Isolation of Microorganisms and Production of Microbial Enzymes from the Waste of *Catla catla* Fish

S. PAVITHRA

(13PBT007)

Thesis submitted to

Avinashilingam Institute for Home Science and Higher Education for Women,

Coimbatore – 641043

In partial fulfillment of the requirement for the degree of

MASTER OF SCIENCE IN BIOTECHNOLOGY

MARCH 2015

CERTIFICATE

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Signature of the Head of Department

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Signature of the Supervisor

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‘‘GRATITUDE IS THE MEMORY OF THE HEART’’

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LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
CI	Catla Intestine
CV-I	Crystal Violet-Iodine Complex
FAO	Food and Agriculture Organization
MR-VP	Methyl red- Voges proskaur
TSI	Triple Sugar Iron
VP	Voges Proskaur

INTRODUCTION

1.0. INTRODUCTION

In recent years, there has been constant increase in the exploitation of fish resources. Globally, the estimated quantity used for human consumption (105.6 million tons) is 75% of the world's fish production. The remaining 25% are considered as wastes (Rebah and Miled, 2013).

The commercial fish processing industry generates large quantities of solid wastes which represents 20-60% of the initial raw materials. The solid fish waste consist consists of head, tail, gut, skin, fins and frames (Ramakrishan, 2013). These are not utilized in many countries and dumped at sea causing environmental problems (Bozzano and Sarda, 2002).

Fish waste may be considered as an important source of proteins (58%), fat (19%) and minerals with a high biological value (Ramakrishan, 2013). The by-products of fish processing industry have a great source of organic, inorganic and metal based compounds which are present in high amounts (Abdelmoez *et al.,* 2007).

Being rich in nutrients, the digestive tract of fish confers a favorable culture environment for the microorganisms (Sumathi *et al.,* 2011). It provides an excellent source for microbial growth which can be exploited in various metabolites like lysines, enzymes and so on (Coello *et al*., 2000).

Gut flora plays a major role in the digestive process, growth and disease of the host. The probiotics are live microbial cells that are administered in to the gastrointestinal tract of the host as a feed supplement and to improve the intestinal microbial balance and health (Ramesh *et al.*, 2013).

Microorganisms including bacteria, yeast and fungi produce different groups of enzymes (protease, lipase, amylase and so on) (Rebah and Miled, 2013). Enzyme is a biocatalyst which is used to speed up a favorable chemical reaction (Archer *et al*., 2001). Several biotic and abiotic factors influence the levels of digestive enzymes, such as age of the fish, pH, temperature, salt concentration and so on (Muyan *et al.,* 2006).

Enzyme production is a fast growing field in biotechnology. The industrial enzyme market is divided into three application sectors: (i) technical enzymes, (ii) food enzymes and (iii) animal feed enzymes (among the specific types of industrial enzymes, protease and amylase lead the market with current shares of 25% and 20% respectively) (Krishna, 2005).

Fish derived enzymes are commercially used in fish processing application to remove scales, skin and membranes that are otherwise difficult to remove e.g. tuna are skate skin. It is also used in baking, meat tenderization, leather production, milk, fish sauce and fish flavoring production. Other fish derived enzymes are used as an additive in aquaculture feed to aid fish digestion (Archer *et al*, 2001).

These fish enzymes have a very high biotechnological interest in food processing, detergent industry, textile industry, pharmaceutical products, medical therapy and so on (Rebah and Miled, 2013). Protease is a group of enzymes whose catalytic function is to hydrolyze or breakdown proteins. They are also called as proteinases or proteolytic enzymes (Shobana and Subash, 2013).

Proteases are commercially very important and it is isolated from various living sources such as plants, animals, fungi and bacteria. However, proteases from microbial sources are mostly preferred for the production of enzymes (Geethanjali and Subash, 2006). Proteases have a large variety of biotechnological applications because of their vast diversity and their specificity of action (Gupta *et al.*, 2002).

It represents one of the three largest groups of industrial enzymes. They have many applications in leather industry and also in bio processing of silver recovery from used X-ray films (Nadeem *et al.*, 2013).

Amylase is also one of the most important enzymes used in biotechnology, particularly in starch hydrolysis. Amylases originate from different sources such as plants, animals and microorganisms. The microbial amylases are the most produced and used in industry, due to their productivity and thermo stability (Burhan *et al.*, 2003).

Two major classes of amylases have been identified in microorganisms namely α- amylase and glycol amylase. Among various extracellular enzymes, α-amylase ranks first in commercial exploitation (Pandey *et al.,* 2002).

Amylase having approximately 25% of the enzymes market has almost completely replaced chemical hydrolysis of starch in starch processing industry. Thermostable α-amylases have extensive commercial applications in starch processing, brewing and sugar production (Leveque *et al*., 2000), desizing in textile industries, baking and in detergent manufacturing processes (Gangadharan *et al*., 2006).

Lipases are class of enzymes which catalyze the hydrolysis of long chain triglycerides (Hasan *et al*., 2006). With enzyme technology, lipases are currently receiving much attention involving various selected microorganisms especially from fungi, yeasts and bacteria.

Fish processing by-products contain growth factors offering good potential as culture media and high level of lipase activity produced by some microbial strains (Rebah *et al*., 2008).

Lipases have a large variety of commercial interest and various industrial applications such as detergent, food, flavor industry, esters and amino acids derivatives, biocatalytic resolution of pharmaceuticals, agro chemicals, bioremediation, use as biosensor, cosmetics, perfumery and so on (Rebah *et al*, 2008).

Esterases catalyzing the cleavage of ester bonds are known as α/β hydrolases. These are widely distributed in plants, animals and microorganisms (Faiz *et al.*, 2007). Microbial esterases have potential applications in biotechnology. The major reason of limiting industrial usage of esterases is their limited thermostability, mainly at high temperatures and pH stability in operating industrial conditions (Owusu and Cowan, 1989).

It has various applications like synthesis of flavor ester for food industry, modification of triglycerides for fat and oil industry, resolution of racemic mixtures used for the synthesis of fine chemicals for the

pharmaceutical industry and the carboxyl esterase has been used in the synthesis of naproxen as a non steroidal anti-inflammatory drug (Faiz *et al.*, 2007).

