has to be separated via dialysis;

(b) Reaction of immobilized enzyme with substrate and formation of product, which can be separated via filtration or using a fluidized magnetized bed reactor system

2.7 Uses of immobilized enzyme

Immobilization techniques can be divided into as physical, chemical, enzymatic and genetic engineering methods. Enzyme or active molecule with a polymer after crosslinking the polymer to form a lattice structure that traps the enzyme.

Table-3

Industrial Applications of Immobilized Enzyme(Forde and Fagain, 2008)

Industry	**Enzyme class**	**Application**
Food	Glucose Isomerase	Production of HFCS
	β Galactosidase	Hydrolysis of lactose in dairy products
	Lipases	Dairy, Baking, Fats/Oils
	Transglutaminase	Modify visco-elastic properties strengthens dough
Pharmaceutical	Penicillin acylase	Synthesis of 6-APA for production of penicillin
Chemical	Lipase	Resolution of chiral alcohols and amines
	Subtilisin	Protein stain removal, Detergent formulations
Detergents	Lipase	Fatty acid and oil stain removal
	Cellulose	Colour clarification, cleaning
	Peroxidase	Dye removal
Fuel	Lipases	Biodiesel production
	Glycosidase	Saccharification

2.7.1 Biosensor

The use of immobilized enzymes in biosensors the reactors used to efficiently and enzymes cost-effectively carry out the reaction**.**Immobilized enzymes in different field such as medicine, antibiotic resistance, drug metabolism, food industry, biodiesel production and bioremediation

Biosensors with the principle of immobilized enzyme inhibition have been used for a wide range of analytes such as

1. Organophosphorus pesticides
2. Organochlorine pesticides
3. Derivatives of insecticides
4. Heavy metals
5. Glycoalkaloids

The development of biosensor by using immobilized enzymes that is solve the many problems loss of enzymes, Maintains of enzyme stability, shelf life of biosensor additional reduce the time of enzymatic reaction(Khan and Alzohairy, 2010).

2.7.2 Medicine

Many immobilized enzymes are used treatment of disease and diagnosis such as example for Extracorporeal therapy,Hyperuricemia,Glycogen storage disease,Leukemia, Cancer,Artificial kidney and uraemic disorders. Used magnetic iron nanoparticle with cisplatin adsorbed drug release in magnetic heating treatment of cancer. Finally medicine has benefit from immobilized enzymes (Abdelmajeed*et al.,* 2012).

2.7.4 Food industry

Immobilized enzymes are used mainly in the processing of food sample and its analyses. Enzymes as immobilized are used for lactose hydrolysis, skinned milk production, etc.. The greatly facilitated immobilized glucose isomerase is used for the production of high fructose corn syrups. One of the new concepts using immobilized enzyme in food industry is single matrix for immobilizing.

The recently used enzymes for food processing are glucoamylase, lactase, protease and flavor modifying enzymes**.** (Carpio*et al.,* 2000)

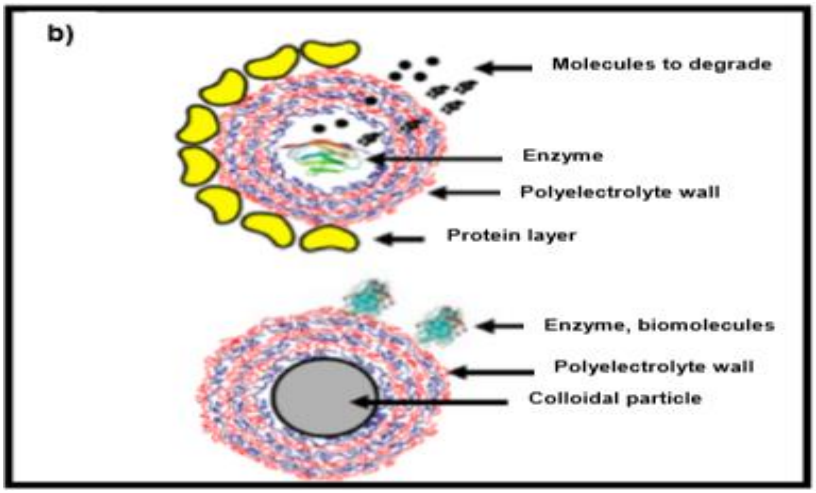
The food industry is increasing gradually by using immobilized enzymes. Starch contain in the food that can be manufactured with immobilized enzymes so as introduce sweetness by conversion of fructose and to decrease by partial conversion to glucinate.

Production of high fructose syrup-One of the industrial processes that makes use by immobilized enzymes is the result of transformation of glucose to fructose. Enormousgrowth of the controversies regarding production of artificial sweeteners in the last few years.As a result change from cyclamates and saccharine to the use of sucrose and fructose .At present immobilized glucose isomerase which contains 42% fructose.50% glucose and 8% of other sugars that can be use of sucrose syrup**.**In the future, amyloglucosidase based immobilization process could be used to meet the increased demand of glucose as a substrate for the production of high fructose syrup(Rello*et al*., 2010)

2.7.5 Biodiesel

Biodiesel is usually defined as methyl (or ethyl) esters of fatty acids obtained by transesterification (alcoholysis) of triglycerides. Typically, biodiesel encompasses alkyl fatty acid (chain length C14– C22) esters of short-chain alcohols, primarily, methanol or ethanol. Biodiesel has the most compatible characteristics with the fossil fuel due to its higherheating value, flash point, cetane numberand kinematic viscosity(Demirbas, 2009)

Figure 4
Supramacromolecularstructures usable to immobilize biomolecules
3D structures



Source: Couco and Herrera, (2006)

2.8 Industrial application of protease

The bioactive compound, protease, was purified from the visceral waste of different fish species. The crude enzyme from the visceral waste of the Red snapper and Great barracuda removed the blood stains effectively within 20 min, without any detergents and also used in dehaired the goat hide after 22 hours of incubation, without use of sodium sulfide.

The direct use is acceptable as substitute for the commercial ones (Sabtecha*et al.*, 2014). Therefore, in recent decades, marine scientists are looking into the composition of lipids, polysaccharides, enzymes, vitamins, and proteins in different fish visceral source
(Sekar *et al.*, 2016).

2.8.1 Detergent industry

Proteases are the highest selling industrial enzymes. Their sales are projected to increases in the recent application of coming years in detergent formulations, peptide synthesis and protein processing (Chandel and Anuj 2007).

The protease was used to destain blood, ink, coffee and was active and stable under more than one extreme condition of high salt, pH, and temperature (Sekar*et al.*, 2016).

The decolorization of dye in wastewater using from microbial enzymes has been a studies in recent years due to their low cost of production and efficient application. In the present study clearly reported crude protease enzyme isolate from Bacillus Cereus Stain wwcp1 produced novel alkaline protease properties that ability to decolorize and degrade MG (Malachite Green) dye removal in wastewater treatment processes (Wanyonyi*et al.*, 2014).

With this background information, the experimental design for the study was formulated as given in the following

3.0 EXPERIMENTAL PROCEDURE

Enzymes are the biocatalysts which enhance biochemical reactions that occur in living organisms. Most of the enzymes are protein in nature and is necessary for the life (Dam *et al.,* 2013).

Fish Industry waste are important environmental contamination source, Research has been carried out in order to develop methods to convert wastes into useful products. Fish waste proves to be a great source of minerals, proteins (58% dry matter (d.m) fat (19% d.m). Fatty acids (monosaturated acids, Palmitic acid and oleic acids) are found in fish waste, while the high ash content (22% d.m) indicates (Subash*et al.,* 2011).

Fish waste management is very important, because Treated fish waste was found to have many applications among which the most important are animal feeds, biodiesel/biogas, dietetic products, natural pigments, foods-packagings, cosmetics, enzymes, soil fertilizer and moisture maintenance in food (Zhau*et al.,* 2008).

Fish processing by products and the under- utilized discards are traditionally recognized as low- value resources. Many of them are developed as plant fertilizer and livestock feeds. However these rest raw materials consist of give valuable components such as fish oil, proteins, collagen and gelatin, enzymes and minerals and used in the development of human food products(Liu *et al.,* 2015).

The potential application of immobilized alkaline protease of *B. amyloliquefaciens SP1* in the detergent industry and the need of development of economic methods for improved enzyme production make whole cell and enzyme immobilization excellent alternative methods (Guleria et al.,2016).

The present study entitled**"Application Studies of Immobilized Protease from the Waste of Fish in Detergent Industry"** was carried out with the following experimental design.

3.1Collection of sample

3.2 Preparation of crude homogenate

3.3 Precipitation of protease

3.3.1Precipitation with ammonium sulphate

3.4Immobilization of fish waste protease

3.4.1 Immobilization by entrapment

3.4.2 Determination of immobilized enzyme activity

3.4.3 Optimization of immobilization condition

3.4.3.1 Effect of sodium alginate concentration

3.4.3.2 Effect of calcium chloride concentration

3.4.4 Characterization of immobilized enzyme

3.4.4.1 Effect of pH

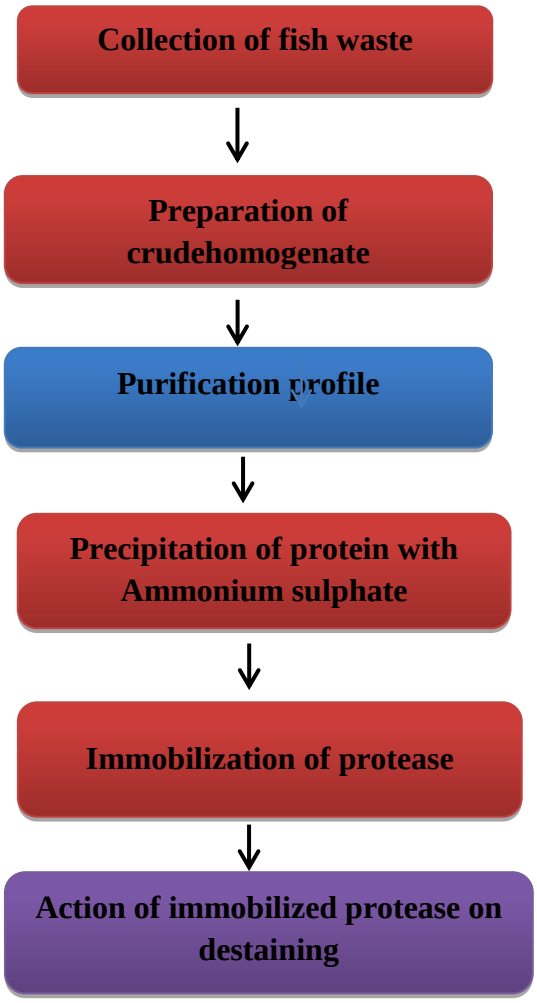
3.4.4.2 Effect of temperature

3.5Qualitative detection of immobilized enzyme

3.5.1 Action of immobilized enzyme on destaining

Figure-5

Experimental Design



3.1 Collection of samples

Fish waste was collected from fish industry in Cochin. The visceral organ, head and tail wastes of the fish were collected soon after cutting, placed in separate clean plastic bags, maintained in ice and brought to the place of study.