Since the protein content of fish is very high, this fish protein is used in human food and also in animal feed, which is highly suitable for human consumption (Ghaley *et al*, 2013).

Hence, the present investigation entitled **‘‘Isolation of Microorganisms and Production of Microbial Enzymes from the Waste of *Catla catla* Fish’’** has been selected with the following objectives:

- Isolation and identification of enzyme producing microorganisms from the selected source.
- Culture and maintenance of the selected microbial sp.
- Production and estimation of the enzymes produced by the selected microbial sp.
- Determination of enzyme activity
- Optimization of various conditions for obtaining the maximum enzyme activity.

REVIEW OF LITERATURE

2.0 REVIEW OF LITERATURE

Fish processing industries generate large amounts of by-products. It is an important environmental contaminant. 50% of the remaining material from the total fish capture is not used as food (Arvanitoyannis and Kassaveti, 2008).

Enzymes are naturally occurring high molecular weight proteins which is capable of catalyzing the chemical reactions of biological processes. They are the vital parts of all the living processes and are believed to be as life itself (Paul and Pardeshi, 2002). Enzymes can be derived from several sources. But microbial enzymes generally meet industrial demands (Pandey *et al*., 2002).

The review of literature pertaining to this present study entitled **''Isolation of Microorganisms and Production of Microbial Enzymes from the Waste of *Catla catla* Fish''** is discussed under the following headings.

2.1 Microbes and Microbial Enzymes

2. 2 Microbes from Fish Intestine

2.3 Enzymes

2.3.1 Protease

2.3.2 Amylase

2.3.3 Lipase

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2.4 Application of Enzymes

2.4.1 Application of Protease

2.4.2 Application of Amylase

2.4.3 Application of Lipase

2.4.4 Application of Esterase

2.5 *Catla catla*

2.1 Microbes and Microbial Enzymes

Microorganisms have been regarded as treasure sources of useful enzymes owing to their broad biochemical diversity and susceptibility to genetic manipulation. Bulk production capacity of microbes is high and they are easy to manipulate to obtain enzymes of desired characteristics (Kathiresan and Maivaean*,* 2006).

Isolation of aerobic bacterial flora in the gut of culturable fresh water teleosts, catla, rohu, mrigal, grass carp, tilapia and murrel has been carried out. Each single strain of organism produces a large number of enzymes (Bairagi *et al.,* 2002).

Microorganisms also have some beneficiary effect on the digestive process of fish (Gosh *et al.,* 2003). They are capable of digesting insoluble nutrient materials such as cellulose, protein and starch (Suji *et al*., 2014).

Enzymes are wide spread in nature and microbes serve as a preferred source of these enzymes because of their rapid growth and the limited space required for their cultivation. It can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications (Alnahdi, 2012).

Previous studies indicate that there is a distinct microbial source of the digestive enzymes such as amylase, cellulose, lipase and protease from endogenous sources in fish gut (Bairagi *et al.,* 2002).

The enzyme producing micro biota can be used as the probiotic supplements. Probiotics are supplementary microbes which have positive effect on host. It has an increasing feed nutritional value and improving the host immune response towards disease (Marlida *et al.,* 2014).

Most commonly studied digestive proteolytic enzymes from aquatic organisms include pepsin, trypsin, gastricin and elastase (Sovik and Rustad, 2005).

2.2 Microbes from Fish Intestine

Gastro intestinal (GI) tract of fish is a complex eco system and it contains large number of microorganisms. The microbial populations of the GI tract are much higher than the surrounding water. This indicates that the intestine provide a favourable conditions for microorganisms.

From the studies of the intestinal microbiota, the resident bacterial population of the intestine influences the establishment of pathogenic microorganisms in the intestinal tract and disease preventive effect (Krishnan, 2014).

Several studies have reported the presence of amylase activity in stomach extracts of various fishes. The presence of digestive carbohydrases was determined in sea bream intestines and it has high amylase activity (Alasco *et al.,* 2001).

The microbial populations of GI tract provide a protection against pathogenic visitors to the GI tract and aid host digestive function via the production of exogenous digestive enzymes and vitamins.

One of the most important aspects of GI tract micro biota is the understanding of how dietary factors influence the GI tract micro biota. The resident micro biota provides fermentation products and exogenous enzymes to fish with different feeding habits (Gosh and Ray, 2014).

From the studies, it appears that different species of fish and crustaceans have a specific resident gut microbiota and the isolated species includes *Acinetobacter* spp, *Enterobacter* spp. and *Pseudomonas* sp. in trout (*Oncorhynchus* sp.); *Aeromonas* spp., *Flavobacterium* spp. and *Lactobacillus* spp. in Arctic charr (*Salvelinus alpines*); *Enterovibrio* spp. from the intestinal tract of turbot (*Scophthalamus maximus*); *Vibrio* spp., *Bacillus* sp., *Pseudomonas* sp., *Photobacterium* sp. and *Plesiomonas* sp. in white shrimp (Sumathi *et al.,* 2011).

2.3 Enzymes

Enzymes are biocatalysts produced by living cells. It has specific biochemical reactions generally forming parts of the metabolic process of the cells. Enzymes are the most important bio product and are utilized in a large number of process in the areas of environmental, industrial and food biotechnology (Suji *et al*., 2014).

They can serve as an important molecular device for bioconversion of hazardous waste into biofriendly compost. It can be used to fertilize soil without affecting the ambience. The most important characteristics of enzyme are specificity and catalytic power (Samanta *et al*., 2013).

Fish waste has wide biotechnological potential as a source of digestive enzymes (Kishimura *et al.,* 2005). The Industrial enzyme producers sell the enzymes for a wide variety of applications. The estimated world market value is presently about US$ 2.7 billion and it is estimated to increase by 4% annually through 2012.

The main industries like detergents (37%), textile (12%), starch (11%), baking (8%) and animal feed (6%), use about 75% of industrially produced enzymes (Singh *et al*., 2011).

2.3.1 Protease

Proteases (E.C 3.4.2.1) are group of enzymes, which catalyzes the hydrolysis of peptide bonds of proteins and break them into polypeptides or free amino acid (Demir *et al.,* 2007). It constitutes 59% of the global market of industrial enzymes.

It has wide range of commercial usage in leather, detergent, food and pharmaceutical industries. Many sources are used for proteases including all forms of life that is plants, animals and microorganisms.

Protease is classified into three groups based on the pH as, acid, neutral and alkaline proteases. Acid proteases have pH optima in the range of 2.0-5.0 and are mostly produced by fungi. Protease performing best at pH range of 7.0 or around is called neutral protease and it is mainly of plant

origin. Proteases having optimum activity at pH range of 8.0 and above are classified as alkaline protease and it is mainly produced from microorganisms. Microorganism producing proteases play important roles in several industries (Alnahdi, 2012).

Alkaline proteases in the intestine of marine animals help in the digestion of protein in the compound diet. Therefore alkaline protease from the gut of marine animals has attracted much attention in recent years (Shanmugapriya *et al.,* 2007).

2.3.2 Amylase

Amylase is an important biocatalyst and its catalytic function is to hydrolyze alpha-1, 4-glycosidic linkages of polysaccharide starch to yield oligosaccharides, dextrin, maltose and D-glucose. Three types of amylases are mainly found based on the differences in the glycoside bond they attack, α amylase (EC 3.2.1.1), β amylase (EC 3.2.1.2) and γ amylases (EC 3.2.1.3) (Samanta *et al*., 2013).

Sources of amylase include animal, plant and microorganisms. The microbial amylases are having higher yield and are more thermostable. Both intracellular and extracellular amylases are produced by diverse group of fungal strains, but bacteria are the suitable source for the maximum amount of generated enzyme within a very short period of time (Samanta *et al*., 2013).

Bacteria and fungi secrete amylase to the outside of the cells to carry out extracellular digestion. When they have broken down the soluble starch and the soluble end products (glucose or maltose) are absorbed into their cells (Sudhaesa *et al.,* 2007).

Bacteria producing extracellular products are high compared to other microorganisms and are easy to isolate. Bacterial amylase provides an exquisite alternative to the chemical hydrolysis of starch. In fact, the bacterial amylase already has a profound importance in the field of biotechnology, textile industry, pharmaceutical and food processing industries. It can be utilized for waste management as well (Samanta *et al*., 2013).

2.3.3 Lipase

Lipases (EC 3.1.1.3) catalyze the hydrolysis and the synthesis of esters and it is formed from glycerol and long chain fatty acids. Lipases occur widely in nature but the microbial lipases are commercially used (Sharma *et al*., 2001).

In recent advancements in microbiology and biotechnology, lipases are the key enzymes owing to their multifaceted properties which find use in a wide array of industrial applications (Sagar *et al.,* 2013).

When adsorbed to an oil–water interface, the lipases are activated and do not hydrolyze dissolved substrate in the bulk fluid. Lipases are serine hydrolases and it will spilt emulsified ester of glycerin and long chain fatty acids such as triolein and tripalmitin. Lipases are involved in various stages of lipid metabolism in eukaryotes, including fat digestion, reconstitution, lipoprotein metabolism and fat digestion (Sharma *et al*., 2001).

Thermophilic lipases has higher thermostability, higher activity at elevated temperatures and it shows more resistance to chemical denaturation, ideal tools in industrial and chemical processes where relatively high reaction temperatures and organic solvents are used (Lelie *et al.,* 2005).

2.3.4 Esterase

Esterases (E.C 3.1.1.1) represent a diverse group of hydrolases catalyzing the cleavage and formation of ester bonds. There are widely distributed in plants, animals and microorganisms (Kumar *et al.,* 2012).

Esterases are the class of hydrolytic enzyme catalyzing the hydrolysis of ester bonds in organic media. It catalyzes the reactions like esterification and transesterification. Esterases hydrolyse simple esters (e.g. ethyl acetate) and usually only triglycerides bearing fatty acids shorter than C_6.

They are expensive enzymes and currently used in a wide range of industrial applications, such as organic chemical processing, synthesis of biosurfactants, detergent formulations and oleo chemical industry (Esakkiraj *et al*., 2012).

2.4. Application of Enzymes

2.4.1. Application of Protease

Proteases execute a large variety of functions and have important biotechnology applications. It represents one of three largest groups of industrial enzymes. It is used in leather, detergent, pharmaceutical and food industry and also in bioremediation process.

In textile industry, it is used to remove the stiff and dull gum layer of sericine from the raw silk fiber to achieve improved luster and softness. Protease treatments can modify the surface of silk and wool fibers. (Najafi and Deobagkar, 2005).

In leather industry, it is also used in hide dehairing process, were the processes are carried out at pH values between 8 and 10. The interesting application of alkaline protease is to decompose gelatinous coating of X-ray films, from which silver was recovered.

It is also important in biopharmaceutical products such as contact lens enzyme cleaners and enzymic debriders. Proteolytic enzymes also offer a gentle and selective debridement, supporting the natural healing process in successful management of skin ulcerations by the efficient removal of the necrotic material (Najafi and Deobagkar, 2005).

Microbial protease has been used for centuries in the production of soy sauce, tamari sauce and miso, a traditional food of Japanese. One of the largest uses for microbial proteases is in baking bread and crackers. Protease action reduces mixing time and increases extensibility of doughs and improves grain texture and loaf volume.

It is used throughout the dry cleaning industry and where the dry cleaning solvents will not remove the proteinaceous stains, such as milk, blood and egg from clothing and it is also used for desizing and degumming (Underkofler *et al.,* 1957).

2.4.2 Application of Amylases

Amylase was the first enzyme to be discovered and isolated. It is a class of industrial enzyme which is having approximately 25% of the enzyme market (Singh *et al*., 2011).

Dr. Jhokichi takmine, Japanese father of American Biotechnology, developed diastase from koji culture of *Aspergillus oryza* (a fungus used in the manufacture of soy sauce and miso) in 1894. The industrial production of dextrose crystal and dextrose powder from starch using α-amylase and gluco amylase began in 1959 (Aiyer, 2005).

The extracellular amylase, specifically starch digesting amylase has important application in bioconversion of starches and starch based substrates. The alpha amylase activity in various human body fluids is of clinical importance e.g. in diabetes, pancreatitis and cancer research.

Plant and microbial alpha-amylases are used as industrial enzymes. Starch degrading amylolytic enzymes has great significance in biotechnological applications ranging from food fermentation, textile to paper industries.