3.2 Preparation of Crude Homogenate

The collected fish wastes were washed well with distilled water and stored at - 20^0C until use. They were then weighed, cut into small pieces and homogenized in 20 mMTris-HCl buffer, pH 7.8 to get the crude homogenate. The details of the procedure were given in Appendix I.

3.3 Precipitation of protease

The crude enzyme extract obtained from homogenization was precipitated using. Ammoniumsulphatein the following fraction 0-30, 30-60 and 60-90.The fraction which is highest specific activity, recovery percentage and purification fold were using for further process.

3.3.1 Precipitation with Ammonium sulphate purification

Ammonium sulphate precipitation is a simple method and is generally employed as an initial step to remove other proteins from crude extract. The crude enzyme waspartially purified with20% - 100% of ammonium sulfate(Simpson, 2004).The procedure was explained in the Appendix-II.

3.4 Immobilization of purified fish industrial waste protease

3.4.1 Immobilization by entrapment

Enzyme immobilized could make the enzyme reusable, thereby reducing the processing cost. The enzyme was immobilized with sodium alginate and calcium chloride.The beads thus formed were then stored at -20^0C Appendix- IV.

3.4.2Determination of immobilized protease activity

The activity of the immobilized protease was colorimetrically assayed. Appendix-IV gives the procedure for the same

3.4.3 Optimization of immobilization condition

Immobilization parameters should be studied and optimized to preserve the native activity of biomolecules and to achieve high immobilization efficiency (Lu et al.,2009).For this purpose varying concentration of sodium alginate (1 – 5%) and calcium chloride (0.1 – 0.5) were used.

3.4.3.1. Effect of sodium alginate concentration

The optimum concentration of sodium alginate required for enzyme immobilization was determined by using various concentration of sodium alginate (1,2,3,4 and 5%) to immobilize the isolated protease and then study its activity. The detailed procedure was given in Appendix-VII.

3.4.3.2 Effect of calcium chloride concentration

The optimum concentration for enzyme immobilization was studied by using concentration of calcium chloride (0.1 – 0.5) to immobilize the isolated protease.The Procedure recorded in Appendix-VIII.

3.4.4Characterization of immobilized protease

3.4.4.1 Effect of pH

The optimum pH for the immobilized protease was determined using the substrate casein prepared in buffer of varying pH ranging from (4.0 - 12.0) according to the protocol in Appendix-IX.

3.4.4.2 Effect of temperature

The activity of the immobilized protease was assayed at different temperature determined ranging from (20- 80^{o}C) according to the method described in Appendix –X.

3.5 Qualitative detection of immobilized enzyme

3.5.1 Action of protease on different Stain

The destaining activity of purified protease was examined with a piece of soya,eggyolk chocolate,and blood stained cloth, which was incubated with the purified protease enzyme samples. The detailed procedure is recorded in Appendix -XI

4.0 RESULT AND DISCUSSION

Fisheries generate a large amount of solid fish waste such as fish head, tail, viscera, skin, liver, gonads, bones, guts and some muscle tissue and liquid waste consists of waste water used during fish processing. These wastes are rich in organic contents such as bioactive peptides, protein, collagen,oil, gelatin, calcium and enzyme which make this more expensive. Fish wastes acts as a potential medium for protease production. Proteases play an important role in industrial applications like leather and detergent industry, food and pharmaceutical industries and also in bioremediation processesRamakrishnan,et al.,2013.

A protease enzyme hydrolyses the peptide bonds that link amino acids together in the polypeptide chain which is the back bone of a protein molecule(Genckal*et al.,* 2006). Protease has the high capability for removing protein stain from clothes and it could also be used along with detergent powder or solution. Anwar and Saleemuddin, (1997) reported the usefulness of protease from *Spilosomaobliqua* for removal of blood stains from cotton cloth in the presence and absence of detergents where it was believed that the protease was more effective

The results of the present study entitled **"Application Studies of Immobilized Protease from the Waste of Fish in Detergent Industry"** was discussed under the following headings

4.1. **Quantitative assay of enzymes**

4.1.1. Isolation and purification of protease

4.1.1.1. Isolation of crude protease

4.1.1.2. Purification by ammonium sulphate precipitation

4.2. Immobilization studies of protease from fish wastes

4.2.1. Optimization of immobilization parameters

4.2.1.1. Effect of sodium alginate concentration

4.2.1.2. Effect of calcium chloride concentration

4.2.2. Effect of pH on the activity of immobilized protease

4.2.3. Effect of temperature on the activity of immobilized protease

4.3. Qualitative studies on immobilized protease from fish waste

4.3.1. Action of immobilized protease on soya sauce stained cloth

4.3.2. Action of immobilized protease on egg yolk stained cloth

4.3.3. Action of immobilized protease on blood stained cloth

4.3.4. Action of immobilized protease on chocolate stained cloth

4.1 Quantitative assay of enzymes

The crude enzyme was extracted from the fish waste and used as enzyme source to determine activity of protease.

4.1.1 Isolation and Purification of protease

The crude protease extract isolated from the fish waste.

4.1.1.1. Purification profile of crude protease

50 ml of the crude enzyme extract was taken and the purification profile was checked. The total protein content, protease activity, specific activity, recovery percentage and purification fold of the crude protease sample was recorded in Table 4.

Table-4

Purification profile of crude protease

Parameter	Crude extract
Total activity (U/ml)	3.02
Protein content (mg/ml)	6.52
Specific activity (U/mg)	1.42
Recovery percentage (%)	100
Purification fold	0.95

The protease activity was 3.02 U/ml in the crude extract and its specific activity was found to be 1.42 U/mg with recovery percentage 100 and purification fold 0.95. the protein content of the crude extract was found to be 6.52 mg/ml.

Sabtecha*et al*. (2014) have reported that the visceral waste of Red snapper and Seer fish showed high proteolytic activity (27.40±0.05 U & 23.21±0.61 Urespectively), protein content (7.22± 0.21& 9.44± 0.13 mgrespectively) and specific activity (3.79± 0.19 & 2.46± 0.31 U/mgrespectively) in the crude sample

4.1.1.2. Purification by ammonium sulphate precipitation

The crude protease extract from fish waste was partially purified by ammonium sulphate precipitation method. Different concentration of ammonium sulphate ranging from 0-30, 30-60 and 60-90 percent were used and checked for the highest specific activity.

The purification profile for the protease purified by ammonium sulphate precipitation at different concentration was summarized in the Table -5

Table-5

Purification profile of ammonium sulphate precipitated protease

Sample	**Total Activity (U/ml)**	**Protein content (mg/ml)**	**Specific activity (U/mg)**	**Recovery Percentage (%)**	**Purification fold**
0-30	0.88	0.53	0.63	24	0.63
30-60	**0.91**	**1.0**	**1.02**	**38**	**1.98**
60-90	0.60	0.88	0.51	20	0.83

30-60 % ammonium sulphate precipitated protease sample showed highest total activity (0.91 U/ml), Protein content (1.0mg/ml) and Specific activity (1.02 U/mg) with Recovery Percentage of 38 and 1.98 Purification fold, when compared with the other precipitations.

30-60% concentration shows increased total activity and specific activity. Similar studies were reported by Subash*et al*. (2012), where the protein content was found to be highest in the crude sample when compared to the dialyzed fish waste samples. The protease activity and specific activity of the 40-80% dialyzed sample was found to be higher when compared to crude.

The results are similar to reports of El-Beltagy*et al*. (2005), where he has studiedabout alkaline protease purification from Bolti fishsamples (*Tilapia nilotica*) showing a high recovery percentage (62) at 40-60% through ammonium sulphate precipitation.

Summary of Purification profile

The summary of purification profile was recorded in table-6

Table-6

Summary of the purification profile

Sample	Total protease activity (U/ml)	Protein Content (mg/ml)	Specific Activity (U/mg)	Recovery Percentage (%)	Purification Fold
Crude extract	3.02	6.52	1.40	100	0.95
Ammonium Sulphate Precipitation (30-60%)	1.0	0.91	1.217	38	1.98

Highest specific activity was noted in crude (1.40 U/mg) and in 30-60% ammonium sulphate precipitation sample it was 1.217 U/mg.

From table -6, it was noted that after ammonium sulphate precipitation there was in increase in the Purification Fold (0.95-1.98), which indicate that the protease was partially purified from the crude homogenate. It was also noted that the protein content reduced, noting that protease has been purified partially.

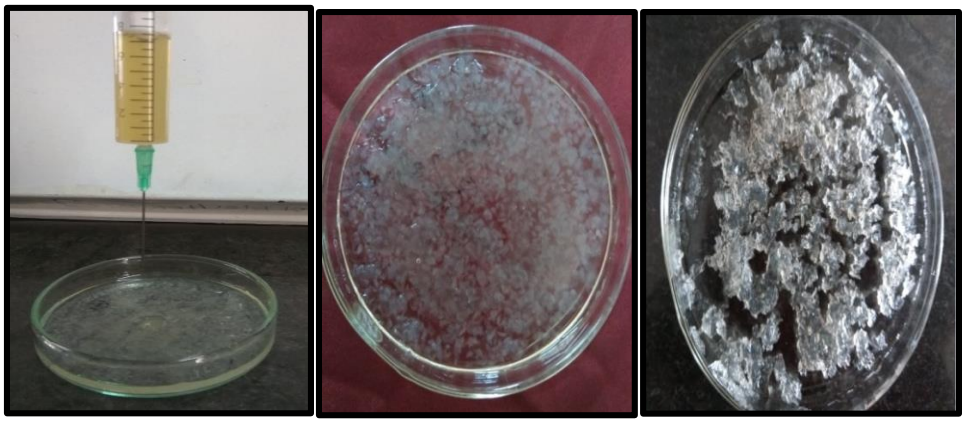
4.2 Immobilization studies of protease from fish wastes

4.2.1 Immobilization by entrapment

The crude enzyme was entrapped in calcium alginate gel. After the completion of reaction, the calcium alginate beads got swollen and prolonged reaction time the beads got cracked. Since the alginate beads were found to be stable in calcium chloride solution, the reaction with protease enzyme was carried out in it.

Figure-6

Beads of calcium chloride containing immobilized fish waste protease



(a) **(b)** **(c)**

sodium alginate (2%) and purified enzyme in0.2M $CaCl_2$ solution

Calcium alginate beads

Entrapped protease .

Figure-6 shows the effect of concentration of calcium chloride solution on the activity of enzyme entrapped in the freshly prepared beads. Figure-6 **(a)** shows mix equal volume of sodium alginate (2%) and purified enzymesolution in through needle into 0.2M $CaCl_2$ solution.Figure-6 **(b)** shows calcium alginate beads preparedFigure-6 **(c)** shows rinse with water entrapped protease ready for use. When the reaction was carried out in 0.2 M calcium chloride solution, the activity of entrapped enzyme was found to be maximum

4.2.2. Optimization of immobilization parameters

For the preparation of beads with proper permeability and rigidity, parameters such as sodium alginate concentration and molarity of the calcium need to be optimized. The following parameter depict the immobilization efficiency of the protease from fish wastes.

4.2.1.1. Effect of sodium alginate concentration

The effect of sodium alginate concentration ranging from 1-5% and 0.2M calcium chloride on immobilized protease from fish waste was shown in Table-7and Figure-7

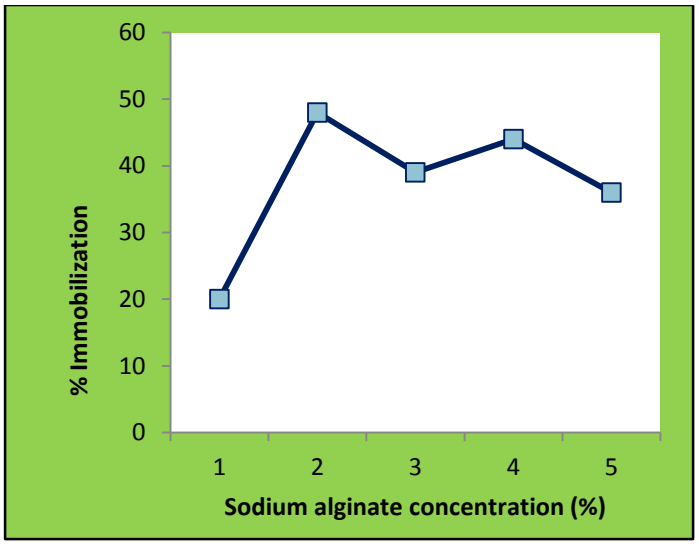
Table-7

Effect of sodium alginate concentration on immobilized fish waste protease

Sodium alginate concentration (%)	% Immobilization
1	20
2	49
3	39
4	44
5	36

Figure-7

Effect of sodium alginate concentration on immobilized fish waste protease



The data from the figure-7 and table-7 confirm that highest immobilization (49%) of the enzyme was obtained with beads prepared from 2% (w/v) of sodium alginate followed by 4% and 3%. The lowest percent immobilization was observed with 1% sodium alginate, Maximum leakage of enzymes from the immobilized beads due to the larger pore size of the beads and decrease in the tight cross links with the calcium chloride which results in a lower immobilization percentage of 20.

Similar findings were observed by Sankaralingam*et al.* (2012) production of protease by Immobilized cells in sodium alginate. The enzyme production reached maximum level in *Pseudomonas sp*. (550.13±1.63 U/ml) by 48 hours. Dey*et al*, (2003) reported that sodium alginate 4% gave the highest immobilization yield 75% of *B. circulans* α-amylase. Singh *et al.* (2012) reported that immobilization of α-amylase by entrapment was recorded with highest immobilization yield (61.4%) obtained with sodium alginate 2%.Immobilization of *B. licheniformis* ATCC21415 alkaline protease with Sodium alginate had the highest immobilization yield (23.2%) (Samia*et al.,* 2007).

4.2.1.2. Effect of calcium chloride concentration

Alginate beads were prepared with varying concentration of calcium chloride (0.1,0.2,0.3,0.4 and 0.5 M) mixed with 2% sodium alginate since 3% showed the highest immobilization efficiency.

The effect of calcium chloride concentration on the rigidity of the beads is shown in Table-8 and Figure-8

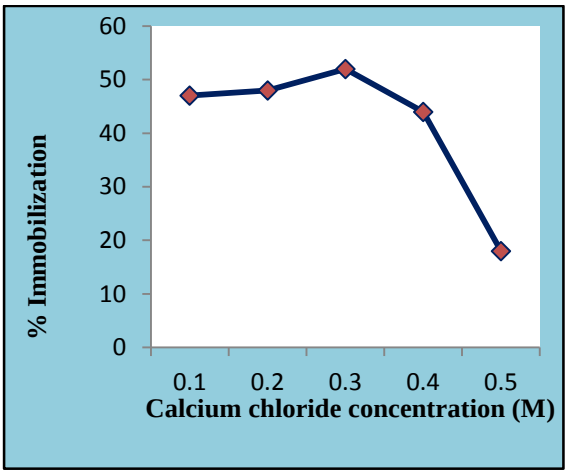
Table-8

Effect of calcium chloride concentrationon immobilization of fish waste protease

Calcium chloride concentration (M)	% Immobilization
0.1	46
0.2	48
0.3	52
0.4	44
0.5	18

Figure-8

Effect of calcium chloride concentration on immobilization of fish waste protease



From the table-8, it was obvious that it can be concluded that all concentrations of sodium alginate and calcium chloride showed entrapped enzyme activity. A concentration of 2% sodium alginate and 0.3 M calcium chloride was found to be the optimum concentration for formation of stable beads with entrapped activity followed by 0.2 (48%), 0.1(46%) and 0.4 (44%)respectively.

DEAE-Cellulose DE-52 was the most suitable for enzyme immobilization which gave the highest activity (25.4U/g carrier) with the highest immobilization yield (44.6%). Entrapment of enzyme in agar and Calcium alginate maximal bound enzyme (68.9U/10ml) and immobilization yield of 35.2% (Samia*et al*., 2007).

4.2 Characterization of purified protease from fish waste

4.2.2 Effect of pH on activity of immobilized protease

The effect of pH on the relative activity immobilized protease enzyme of fish waste is compared in Table-9 and figure-9

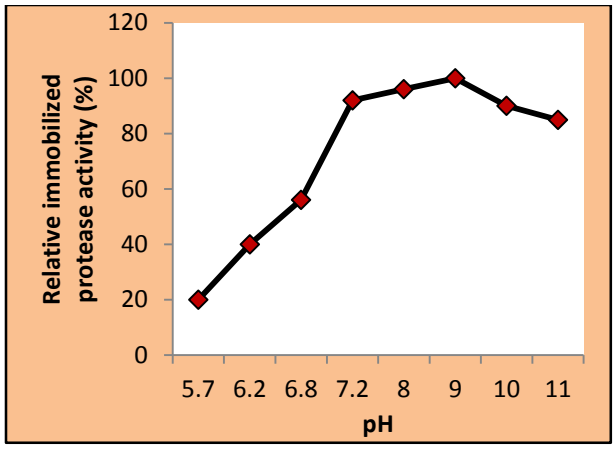
Table-9

Effect of pH on activity of immobilized fish waste protease

pH range	Crude immobilized protease activity of the samples (%)
5.7	20
6.2	40
6.8	56
7.2	92
8.0	96
9.0	100
10.0	90
11.0	85

Figure-9

Effect of pH on activity of immobilized fish waste protease



The activity was studied at pH range of 5.7-11.0 in room temperature. From the table-8 and figure-8, it was evident that, there was a gradual increase in the enzyme that activity from pH 5.7 to pH 9.0and then a gradual decrease wasnoted from 9.0 to 11.0 which concluded the optimum pH for the activity of the crude immobilized protease enzyme is 9.0.

Maghsoodi*et al*., (2013) studied the effect of pH and reported that maximum protease production was obtained at pH=7.6 at 37^0C. The pH ranges used was 7 to 11, and the maximum production of protease was obtained at pH=10. Similar optimum pH has been reported for other proteases in literarure, optimum pH of 9 has been reported for alkaline protease from *Virgibacilluspantothenticus*(Thomas *et al.,* 2007).

4.2.3. Effect of temperature on activity of immobilized protease

Alginate entrapped enzyme was assayed at different temperature ranging 20,30,37, 45,50,60,70 and 80^0C at an optimum pH of 9.0.The properties of the crude immobilized protease enzymes are depicted in Table-10 and Figure-10.

Table-10

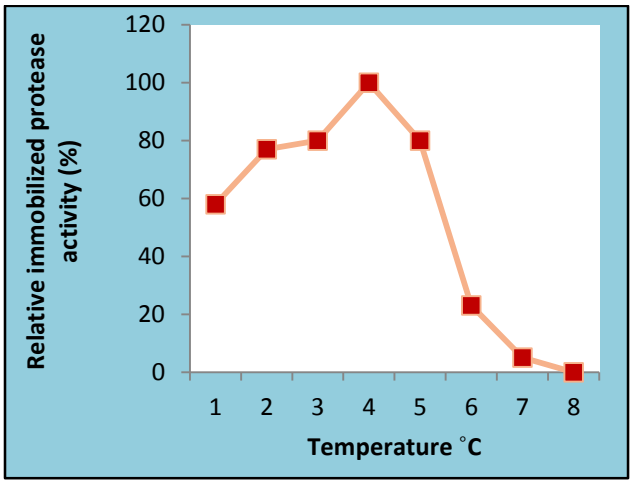
Effect of temperature on activity of immobilized fish waste protease

Temperature ^{0}C	**Crude immobilized protease activity of the samples (%)**
20	58
30	77
37	80

45	100
50	80
60	23
70	10
80	-

Figure-10

Effect of temperature on the activity of immobilized fish waste protease



From the table-10, it was clearly understood that the activity of crude immobilized protease increase from 20^0C to 45^0C and started to from decrease 50^0C. Beyond this temperature, there was gradual decrease in the activity of 80% was absolutely no activity detected. Therefore, it can be concluded that the optimum temperature of the immobilized enzyme 45^0C (100).

Kumari*et al*., (2015) reported that the effect of temperature on protease immobilized on SBA-15 and Nano-ZSM-5. Soluble protease from brinjal exhibited an optimum temperature of 70^0 C whereas immobilized protease exhibited the optimum temperature of 75^0 C.Sahu*et al*., 2016 reported that immobilized protease catalyzed synthesis of AMD-1,AMD-2 and AMD-3 respectively. Observing concluded that an increase the reaction temperature up to the optimum point (50^0 C). NB34 culture and incubated at different incubation temperature viz 30°C, 37°C,40°C for 24 h and the enzyme production was found to be maximum at 37°C (Bharadwaj*et al*., 2014).