Amylases can be derived from many sources, such as animals, plants and microorganisms. The enzymes from microbial sources generally meet industrial demands. It has made significant contribution to the production of foods and beverages in the last three decades. The microbial amylases have completely replaced chemical hydrolysis of starch in starch processing industry (Singh *et al*., 2011).

Conversion of starch in to sugar, dextrins and syrups forms the major part of starch processing industry. In fermentation the hydrolysates are used as carbon source as well as sweetness of manufacture food products and beverages. Hydrolysis of starch to products containing maltose, glucose etc. is made by controlled degradation (Aiyer, 2005).

It has potential application in a number of industries such as food, paper industries, textiles, glucose and fructose syrups, detergents, bread

making, fruit juices, fuel ethanol from starches, alcoholic beverages, sweetness, digestive aid and spot remover in dry cleaning.

Bacterial alpha amylases are used in clinical, medicinal and analytical chemistry. Widely used thermo stable enzymes are amylases in the starch industry (Singh *et al*., 2011).

2.4.3 Application of Lipase

A hydrolytic enzyme like lipases has the greatest share in the industrial enzyme market. In recent advancement in microbiology and biotechnology, lipases owe to their multifaceted properties which find use in a wide array of industrial applications. Lipases are derived from many sources like fungi, bacteria, plant and animal sources. However the bacterial lipases are economical and stable (Sagar *et al*., 2013).

Many lipases are active in organic solvents. They catalyze a number of useful reactions including esterification, transesterification, synthesis of peptides and other chemicals, regioselective acylation of glycols and methanols. The expectation is that lipases will be as important industrially in the future like the current enzymes such as proteases and carbohydrases (Lelie *et al.,* 2005)

It has promising applications in organic chemical processing, synthesis of biosurfactants, detergent formulations, dairy industry, oleo chemical industry, agro chemical industry, paper manufacturer, nutrition, cosmetics and pharmaceutical processing.

The lipase based technology development for the synthesis of novel compound is rapidly expanding the use of the enzymes. The major commercial application of hydrolytic lipase is its use in laundry detergent.

Lipases for the detergent needs, it has to be thermostable and should be active in the alkaline environment of a typical machine wash. It is estimated that thousand tons of lipases are used to produce 1.3 billion tons of detergents each year (Sharma *et al*., 2001).

Lipases play a major role in the processing of γ–linolenic acid, a poly unsaturated fatty acid, astaxanthine - a food colorant and methyl ketones-flavor molecules characteristics of blue cheese. Also in inter esterification of cheaper gylcerides to more valuable forms and modification of vegetable oils at position two of the triglyceride, to obtain fats similar to human milk fat for use in baby feeds. Lipases also used in the production of cosmetics emulsifiers in food and pharmaceutical applications (Sharma *et al*., 2001).

2.4.4 Application of Esterase

Esterases are the diverse group of hydrolases catalyzing the cleavage and formation of ester bonds. It is widely distributed in plants, animals and microorganisms.

Lipases and esterases are the class of hydrolytic enzymes catalyzing the hydrolysis of ester bonds in organic media. But esterases differ from lipases on the basis of substrate specificity by the phenomenon of interfacial activation, which was only observed in lipases (Esakkiraj *et al.,* 2012).

Esterases show a wide substrate tolerance. It also shows high regio- and stereo specificity, which makes them attractive biocatalysts for the production of pure compounds in fine chemical synthesis. The interesting fact about this enzyme is that they do not require cofactors, are usually stable and are even active in organic solvents (Bornscheuer, 2002).

It has wide range of industrial applications such as organic chemical processing, synthesis of biosurfactants, detergent formulation and oleo chemical industry. Fermentation techniques are used for the production of specific esterases (Esakkiraj *et al.,* 2012).

Esterase producing bacteria have been found in diverse habitats such as soil contaminated with oil, dairy waste, industrial wastes, oil seeds and decaying food, compost heaps, coal tips and hot springs (Kumar *et al.,* 2012).

Solid or submerged state fermentation are used for the production of different types of esterases, like feruloyl esterases, ferulic acid esterases and cinnamic acid esterases produced from *Aspergillus sp*. Among the waste

products marine fish processing wastes are widely used as a good substrate for the production of esterases (Esakkiraj *et al*., 2012).

Few of the carboxyl esterases are used for the synthesis of optically pure compound because of their limited commercial availability. Best studied enzyme is carboxyl esterase NP (NP from naproxen steroidal anti inflammatory drug). This is produced as intracellular protein and its structure is unknown. Vanillin is a major flavor compound and it is produced with the use of carboxyl esterase (Bornscheuer, 2004).

2.5 Catla fish (*Catla catla*)

PLATE 1

Catla catla

Scientific classification

Kingdom: Animalia

Phylum: Chordata

Family: Cyprinidae

Genus: ***Catla***

Species: ***Catla catla***

Catla is the major Indian carp. It is commonly found in rivers and lakes in northern India, Indus plain, Pakistan, Bangladesh, Nepal and Myanmar. It is the second most important species after rohu. Catla has large and broad head, a large protruding lower jaw and upturned mouth and greyish scales on

dorsal side and whitish on belly. The natural distribution of catla seems to be governed by temperature dependency rather than latitude and longitude. It has higher growth rate and compatibility with other major carps, specific surface feeding habit, and consumer preference have increased its popularity in carp polyculture systems among the fish farmers in India, Bangladesh, Myanmar, Laos, Pakistan and Thailand. The species has also been introduced including Sri Lanka, Israel, Japan and Mauritius (FAO, 2006).

EXPERIMENTAL PROCEDURE

3.0 EXPERIMENTAL PROCEDURE

Fish receive bacteria in the digestive tract through water and food from the aquatic environment that are populated with bacteria. The digestive tract of fish gives a suitable culture environment for the microorganisms, since it is rich in nutrients. The intestinal bacteria provide importance in the nutrition and well–being of their hosts which has been established for homeothermic species, such as birds and mammals.

Gut micro flora plays a major role in the digestive process, growth and disease of the host. Some bacteria which possess the ability to tolerate the low pH in gastric juices resist the action of bile acids, lysozyme secreted in intestines and immune responses. It also adheres to the mucus or enteric wall surface that could persist for a relatively longer time and eventually make intestinal micro flora specific to each host animal (Kar and Ghosh, 2008).