4.3. QUALITATIVE STUDIES ON IMMOBILIZED PROTEASE FROM FISH WASTE

The immobilized protease enzymeisolated from fish waste was subjected to few qualitative tests to confirm destaining studies on the different types of cloths namelyPure Cotton,Trapery Fabric Lining and Kala Cotton Fabric and Fabricstained withSoya Sauce, Egg yolk, Blood and Chocolatestained.

4.3.1. Action of immobilized protease on soya sauce stained cloth

The immobilized protease isolated from the fish waste has the ability to digest protein. The result of incubating crude immobilized protease and purified immoblized protease with soya sauce on different cloths were shown in plate-1, plate-2 and plate-3.

Plate-1

Destaining of Soya Sauce on Pure cotton fabric

(a) Soya Sauce Stained cloth **(b) 1% Ariel** **(c) Crudeimmobillized protease**

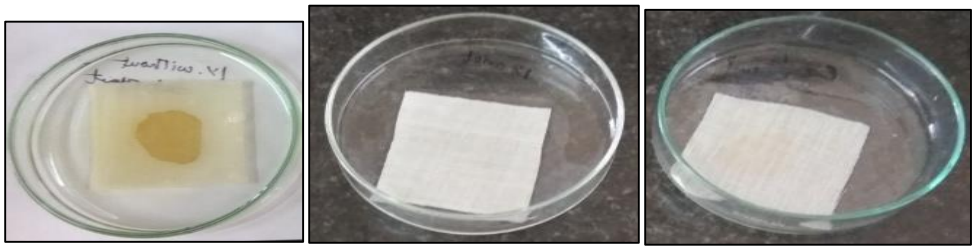
(e)Without protease detergent

(d)purified immobilized protease

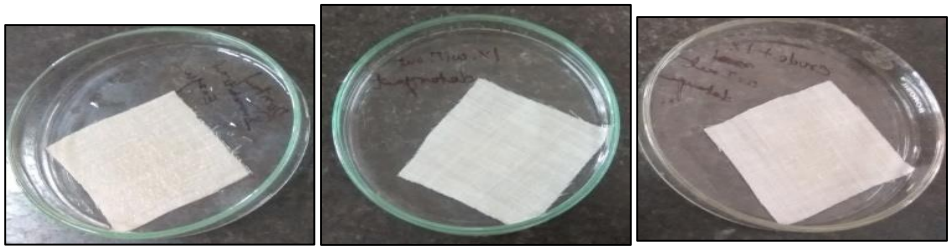
(f)Without protease detergent+crude immobilized

Plate-2

Destaining of Soya Sauce on Trapery fabric lining



(a) Soya Sauce Stained cloth **(b) 1% Ariel** **(c)Crude immobillized protease**



(d) Purified immobilized protease(e) Without protease detergent **(f) Without protease detergent+crude immobilized**

Plate-1 shows the photograph**Soya Sauce**stained **pure cotton cloth** piece

- **Plate-1 (a)** shows the photograph soya sauce stained pure cotton cloth piece that is control
- **Plate-1 (b)** shows the photograph soya sauce stained pure cotton cloth pieces and 1% arielwhich present protease primarily
- **Plate-1 (c)** shows the photograph soya sauce stained pure cotton cloth pieces and crude immobilized protease.
- **Plate-1 (d)** shows the photograph soya sauce stained pure cotton cloth pieces and partiallypurified protease enzyme
- **Plate-1 (e)** shows the photograph soya sauce stained pure cotton cloth pieces and without protease detergent (sun light)
- **Plate-1 (f)** shows the photograph soya sauce stained pure cotton cloth pieces and crude immobilized protease with without protease detergent (sun light)

From above, it was noted that the stains was completely removed when treated which without protease detergent (sun light) with crude immobilized protease and partial purified immobilized protease followed by 1% ariel, without protease detergent alone, crude immobilized protease. It can be concluded that protease has ability to remove the stain within 20 minutes when incubated at room temparature

Plate -2 shows the photograph**Soya Sauce**stained**Trapery Fabric Lining** piece

- **Plate-1 (a)** shows the photograph soya sauce stained pure cotton cloth piece that is control
- **Plate-1 (b)** shows the photograph soya sauce stained pure cotton cloth pieces and 1% arielwhich present protease primarily
- **Plate-1 (c)** shows the photograph soya sauce stained pure cotton cloth pieces and crude immobilized protease.
- **Plate-1 (d)** shows the photograph soya sauce stained pure cotton cloth pieces and partiallypurified protease enzyme
- **Plate-1 (e)** shows the photograph soya sauce stained pure cotton cloth pieces and without protease detergent (sun light)
- **Plate-1 (f)** shows the photograph soya sauce stained pure cotton cloth pieces and crude immobilized protease with without protease detergent (sun light)

From above, it was noted that the stains was completely removed when treated which without protease detergent (sun light) with crude immobilized protease, partial purified immobilized protease and crude immobilized protease followed by 1% ariel, without protease detergent alone,. It can be concluded that protease has ability to remove the stain within 20 minutes when incubated at room temparature

Plate -3 shows the photograph**Soya Sauce**stained**Kala Cotton** piece

- **Plate-1 (a)** shows the photograph soya sauce stained pure cotton cloth piece that is control
- **Plate-1 (b)** shows the photograph soya sauce stained pure cotton cloth pieces and 1% arielwhich present protease primarily
- **Plate-1 (c)** shows the photograph soya sauce stained pure cotton cloth pieces and crude

immobilized protease.

- **Plate-1 (d)** shows the photograph soya sauce stained pure cotton cloth pieces and partiallypurified protease enzyme
- **Plate-1 (e)** shows the photograph soya sauce stained pure cotton cloth pieces and without protease detergent (sun light)
- **Plate-1 (f)** shows the photograph soya sauce stained pure cotton cloth pieces and crude immobilized protease with without protease detergent (sun light)

From above, it was noted that the stains was completely removed when treated which without protease detergent (sun light) with crude immobilized protease and partial purified immobilized protease followed by 1% ariel, without protease detergent alone, crude immobilized protease. It can be concluded that protease has ability to remove the stain within 20 minutes when incubated at room temparature

The remarkable increase in washing efficiency of detergents on addition of enzymes to it was also agreed upon by Kanmani et al. (2011). Esposito et al, (2009) who reported effectiveness of fish protease in laundry detergents.

Plate-3

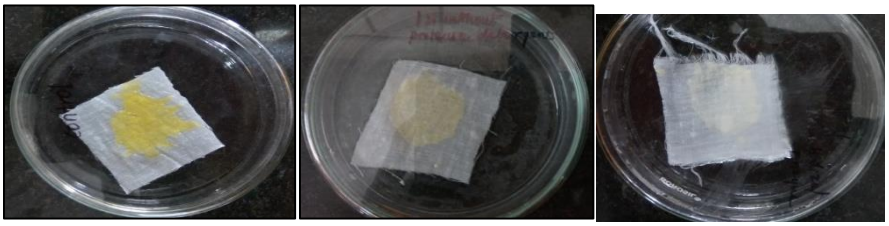
Destaining of Soya sauce on Kala cotton

(a)Soya Sauce Stained cloth(b) 1% Ariel **(c)Crude immobillized protease**

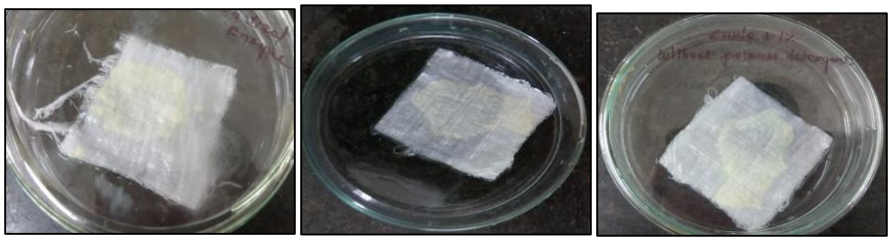
(d) Purified immobilized protease (e) Without protease detergent (f) Without protease detergent+crudeimmobilized

Plate-4

Destaining of Egg yolk on Pure cotton cloth



(a)Egg yolk Stained cloth (b) 1% Ariel (c)Crudeimmobillized protease



(d) Purified immobilized protease (e)Without protease detergent (f)Without protease detergent+crude immobilized

4.3.2. Action of immobilized protease on egg yolkstained cloth

The immobilized protease isolated from the fish waste has the ability to digest albumin. The result of incubating crude immobilized protease and purified immoblized protease with **egg yolk**is shown in plate-4, plate-5 and plate-6

Plate-4 shows the photograph**Egg yolk** stained**Pure cotton cloth** piece

- **Plate-1 (a)** shows the photograph Egg yolkstained pure cotton cloth piece that is control
- **Plate-1 (b)** shows the photograph Egg yolkstained pure cotton cloth pieces and 1% arielwhich present protease primarily
- **Plate-1 (c)** shows the photograph Egg yolkstained pure cotton cloth pieces and crude immobilized protease.
- **Plate-1 (d)** shows the photograph Egg yolkstained pure cotton cloth pieces and partiallydpurified protease enzyme

- **Plate-1 (e)** shows the photograph Egg yolkstained pure cotton cloth pieces and without protease detergent (sun light)
- **Plate-1 (f)** shows the photograph Egg yolkstained pure cotton cloth pieces and crude immobilized protease with without protease detergent (sun light)

From above, it was noted that the stains was completely removed when treated which without protease detergent (sun light) with crude immobilized protease and partial purified immobilized protease followed by 1% ariel, without protease detergent alone, crude immobilized protease. It can be concluded that protease has ability to remove the stain within 20 minutes when incubated at room temparature

- **Plate-5** shows the photograph **Egg yolk** stained **Trapery Fabric Lining** piece
- **Plate-1 (a)** shows the photograph Egg yolkstained pure cotton cloth piece that is control
- **Plate-1 (b)** shows the photograph Egg yolkstained pure cotton cloth pieces and 1% arielwhich present protease primarily
- **Plate-1 (c)** shows the photograph Egg yolkstained pure cotton cloth pieces and crude immobilized protease.
- **Plate-1 (d)** shows the photograph Egg yolkstained pure cotton cloth pieces and partiallypurified protease enzyme
- **Plate-1 (e)** shows the photograph Egg yolkstained pure cotton cloth pieces and without protease detergent (sun light)
- **Plate-1 (f)** shows the photograph Egg yolkstained pure cotton cloth pieces and crude immobilized protease with without protease detergent (sun light)

From above, it was noted that the stains was completely removed when treated which without protease detergent (sun light) with crude immobilized protease and partial purified immobilized protease followed by 1% ariel, without protease detergent alone, crude immobilized protease. It can be concluded that protease has ability to remove the stain within 20 minutes when incubated at room temparature.