In the present study entitled **''Isolation of Microorganisms and Production of Microbial Enzymes from the Waste of *Catla catla* Fish''** procedure was carried out under the following headings.

3.1 Collection of Microbial Source

3.2 Isolation, Maintenance and Identification of the Bacterial Strain

- **3.2.1 Isolation of the Bacterial Strain**
- **3.2.2 Maintenance of the Bacterial Strain**
- **3.2.3 Identification of the Bacterial Strain**
  - **3.2.3.1 Gram Staining**
  - **3.2.3.2 Biochemical Tests**

3.3 Growth of the Bacterial Strain for Enzyme Production

3.4 Extraction and Estimation of the Activity of Enzymes

- **3.4.1 Protease**
- **3.4.2 Amylase**
- **3.4.3 Lipase**

3.4.4 Esterase

3.5 Determination of Protein Content

3.6 Optimization of Enzyme Production

3.6.1 Incubation period

3.6.2 Inoculum concentration

3.6.3 Carbon source

3.6.4 Nitrogen source

3.6.5 pH

3.6.6 Metal salts and metal ions

3.1 Collection of Microbial Source

Fish intestine was collected from the local fish market. It was sealed in a plastic bag, kept in ice and transported from the fish market to the work place and stored in a deep freezer (-20˚C) until use.

3.2 Isolation, Maintenance and Identification of the Bacterial Strain

3.2.1 Isolation of the Bacterial Strain

Homogenate solution was made by adding intestine and 0.9% NaCl solution (1:10 w/v). This homogenate solution was serially diluted in sterile distilled water from 10^{-1} to 10^{-7} dilutions. 1ml of the sample from each dilution was poured into tryptone soy agar and incubated at 37˚C for 24 hours. Well grown individual colonies were sub cultured on nutrient agar slants. Composition of tryptone soy agar medium and nutrient agar medium was given in Appendix I. These colonies were analyzed on agar plates with selective media. Media composition and enzyme screening procedure was given in Appendix I and II respectively.

3.2.2 Maintenance of the Bacterial Strain

Nutrient agar slants were used to grow bacterial cultures. Periodical sub culturing was done and stored at 4˚C.

3.2.3 Identification of the Bacterial Strain

3.2.3.1 Gram Staining

The bacterial colonies were identified by Gram staining technique. The procedure is detailed in Appendix III

3.2.3.2 Biochemical Tests

Biochemical tests were performed to characterize the bacterial colonies as described in Appendix IV.

3.3 Growth of the Bacterial Strain for Enzyme Production

Conical flask containing 250 ml of nutrient broth was used to culture the bacterial colonies. The flask was placed on a rotary shaker at 37˚C for 24 hours. The fermented medium was used as the enzyme source.

3.4 Extraction and Estimation of the Activity of Enzymes

The enzymes were extracted from 24 hours old culture. Centrifugation (1000 rpm, 4˚C, 20 minutes) was performed to separate the cells and the cell free supernatant serves as the crude enzyme extract.

3.4.1 Protease

Protease activity of the crude enzyme extract was determined by the method of Tsuchida *et al.* (1986). This was given in Appendix VI.

3.4.2 Amylase

The activity of amylase was determined by the method put forward by Bernfield (1955) as explained in Appendix VII.

3.4.3 Lipase

The activity of lipase was assayed by titrimetric method as described by Selvam *et al.* (2011). Determination of lipase procedure was given in Appendix VIII.

3.4.4 Esterase

Esterase activity was determined by the method of Esakkiraj *et al*. (2012). This was explained in Appendix XI.

3.5 Determination of Protein Content

The protein concentration in the crude enzyme extract was determined using Lowry's *et al.* (1951) method. The procedure has been detailed in Appendix X.

3.6 Optimization of Enzyme Production

3.6.1 Incubation period

Inoculated 24 hours old culture into four test tubes containing 15 ml of nutrient broth labeled as 24 hrs, 48 hrs, 72 hrs and 96 hrs were used to determine optimum time or incubation period required for maximum enzyme activity. Tubes were incubated at 37˚C on a rotary shaker. After each incubation period the cultures were used for biomass determination. Biomass determination procedure was given in Appendix XI.

3.6.2 Inoculum concentration

The inoculum size was standardized by evaluating the effect of varying inoculum size for seeding the basal medium. Culture used as the inoculum was 24 hours old. Six conical flasks containing 100 ml of nutrient broth were taken and inoculated with 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 ml of the inoculum. The obtained culture was used for the determination of biomass.

3.6.3 Carbon source

The carbon source's effect on the enzyme yield was analyzed by adding different carbon sources like dextrose, sucrose, glucose, lactose, maltose, starch in the basal medium. After 48 hours the biomass was determined.

3.6.4 Nitrogen source

The medium was supplemented with different nitrogen sources and analyzed for the maximum enzyme production. Nutrient broth usually contains peptone and beef extract as the nitrogen source. Instead of using peptone in nutrient broth, other nitrogen sources like tryptone, casein, ammonium oxalate and instead of using beef extract, ammonium sulphate, ammonium nitrate and potassium nitrate were used (1% w/v). Biomass was determined after 48 hours.

3.6.5 pH

The optimum pH for enzyme production was determined using various pH ranges. Six conical flasks containing 100ml of nutrient broth were taken at varying pH range from 5.0 - 10 respectively using 0.1 N HCl. 24 hours old culture was used as the inoculums. After 48 hours of incubation, biomass determination was carried out.

3.6.6 Metal salts and metal ions

Various metal ions such as ferric chloride, lead acetate, potassium chloride and calcium chloride were supplemented separately at a concentration of 10mM in the nutrient broth. The concentrations of 5%, 10%, 15% NaCl (metal salt) were added in the medium. 24 hours old culture was used as the inoculums. After 48 hours of incubation, the biomass was determined.

RESULTS AND DISSCUSSION

4.0 RESULTS AND DISCUSSION

Enzymes are reaction catalyst of biological system with extraordinary catalytic power. It has high degree of specificity to their substrates and it speed up a particular chemical reaction by lowering the activation energy for the reaction. They achieve it by forming an intermediate enzyme substrate complex, which alters the energy of the substrate and it can be more readily converted into the product (Schmid *et al.,* 2001).

Most of the microbial enzymes are extracellular in origin and are influenced externally by various factors like pH and temperature. Optimum activity and stability are very much close to optimum conditions for microbial growth (Purohit, 1997). In the present study an attempt was made to isolate and identify enzyme producing microorganisms from waste of the fish *Catla catla*, followed by the optimization of conditions for maximum production of esterase.

The results observed and obtained for the study entitled **''Isolation of Microorganisms and Production of Microbial Enzymes from the Waste of *Catla catla* Fish''** are presented and discussed as follows:

4.1 Isolation and Identification of Microbes from the Waste of *Catla catla* Fish

4.1.1 Isolation of Microbes Producing Enzymes

4.1.2 Screening of Microorganisms for the Production of Enzymes

4.1.2.1 Screening of Protease

4.1.2.2 Screening of Amylase

4.1.2.3 Screening of Lipase and Esterase

4.2 Identification of Microbes

4.2.1 Gram Staining and Morphology

4.2.2 Biochemical Characteristics of the Bacterial Isolates

4.3 Enzymes Produced by Microorganisms

4.4 Optimization of Growth Conditions and Enzyme Production

4.4.1 Effect of Incubation Period on the Growth and Enzyme Production by *Bacillus sp*.

4.4.2 Effect of Inoculum Concentration on the Growth and Enzyme Production by *Bacillus sp*.

4.4.3 Effect of Carbon Source on the Growth and Enzyme Production by *Bacillus sp*.

4.4.4 Effect of Nitrogen Source on the Growth and Enzyme Production by *Bacillus sp*.

4.4.5 Effect of pH on the Growth and Enzyme Production by *Bacillus sp*.

4.4.6 Effect of Metal Salts and Metal Ions on the Growth and Enzyme Production by *Bacillus sp*.

4.1 Isolation and Identification of Microbes from the Waste of *Catla catla* Fish

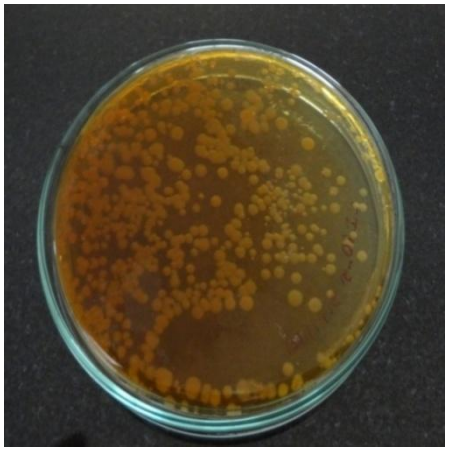
4.1.1 Isolation of Microbes Producing Enzymes

Homogenized and serially diluted fish intestine samples were inoculated on tryptone soy agar medium and bacterial colonies were obtained on the same. Tryptone soy agar medium with bacterial colonies was showed in Plate 2a.

Based on their morphological character, seven colonies were sub cultured on nutrient agar plates and nutrient agar slants which were shown in the Plates 2b and 2c respectively. These nutrient agar slants were stored at 4˚C for further use.

PLATE 2

Colonies Isolated from the Waste of *Catla catla* Fish



2aTryptone Soy Agar Medium

2b Nutrient Agar Medium

2c Nutrient Agar Slant

4.1.2 Screening of Microorganisms for Production of Enzymes

4.1.2.1 Screening of Protease

When 15% mercuric chloride was flooded in the peptone gelatin agar plates containing bacterial culture, the formation of clear zone around the colony indicates the proteolytic activity. All the three organisms namely *Bacillus*, *Staphylocoocus* and *Neisseria meningitidis* have good proteolytic activity shown in Plate 3a.

4.1.2.2 Screening of Amylase

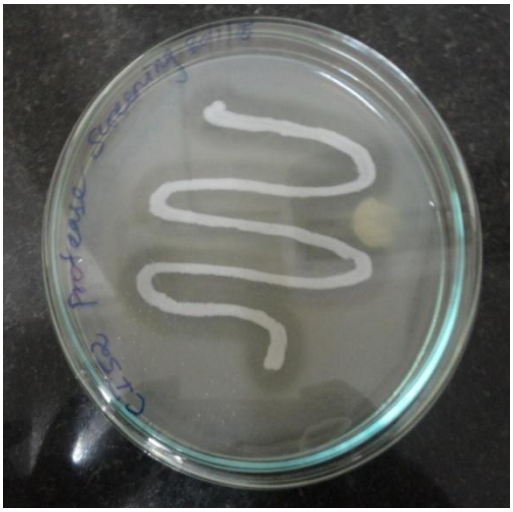
When starch agar plates containing bacterial isolates were flooded with 1% lugol's iodine solution, the formation of transparent zone surrounding the colony indicates the amylase activity. All the three organisms have good amylase activity which is give in Plate 3b.

4.1.2.3 Screening of Lipase and Esterase

Clear zone formation in Tributyrin agar plates containing colonies shows lipase and esterase activity, when the agar plates were incubated at 37˚C for 24-48hrs. Plate 3c shows the lipase activity of the organisms, where *Bacillus* and *Staphylococcus* has good lipase activity and *Neisseria meningitidis* has low lipase esterase activity.

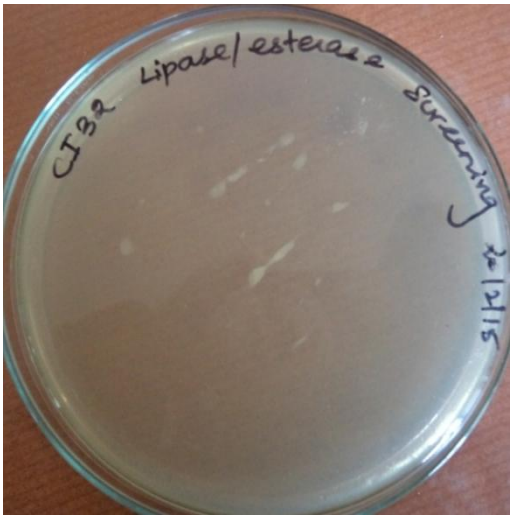
PLATE 3

Screening of Microorganisms



3a Screening of Protease

3b Screening of Amylase



3c Screening of Lipase and Esterase

4.2 Identification of Microbes

The isolated microorganisms were identified based on morphology, Gram staining and their biochemical characteristics.