Plate-6 shows the photograph**Egg yolk** stained**Kala Cotton** piece

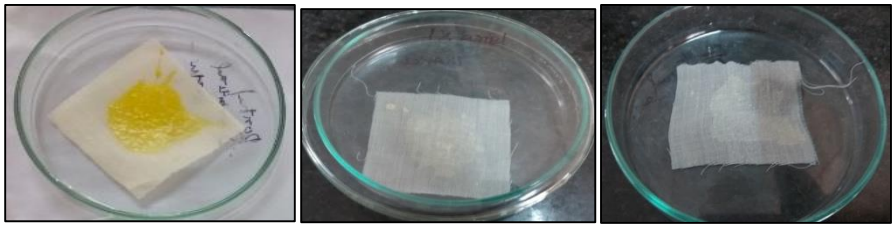
- **Plate-1 (a)** shows the photograph Egg yolkstained pure cotton cloth piece that is control.
- **Plate-1 (b)** shows the photograph Egg yolkstained pure cotton cloth pieces and 1% arielwhich present protease primarily.

- **Plate-1 (c)** shows the photograph Egg yolkstained pure cotton cloth pieces and crude immobilized protease.
- **Plate-1 (d)** shows the photograph Egg yolkstained pure cotton cloth pieces and partiallypurified protease enzyme.
- **Plate-1 (e)** shows the photograph Egg yolkstained pure cotton cloth pieces and without protease detergent (sun light).
- **Plate-1 (f)** shows the photograph Egg yolk stained pure cotton cloth pieces and crude immobilized protease with without protease detergent (sun light).

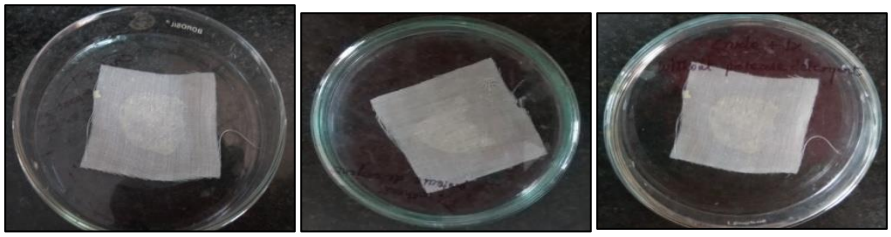
From above, it was noted that the stains was completely removed when treated which without protease detergent (sun light) with crude immobilized protease and partial purified immobilized protease followed by 1% ariel, without protease detergent alone, crude immobilized protease. It can be concluded that protease has ability to remove the stain within 20 minutes..

(Kezia and Naidua, 2016)reported that the destaining activity of the protease from Bacillus subtilis DKMNR as a detergent additive was studied on white cotton cloth pieces measuring 3x3 m2 staining with blood, egg yolk and chocolate. Purified enzyme removed stain within 15 min.This protease solution from *B. subtilis* DKMNR showed high capability for removing stains from cloths therefore it could be used as an alkaline protease additive in detergent manufacturing industry

Plate-5
Destaining of Egg yolk on Trapery fabric lining



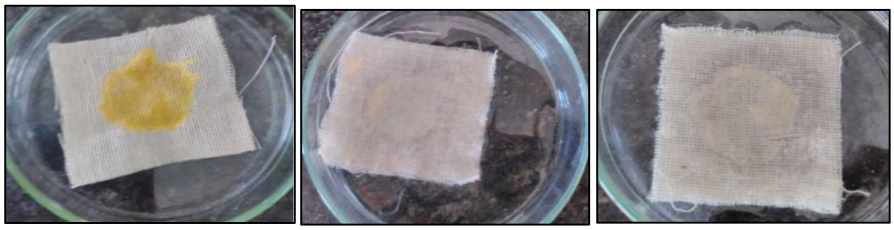
(a)Egg yolkStained cloth **(b) 1% Ariel** **(c)Crudeimmobillized protease**



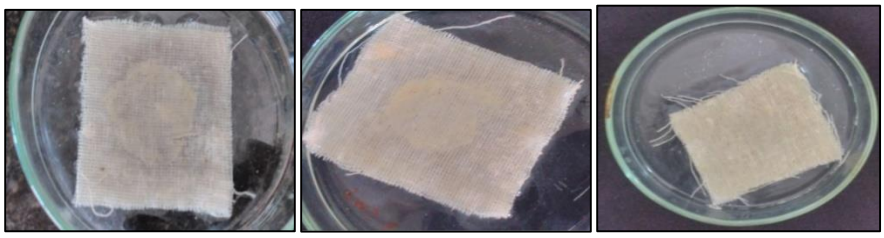
(d) Purified immobilized protease(e)Without protease detergent (f)Without protease detergent+crude immobilized

Plate-6

Destaining of Egg yolk on Kala cotton



(a)Egg yolk Stained cloth (b)1% Ariel (c)Crudeimmobillized Protease



(d) Purified immobilized protease(e)Without protease detergent (f)Without protease detergent+crude immobilized

4.3.3. Action of immobilized protease on blood stained cloth

The immobilized protease isolated from the fish waste has the ability to digest albumin. The result of incubating crude immobilized protease and purified immoblized protease with human blood is shown in plate-7, plate-8 and plate-9.

Plate -7 shows the photograph**Blood** stained **Pure Cotton Fabric** piece

- **Plate-1 (a)** shows the photograph bloodstained pure cotton cloth piece that is control
- **Plate-1 (b)** shows the photograph bloodstained pure cotton cloth pieces and 1% arielwhich present protease primarily

- **Plate-1 (c)** shows the photograph bloodstained pure cotton cloth pieces and crude immobilized protease.
- **Plate-1 (d)** shows the photograph bloodstained pure cotton cloth pieces and partiallypurified protease enzyme
- **Plate-1 (e)** shows the photograph bloodstained pure cotton cloth pieces and without protease detergent (sun light)
- **Plate-1 (f)** shows the photograph bloodstained pure cotton cloth pieces and crudeimmobilized protease with without protease detergent (sun light)

From above, it was noted that the stains was completely removed when treated which without protease detergent (sun light) with crude immobilized protease and partial purified immobilized protease followed by 1% ariel, without protease detergent alone, crude immobilized protease. It can be concluded that protease has ability to remove the stain within 20 minutes when incubated at room temparature

Plate-8 shows the photograph**Blood** stained**Trapery Fabric Lining** piece

- **Plate-1 (a)** shows the photograph bloodstained pure cotton cloth piece that is control
- **Plate-1 (b)** shows the photograph bloodstained pure cotton cloth pieces and 1% arielwhich present protease primarily
- **Plate-1 (c)** shows the photograph bloodstained pure cotton cloth pieces and crude immobilized protease.
- **Plate-1 (d)** shows the photograph bloodstained pure cotton cloth pieces and partiallypurified protease enzyme
- **Plate-1 (e)** shows the photograph bloodstained pure cotton cloth pieces and without protease detergent (sun light)
- **Plate-1 (f)** shows the photograph bloodstained pure cotton cloth pieces and crude immobilized protease with without protease detergent (sun light)

From above, it was noted that the stains was completely removed when treated which without protease detergent (sun light) with crude immobilized protease and partial purified immobilized protease followed by 1% ariel, without protease detergent alone, crude immobilized protease. It can be concluded that protease has ability to remove the stain within 20 minutes when incubated at room temparature

- **Plate-9**shows the photograph **Blood** stained **Kala Cotton** piece
- **Plate-1 (a)** shows the photograph bloodstained pure cotton cloth piece thatis control
- **Plate-1 (b)** shows the photograph bloodstained pure cotton cloth pieces and 1% arielwhich present protease primarily
- **Plate-1 (c)** shows the photograph bloodstained pure cotton cloth pieces and crude immobilized protease.
- **Plate-1 (d)** shows the photograph bloodstained pure cotton cloth pieces and partiallypurified protease enzyme
- **Plate-1 (e)** shows the photograph bloodstained pure cotton cloth pieces and without protease detergent (sun light)
- **Plate-1 (f)** shows the photograph bloodstained pure cotton cloth pieces and crude immobilized protease with without protease detergent (sun light)

From above, it was noted that the stains was completely removed when treated which without protease detergent (sun light) with crude immobilized protease and partial purified immobilized protease followed by 1% ariel, without protease detergent alone, crude immobilized protease. It can be concluded that protease has ability to remove the stain within 20 minutes when incubated at room temparature

Similar findings were observed by the result revealed that bloodstains were completely removed with a combination of polyacrylamide-immobilized enzyme and its most compatible detergent (1 % Ariel) in less than 25 min. However, individual treatment of distilled water, immobilized enzyme and detergent (1 % Ariel) was not able to remove bloodstain even after 30 min. It is similar to the result reported by (Guleria*et al*.,2016).

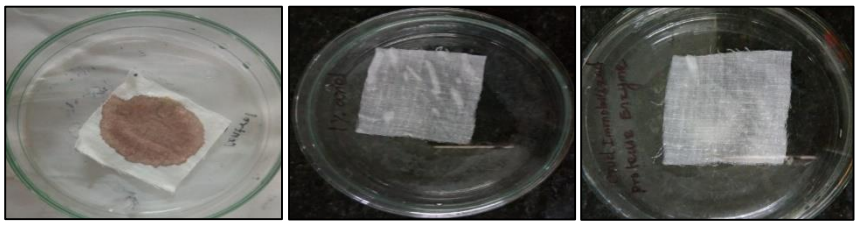
Removing blood and natural pigment stains from cloth, this protease shows great efficiency. Due to its higher potential in removing stain from cloth, it could be used in detergent as a powder or solution was reported by (Vijayalakshmi*et al.,* 2011).

Sabtecha*et al*.,(2014) reported that crude enzyme extract of the Red snapper and Great barracuda removed the blood stains effectively within 20 min, without the usage of any detergents. It was noticed that the alkaline crude extract from fish visceral wastes was more effective in destaining, when compared to partially purified protease observations.

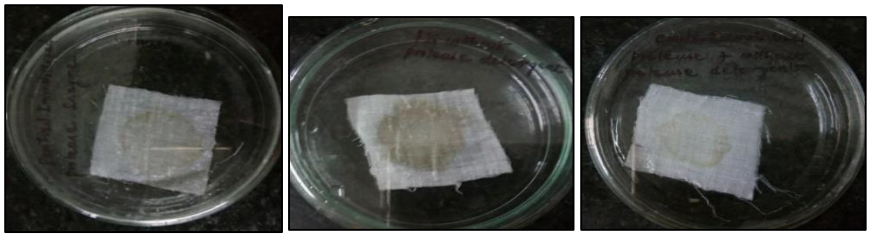
Similar findings were observed bySakpal*et al*. (2015)Blood stained fabric pieces were subjected to wash treatment at 55^0C for 30mins. The supplementation of the thermostable alkaline protease was carried by *Bacillus licheniformis* preparation in detergents like Ariel, Rin and Tide significantly improved the cleansing performance towards the blood stain.

Plate-7

Destaining of Blood on Pure Cotton fabric

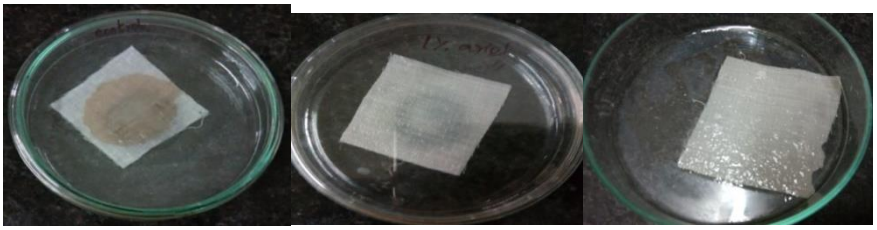


(a)BloodStained cloth **(b) 1% Ariel** **(c)Crudeimmobillized protease**

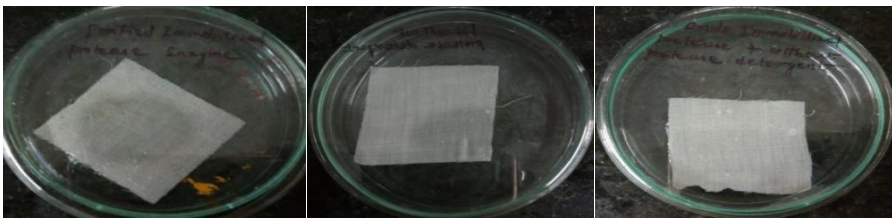


(d)Purified immobilized protease(e)Without protease detergent (f)Without protease detergent+crude immobilized

Plate-8
Destaining of Blood on Trapery fabric lining

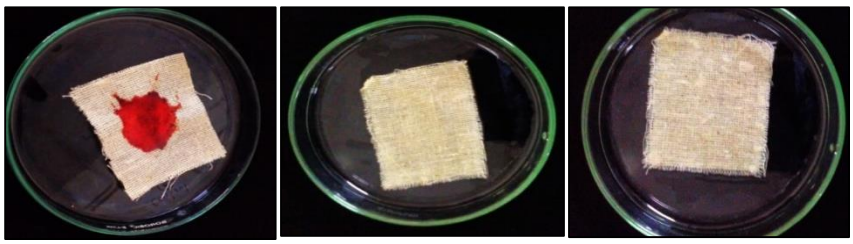


(a) Blood Stained cloth (b) 1% Ariel (c)Crudeimmobillized protease

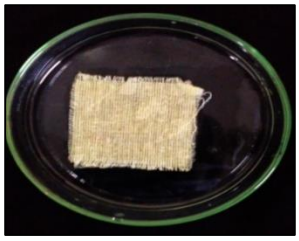
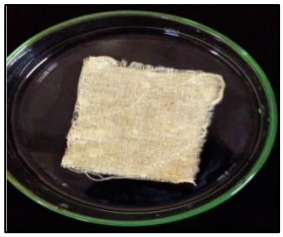
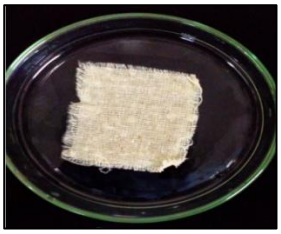


(d) Purified immobilized protease(e)Without protease detergent(f)Without protease detergent+crude immobilized

Plate-9
Destaining of Blood on Kala Cotton fabric

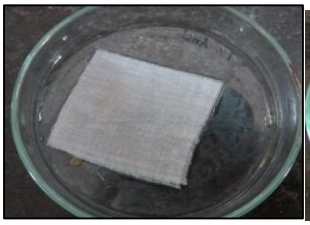
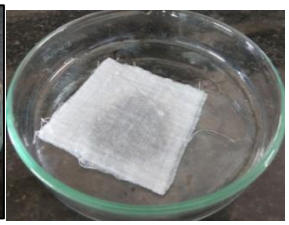


(a)Blood Stained cloth (b) 1% Ariel (c)Crudeimmobillized protease

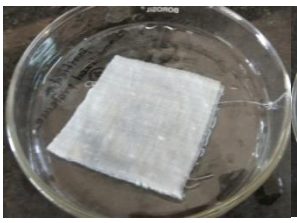
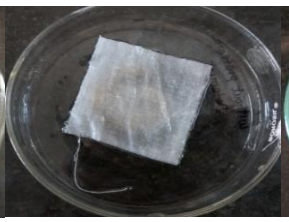
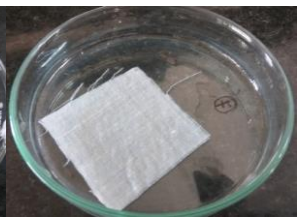




(d)Purified immobilized protease(e)Without protease detergent(f)Without protease detergent+crude immobilized

Plate-10
Destaining of Chocolate on Pure cotton fabric

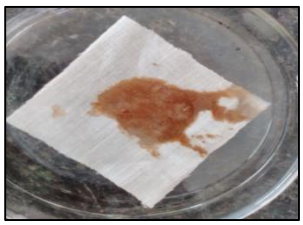
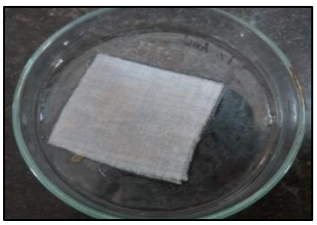
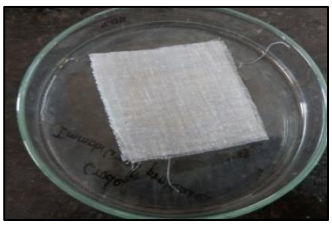




(a) ChocolateStained cloth **(b) 1% Ariel** **(c)Crudeimmobillized protease**

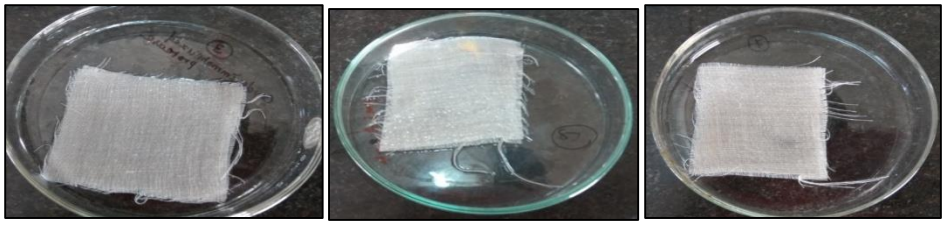




(d)Purified immobilized protease **(e) Without protease detergent** **(f) Without protease detergent+crudeimmobilized**

Plate-11
Destaining of Chocolate on Trapery fabric lining

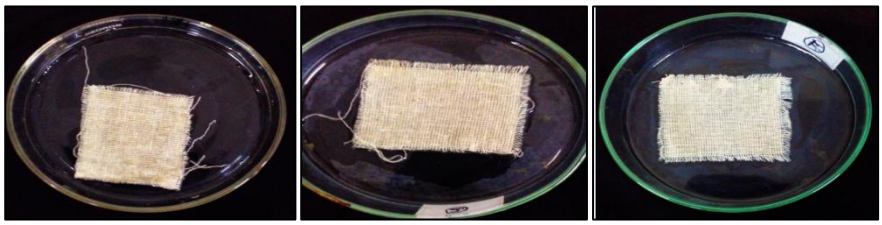
(a)ChocolateStained cloth **(b) 1% Ariel** **(c)Crudeimmobillized protease**



(d)Purified immobilized protease **(e)Without protease detergen** **(f)Without protease detergent+crude immobilized**

Plate-12
Destaining of Chocolate on Kala cotton fabric

(a)ChocolateStained cloth **(b)1% Ariel** **(c)Crudeimmobillized protease**



(d) Purified immobilized protease(e)Without protease detergent(f)Without protease detergent+crude immobilized

4.3.4. Action of immobilized protease on chocolate stained cloth

The immobilized protease isolated from the fish waste has the ability to digest protein. The result of incubating crude immobilized protease and purified immoblized protease with chocolate is shown in plate-10, plate-11 and plate-12.

Plate-10 shows the photograph**chocolate** stained**Pure cotton fabric** piece

- **Plate-1 (a)** shows the photograph chocolatestained pure cotton cloth piece that is control.
- **Plate-1 (b)** shows the photograph chocolatestained pure cotton cloth pieces and 1% ariel

which present protease primarily.

- **Plate-1 (c)** shows the photograph chocolatestained pure cotton cloth pieces and crude immobilized protease.
- **Plate-1 (d)** shows the photograph chocolatestained pure cotton cloth pieces and partially purified protease enzyme.
- **Plate-1 (e)** shows the photograph chocolatestained pure cotton cloth pieces and without protease detergent (sun light).
- **Plate-1 (f)** shows the photograph chocolatestained pure cotton cloth pieces and crude immobilized protease with without protease detergent (sun light).