4.2.1 Gram Staining and Morphology

Morphological characteristics of organisms were identified by Gram staining. Table 1 depicts the morphological characters of the isolated organisms.

TABLE -1

Gram staining and morphology

Sample	Gram Stain	Morphology	Colour	Identified Organism
CI21	-	Cocci	Pink	*Neisseria meningitidis*
CI22	-	Cocci	Pink	*Neisseria meningitidis*
CI31	-	Cocci	Pink	*Neisseria meningitidis*
CI32	+	Cocci	Purple	*Staphylococcus*
CI41	-	Cocci	Pink	*Neisseria meningitidis*
CI51	+	Rod	Purple	*Bacillus sp*
CI52	-	Cocci	Pink	*Neisseria meningitidis*

- **CI – Catla Intestine**

From Table 1, it clearly seen that CI21, CI22, CI31, CI41 and CI52 were found to be gram negative, cocci shaped pink colour bacteria and CI32 was found to be gram positive cocci shaped bacteria and CI51 was gram positive rod shaped bacteria.

CI21, CI22, CI31, CI41 and CI52 were identified as *Neisseria meningitidis*, CI32 was *staphylococcus* and CI51 was identified as *Bacillus sp.*

4.2.2 Biochemical Characteristics of the Bacterial Isolates

The biochemical characteristics of the isolated bacteria were done and recorded in Table 2.

TABLE – 2

Biochemical Characteristics of the Bacterial Isolates

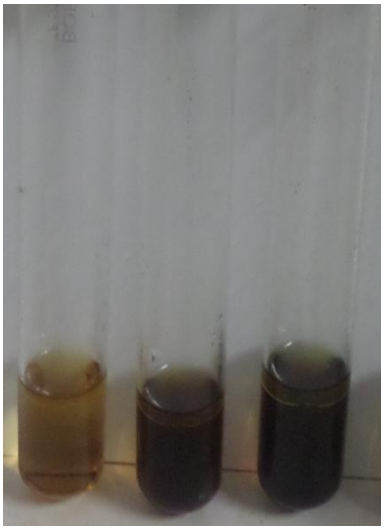
Characteristic features	CI21	CI22	CI31	CI32	CI41	CI51	CI52
Indole	+	-	+	+	-	-	+
VP	+	+	+	+	-	-	+
Citrate	+	+	-	+	+	-	+
Methyl Red Test	-	-	-	-	-	+	+
Nitrate reduction	+	+	+	+	-	+	+
Triple sugar ion	-	-	+	+	-	+	+
Oxidase	+	+	+	+	+	-	+
Urea hydrolysis	-	-	-	-	-	-	-
Catalase	-	-	+	+	+	-	-
Carbohydrate	+	+	+	+	+	-	+
Starch hydrolysis	+	+	+	-	+	+	+

From Table 2 it is noted that Indole test was shown to be positive for the following samples CI21, CI31, CI32 and CI52 and found to be negative for CI22, CI 41 and CI51 which is shown in Plate 4a. Voges proskauer test shows positive results for CI21, CI22, CI31, CI32 and CI52 and negative for CI41 and CI51. Citrate test showed positive results in CI21, CI22, CI32, CI41 and CI52 and negative results in CI31 and CI51. These two were shown in plate 4b and 4c. Methyl red test showed positive results in CI51 and CI52. The other

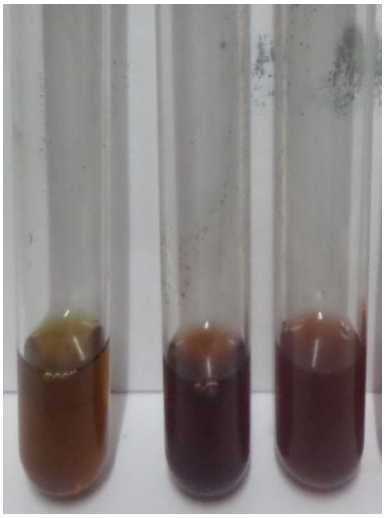
samples indicate the negative results as given in 4d. Nitrate reduction test indicates negative result in CI41 and the other samples showed positive results which is seen in 4e.