From above, it was noted that the stains was completely removed when treated which without protease detergent (sun light) with crude immobilized protease and partial purified immobilized protease followed by 1% ariel, without protease detergent alone, crude immobilized protease. It can be concluded that protease has ability to remove the stain within 20 minutes when incubated at room temparature

Plate- 11 shows the photograph**chocolate** stained**Trapery fabric lining** piece

- **Plate-1 (a)** shows the photograph chocolatestained pure cotton cloth piece that is control
- **Plate-1 (b)** shows the photograph chocolatestained pure cotton cloth pieces and 1% arielwhich present protease primarily
- **Plate-1 (c)** shows the photograph chocolatestained pure cotton cloth pieces and crude immobilized protease.
- **Plate-1 (d)** shows the photograph chocolatestained pure cotton cloth pieces and partiallypurified protease enzyme
- **Plate-1 (e)** shows the photograph chocolatestained pure cotton cloth pieces and without protease detergent (sun light)
- **Plate-1 (f)** shows the photograph chocolatestained pure cotton cloth pieces and crude immobilized protease with without protease detergent (sun light)

From above, it was noted that the stains was completely removed when treated which without protease detergent (sun light) with crude immobilized protease and partial purified immobilized protease followed by 1% ariel, without protease detergent alone, crude immobilized

protease. It can be concluded that protease has ability to remove the stain within 20 minutes when incubated at room temparature

Plate -12 shows the photograph**chocolate** stained**Kala cotton** piece

- **Plate-1 (a)** shows the photograph chocolatestained pure cotton cloth piece that is control
- **Plate-1 (b)** shows the photograph chocolatestained pure cotton cloth pieces and 1% arielwhich present protease primarily
- **Plate-1 (c)** shows the photograph chocolatestained pure cotton cloth pieces and crude immobilized protease.
- **Plate-1 (d)** shows the photograph chocolatestained pure cotton cloth pieces and partiallypurified protease enzyme
- **Plate-1 (e)** shows the photograph chocolatestained pure cotton cloth pieces and without protease detergent (sun light)
- **Plate-1 (f)** shows the photograph chocolatestained pure cotton cloth pieces and crude immobilized protease with without protease detergent (sun light)

From above, it was noted that the stains was completely removed when treated which without protease detergent (sun light) with crude immobilized protease and partial purified immobilized protease followed by 1% ariel, without protease detergent alone, crude immobilized protease. It can be concluded that protease has ability to remove the stain within 20 minutes when incubated at room temparature

Sekar*et al*,(2016) reportedthatprotease produced by *Bacillus sp.* Mk22 in this study significantly removed blood, ink and coffee strains at 40°C

The crude immobilized protease and purified immobilized protease were subjected to qualitative tests to confirm its ability to digest the stains by soya sauce, egg yolk, blood and chocolate on different type of cloth.

The results showed that the immobilized protease dissolved soya sauce, egg yolk, blood and chocolate and removed stains from different types cloth (pure cotton, trapery fabric lining and kala cotton).

5.0 SUMMARY AND CONCLUSION

Fish waste is one of the most important by-products of the fishing industry with a wide biotechnological potential as a 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 have made them suitable for diverse applications in a wide variety of industries such as detergent, food, agrochemical and pharmaceutical industries. In addition, fish enzymes are utilized to produce bioactive peptides

from fish proteins. Considering the specific characteristics of these enzymes, fish processing by-products are currently used for enzyme extraction.

Hence, the present study entitle **"Application Studies of Immobilized Protease from the Waste of Fish in Detergent Industry"** was carried out with the objectives to isolate and purify the protease from the fish wastes, to characterize and immobilize protease and to study the applications of the isolated protease by qualitative tests to confirm destaining studies on different types of cloths. The fish wastes were collected from a fish industry, Cochin, Kerala, India. Crude homogenate was prepared and precipitation was carried out with ammonium sulphate (0-30, 30-60 and 60-90%).

The results showed that the ammonium sulphate precipitation especially 30-60% the highest protease activity (1.0 U/ml), protein content (0.91 mg/ml) and higher specific activity (1.02 U/mg).

Crude and purified protease enzyme was immobilized using various concentrations of sodium alginate and calcium chloride to optimize the best concentration for formation of the beads.Then it was characterized to check the optimal pH and temperature.

The results on immobilization with sodium alginate and calcium chloride showed that a combination of 2 % sodium alginate and 0.3 M calcium chloride were found to be the optimum concentration for the formation of beads.Crude immobilized protease was found to have optimum pH at pH 9.0 and optimum temperature at 45^0C.

The crude immobilized protease and purified immobilized protease were subjected to qualitative tests to confirm its ability to digest the stains by soya sauce, egg yolk, blood and chocolate on different type of cloth.

The results showed that the immobilized protease dissolved soya sauce, egg yolk, blood and chocolate and removed stains from different types cloth (pure cotton, trapery fabric lining and kala cotton).

It can thus be concluded that the fish waste contains protease enzymes could be beneficial thereby reducing the pollution caused by disposal of fish waste.

Recommendations for future study

- Purification of protease by Ion exchange chromatography and Gel filtration

chromatography

- Enzymes other than proteases like amylase, cellulase, chitinase, collagenase and phosphatase can be isolated from fish waste and completely studied.
- Purified protease can be used to different types of other cloths

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APPENDIX I

Preparation of crude homogenate

(El Hadj Ali *et al.*, 2009)

Materials/Reagents

1. Homogenizer
2. Fish waste (from recently killed fish)
3. Homogenization buffer - 20 mMTrisHCl buffer, pH 7.8

Procedure

1. Collect the visceral organs, head and tail wastes from the fish and clean with distilled water
2. Weigh and cut the tissues into small pieces.
3. Homogenize the pieces with 200 ml of homogenization buffer.
4. Centrifuge the homogenate at 8,500×g for 30 minutes at 4^0C.
5. Discard the pellet and collect the supernatant and use as the crude homogenate

APPENDIX II

Precipitation of proteins by ammonium sulphate fractionation

(Simpson, 2004)

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 can be brought about either by adding solid substance or by adding solution of known saturation, generally a fully saturated (100%) solution.

Materials/Reagents

1. Ammonium sulphate – Add ammonium sulphate to the protein solution as solid
2. Resuspension buffer – Resuspend the concentrated protein with 20 mMTris-HCL 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 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 and allow the mixture to stand for 60 minutes 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 10,000 g for 15 minutes at 4^{o}C.
7. Decant the supernatant solution, saving both the protein pellet (0- 30%) and the supernatant.
8. Return the supernatant to the cold room and slowly add ammonium sulphate to the supernatant to achieve a concentration of 30-60%. Stir the mixture for 60 minutes to ensure complete precipitation.
9. Transfer the solution to the appropriate centrifuge tubes and centrifuge the solution at 10,000 g for 15 minutes at 4^{o}C.
10. Decant the supernatant solution, saving both the protein pellet (30- 60% fraction) and the supernatant.
11. Repeat steps 8-10, adding ammonium sulphate to concentrations of 60- 90%, and so on, up to 100% saturation, removing precipitated proteins by centrifugation at each step before increasing the ammonium sulphateconcentration.
12. Resuspend the recovered protein pellets (0-30, 30-60 and 60-100%) in resuspension buffer (20 mMTrisHCl buffer, pH 7.8) and assay them for total proteins.

S.No	**Final concentration (%)**	**Ammonium sulphate (g/ 100 ml aliquots)**
1.	0-30	16.6
2.	30-60	18.4
3.	60-100	20.4

APPENDIX - III

Enzymatic assay of protease

(Anson., 1938; Folin and Ciocalteau, 1927)

Principle

Protease hydrolyses casein in the presence of water to give amino acid.

Casein + H_2O Amino Acids $\rightarrow$

Materials/Reagents

Reagent A: 20 mMTrisHCl buffer, pH 7.8 at 37^0C.

Reagent B: 0.65% Casein solution

0.65 g casein dissolved in 100 ml of Reagent A. Adjust the pH to 7.8.

Reagent C: 110 mMTrichloroacetic acid reagent (TCA)

1.8 g of TCA dissolved in 100 ml of deionized water.

Reagent D: FolinCiocalteu's Phenol Reagent

Dilute 10 ml FolinCiocalteau's Phenol Reagent to 40 mlwith deionized water

Reagent E: 500mM Sodium carbonate Solution.

5.3 g of sodium carbonate dissolved in 100 ml ofdeionized water.

Reagent F: Enzyme solutions: crude extract, ammoniumsulphate precipitated

Reagent G: 1 mM L- Tyrosine Standard

Dissolve 0.0199 g of L-Tyrosine in 100 ml of deionized waterand heat gently (do not boil) until the tyrosine dissolves and cool to room temperature.

Procedure

Pipette the following reagents into suitable vials (in milliliters)

S.No	Reagents	Test (ml)	Blank (ml)
1.	Reagent B (Casein)	5.0	5.0

Equilibrate to 37 °C.			
2.	Reagent F (enzyme solution)	1.0	-
Mix by swirling and incubate at 37 °C for about 10 minutes			
3.	Reagent C (TCA)	5.0	5.0
4.	Reagent F(Enzyme solution)	-	1.0
Mix by swirling and incubate at 37 °C for about 30 minutes			
5.	Filter through Whatman filter paper No. 50 or 0.45 µm and use the filtrate for colour development		

Colour development

Prepare a standard curve by pipetting the following reagents into suitable vials (in milliliters).

S.No	**Reagents**	**Standard 1**	**Standard 2**	**Standard 3**	**Standard 4**	**Blank**
1.	Reagent G (standard solution)	0.05	0.10	0.20	0.40	0.00
2.	Deionized water	1.95	1.90	1.80	1.60	2.00
3.	Reagent E (sodium carbonate)	5.00	5.00	5.00	5.00	5.00
4.	Reagent D (F-C)	1.00	1.00	1.00	1.00	1.00

Sample

Pipette the following reagents into 4 vials (in milliliters):

S.No	Reagents	Test (ml)	Blank (ml)
1.	Test filtrate	2.00	-
2.	Blank filtrate	-	2.00
3.	Reagent E (Sodium carbonate)	5.00	5.00
4.	Reagent D (F-C)	1.00	1.00

2. Mix by swirling and incubate at 37°C for 30 minutes.

3. Remove the vials and allow them to cool to room temperature. Filter through a 0.45 µm filter immediately prior to reading. Read the absorbance at 660 nm for each of the vials in Suitablecuvettes.

Calculation

Standard curve:

δA 660 nm Standard = A660 nm standard – A660 nm standard blank Plot the standard δA 660 nm Standard Vs µmoles of tyrosine

Sample Determination:

δA 660 nm Sample = A 660 nm Test – A 660 nm Sample Blank

Determine the µmoles of tyrosine equivalents liberated using the standard curve

$$\text{Units / ml enzyme} = \frac{(\mu\text{Mole tyrosine equivalents released}) \times (\text{Total volume in milliliters of assay})}{(\text{Time of assay in minutes as per the unit definition}) \times (\text{Volume of enzyme in milliliters}) \times (\text{Volume used in colorimetric Determination})}$$

$$\text{Units / mg solid} = \frac{\text{Units / ml enzyme}}{\text{mg solid / ml enzyme}}$$

$$\text{Specific Activity (or) Units / mg protein} = \frac{\text{Units/ml enzyme}}{\text{mg protein / ml enzyme}}$$

Unit definition:

One unit will hydrolyze casein to produce color equivalent to 1.0 µmole (181µg) of tyrosine per minute at pH 7.5 at 37^0C.

Final Assay Concentration:

In a 6 ml reaction mix, the final concentrations are 42 mM potassium phosphate, 0.54% (w/v) casein, 1.7 mM sodium acetate, 0.8 mM calcium acetate and 0.1 – 0.2 unit protease

APPENDIX -IV

Estimation of protein

(Lowry *et al.*, 1951)

Principle

The blue colour developed by the reduction of the phosphomolybdic – phosphotungstic components in the Folin – Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartarate are measured by the Lowry‘s method .

Reagents

- 2% Sodium Carbonate in 0.1 N in Sodium Hydroxide (Reagent A)
- % Copper Sulphate in 1% Potassium Sodium Tartarate (Reagent B)
- Alkaline Copper Solution : Mix 50 ml Reagent A and 1ml of Reagent B prior to use (Reagent C)
- Folin – Ciocalteau reagent (Reagent D)
- Protein solution (Stock standard)

  Weighed accurately 50 mg of bovine serum albumin (fraction V) and dissolved in distilled water and made up to 50 ml in a standard flask.

- Working standard

Diluted 10 ml of the stock solution to 50 ml distilled water in a standard flask. 1ml of this solution contains 200 µgprotein.

Procedure

1. Pipetted out 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard into a series of test tubes.
2. Pipetted out 0.5 ml of the sample in other tubes. Made up the volume to 1 ml in all the test tubes.
3. A tube with 1ml of water served as the blank. Added 5 ml of Reagent C to each tube including the blank.
4. Mixed well and allowed to stand for 10 minutes. Then added 0.5 ml of Reagent D, mixed well and incubated at room temperature in the dark for 30 minute.
5. Blue colour developed. Took the readings at 660 nm. Drew a standard graph and calculate the amount of protein in the samples.

APPENDIX –V

Immobilized of protease

(Chellapandi, 2007)

Principle

An immobilized is one that has been made insoluble or held in place so that it can be reused many times. Once immobilized, an enzyme's stability is increased, possibly because its ability to change shape is reduced.Once method of immobilized is the entrapment method, in which the enzyme is enclosed within a gel (e.g. alginate beads). The opening in the encapsulating material have to be of sufficient size to permit the substrate or reactant to pass through to the enzyme and not allow the enzyme to leak out .The most common entrapment method is the use of calcium alginate beads. Mixture is dropped into a solution of calcium chloride. The calcium ions replace

the sodium ions and crosslink the polysaccharide. The result is the production of insoluble calcium alginate beads containing trapped enzyme.

$$2Na - Alginate + Ca^{2+} \longrightarrow Ca\ (Alginate)_2 + 2Na+$$

Reagents

1. Purified protease solution
2. 4% Sodium alginate (modified to 2%)
   Dissolve 2 g of sodium alginate in 100 ml hot water. Stir the contents vigorously for 10 min to obtain thick uniform slurry without any undissolved lumps.
3. 0.2 M Calcium chloride (modified to 0.3 M Calcium chloride)
4. Syringe (10 ml) fitted with a wide bore needle approximately 1mm diameter for droplet formation
5. Magnetic stirrer

Procedure

1. Mix equal volumes of sodium alginate (2%) and purified enzyme solution in 1:1 ratio.
2. Introduce the mixture (protease- alginate mixture) drop wise via a 10ml syringe from a height of about 20 cm into an excess of calcium chloride (0.3 M)
3. Leave the beads of calcium alginate- entrapped enzyme to harden in the calcium chloride solution for about 20 minutes.

APPENDIX –VI

Immobilized enzyme assay

(Li *et al.,* 2006 and Anwar *et al*.,2009)

[Modified procedure]

The activity of free protease is determined by the method of Anson*et al,* (1938) using casein as substrate. For immobilized protease, the method has been modified as follows.

1. To 0.5 g of dry immobilized protease, add five milliliters of 0.65% casein solution in 25 mMTris- HCL buffer (pH 8.0)

2. Incubate at 25^0 C for 30 minutes and add 110 mM TCA to precipitate out the undigested protein

3. Neutralized the filtrate with 500mM sodium carbonate and measure the absorbance at 660nm.

Calculation

Initial activity of the free enzyme = 'a'

Volume of enzyme solution ='b'

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

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

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

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

Therefore total enzyme activity after entrapment= 'x %'

APPENDIX-VII

Determination of immobilized enzyme activity with varying concentration of sodium alginate

(Chellapandi, 2007) (Modified procedure)

Reagents / Materials

1.Purified protease solution

2.Sodium alginate (1,2,3,4 and 5%)

3. 0.2 M Calcium chloride

4. Syringe (10ml) fitted with a wide bore needle approximately 1mm diameter for droplet

formation

5. Magnetic stirrer

Procedure

1. Mix equal volumes of sodium alginate (1,2,3,4 and 5%) and purified enzyme solution in 1:1 ratio.
2. Introduce the mixture (protease- alginate mixture) dropwise via a 10ml syringe from a height of about 20 cm into an excess of calcium chloride(0.2 M)
3. Leave the beads of calcium alginate entrapped enzyme to harden in the calcium chloride solution for about 20 minutes
4. Assay the activity of immobilized enzyme as explained in Appendix-VI

APPENDIX-VIII

Determination of immobilized enzyme activity with varying Concentration of Calcium chloride (Chellapandi, 2007) (Modified procedure)

Reagents

1. Purified protease solution (Precipitation of protein by ammonium sulphate fractionation and crude immobilized protease)
2. 2% sodium alginate
3. Calcium chloride (0.1,0.2,0.3,0.4 and 0.5M)
4. Syringe (10ml) fitted with a wide bore needle approximately 1mm diameter for droplet formation
5. Magnetic stirrer

Procedure

1. Mix equal volume of 2% sodium alginate and purified enzyme solution in 1:1 ratio.
2. Introduce the mixture (protease-alginate mixture) dropwise via a 10ml syringe from a height of about 20 cm into excess of calcium chloride (0.1,0.2,0.3,0.4 and 0.5M)
3. Leave the beads of calcium alginate entrapped enzyme to harden in the calcium chloride solution for about 20 minutes.

4. Assay the activity of immobilized enzyme as explained in Appendix-VI

APPENDIX- IX

Effect of pH immobilized protease

(Wang *et al*., 2010) [Modified procedure]

Aim:

To characterize immobilized protease by determining its optimum pH

Reagents / Materials

1. **Substrate :** 1% Starch solution
2. **Activator :** 1% Sodium chloride
3. **Inhibitor :** 2N Sodium hydroxide – 0.8g of Sodium hydroxide dissolved in 100ml of water
4. **Coloring reagents (Dinitrosalicylic acid - DNS) :**Weigh 30.0g of Sodium potassium tartarate, 1.0g of 3,5-DNS and 1.6g of Sodium hydroxide and make up to 100ml with distilled water.
5. **0.2M phosphate buffer (pH 6.8) - Solution A :**Weigh 28.392g of Disodium hydrogen phosphate and dissolve in 1L of water. **Solution B:** Weigh 31.102g of sodium dihydrogen phosphate and dissolve in 1L of water.
6. **Enzyme samples:** Dilute 1.0ml of the enzyme solution to 20.0ml with distilled water.

Procedure

1. Take a series of test tubes for experiment and control. Add 2.5ml of buffer of varying pH (5.7, 6.2, 6.8, 7.2, 8.0, 9.0, 10 and 11.0) followed by 2.5ml of substrate, 1ml of activator and 1ml of water.
2. Preincubated the tubes at 37^0C for 10minutes.
3. Now to the experimental tubes, added 0.5ml of the enzyme and then incubate all the tubes at 37^0C for 15minutes.

4. Arrested the reaction by adding 0.5ml of 2N NaOH to all the test tubes including the control.
5. Then added 0.5ml of enzyme to the control tubes alone followed by 0.5ml of DNS to all the tubes.
6. Heated the tubes in a boiling water bath for 10minutes. Read the color developed at 520nm using a green filter.
7. Plot a graph taking pH on the x-axis and optical density on the y-axis and determined the optimum pH of the enzymes.

APPENDIX – X

Effect of temperature on immobilized protease

(Wang *et al*., 2010) [Modified procedure]

Aim:

To characterize immobilized protease by determining its optimum temperature.

Reagents / Materials

1. **Substrate :** 1% Starch solution
2. **Activator :** 1% Sodium chloride
3. **Inhibitor :** 2N Sodium hydroxide – 0.8g of Sodium hydroxide dissolved in 100ml of water
4. **Coloring reagents (Dinitrosalicylic acid - DNS) :**Weighed 30.0g of Sodium potassium tartarate, 1.0g of 3,5-DNS and 1.6g of Sodium hydroxide and made up to 100ml with distilled water.
5. **0.2M phosphate buffer (pH 6.8) - Solution A :**Weighed 28.392g of Disodium hydrogen phosphate and dissolved in 1L of water. **Solution B:** Weighed 31.102g of sodium dihydrogen phosphate and dissolved in 1L of water.
6. **Enzyme samples:** Diluted 1.0ml of the enzyme solution to 20.0ml with distilled water.

Procedure

1. Take a series of test tubes – one set for the experiment and another set for the control.

2. To these added 2.5ml of buffer prepared at pH 6.8 followed by 2.5ml of substrate, 1.0ml of activator and 1ml of distilled water.
3. Pre incubated the tubes at varying temperature (20^0C, 30^0C, 37^0C, 45^0C and 50^0C) for 10minutes.
4. Now to the experimental tubes added 0.5ml of the enzyme and incubated all the tubes at varying temperature (20^0C, 30^0C, 37^0C, 45^0C and 50^0C) for 15 minutes.
5. Arrested the reaction by adding 0.5ml of 2N NaOH to all test tubes including the controls.

APPENDIX-XI
Testing of protease for destaining
(Najafi*et al.*, 2005)

PRINCIPLE

Protease has the ability to destain the stained cloth

MATERIALS / REAGENTS:

1. Distilled water
2. 2% formaldehyde
3. Detergent powder
4. Soya sauce,blood, chocolate, egg yolk
5. Cotton cloth pieces
6. Immobilized protease enzyme

Procedure

Stain five clean pieces of white cloth (5x5 cm) with soya sauce and prepare the following sets to study the destaining property of protease.

i. stained cloth dipped in flask with distilled water (100 ml)+ 1% Ariel detergent (7mg ml-1)

ii. stained cloth dipped in flask with distilled water (100 ml) + + 2 ml crude immobilized protease enzyme solution.

iii. stained cloth dipped in flask with distilled water (100 ml)+2 ml purified immobilized protease enzyme solution

iv. Stained cloth dipped in flask with distilled water (100 ml) +1 ml without protease detergent (Sun Light)

v.Stained cloth dipped in flask with distilled water (100 ml) +1 ml without protease detergent (Sun Light) + 2 ml crude immobilized protease enzyme solution.

2. Incubate all the four flasks at 60˚C for 15 minutes.

3. After incubation, rinse cloth pieces with water and then dryObserve the cloth for destaining