PLATE 4

Biochemical Characteristics of Microorganisms



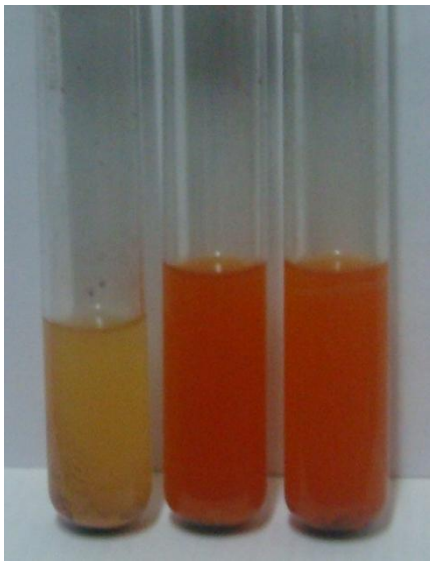
4a Indole Test



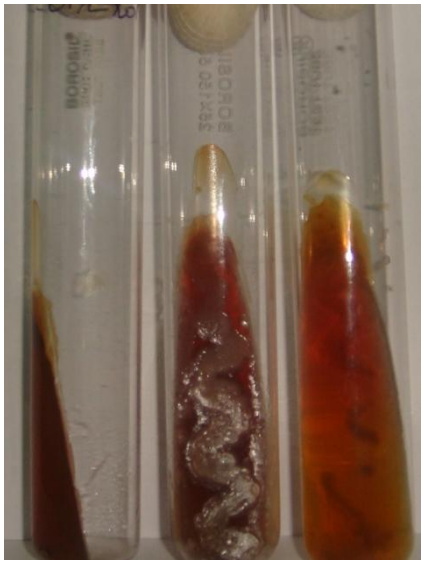
4b Voges proskauer Test

4c Citrate Utilization Test

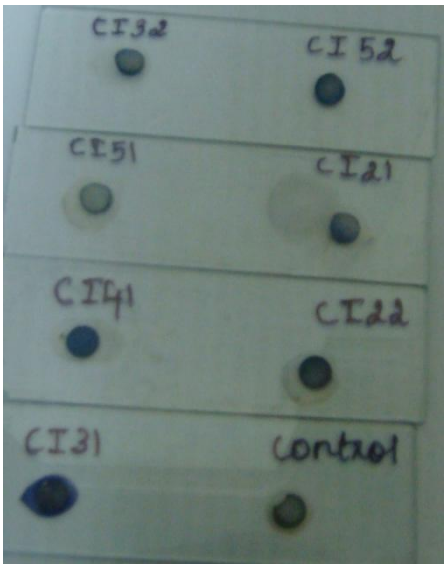
4d Methyl Red Test



4e Nitrate Reduction Test



4f TSI Test



4g Oxidase Test

4h Urea Hydrolysis Test

4i Catalase Test

4j Carbohydrate Test

4k Starch Hydrolysis Test

Triple sugar iron test indicates positive results in CI31, CI32, CI 51 and CI52. Other samples indicate the negative results. CI51 showed negative result in Oxidase test and the other samples showed negative results. Urea hydrolysis test showed negative results in all the samples. These were depicted in Plate 4f, 4g and 4h respectively.

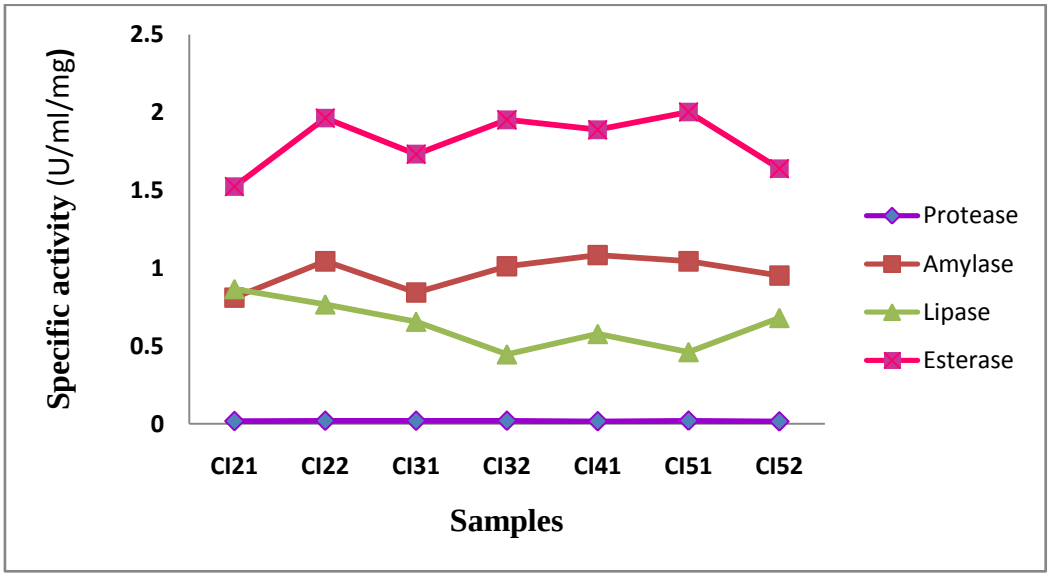
CI31, CI32 and CI41 answered as Catalase positive and the other samples gave negative results. Carbohydrate test showed negative result in CI51 and CI32 indicates negative result in starch hydrolysis. All the other samples showed positive results in carbohydrate and starch hydrolysis test. These were shown in Plate 4i, 4j and 4k.

4.3 Enzymes Produced by Microorganisms

Various enzymes like protease, amylase, lipase and esterase were assayed and its specific activity were presented in Figure 1.

FIGURE – 1

Specific Activity of Various Enzymes



From Figure 1, protease activity was found to be low when compared to the other enzyme activity. Lipase showed moderate specific activity, whereas, specific activity of esterase was highest than the other enzyme activity followed by amylase which showed second highest specific activity.

The highest esterase was produced by *Bacillus sp*. Hence, esterase produced by *Bacillus* sp. was selected further for the optimization.

4.4 Optimization of Growth Conditions and Enzyme Production

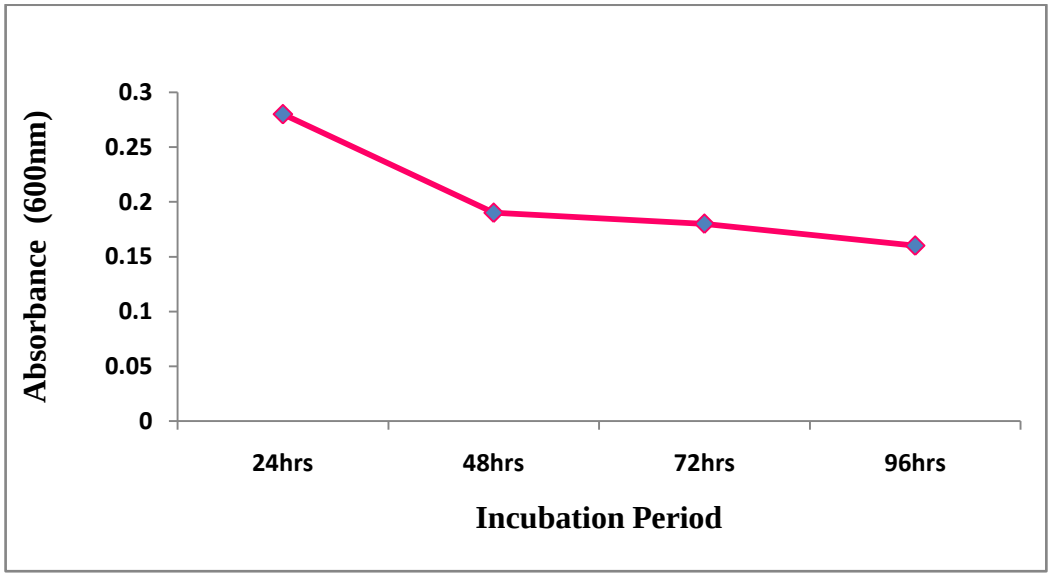
Various conditions like incubation period, inoculum concentration, carbon source, nitrogen source, pH, metal salts and metal ions were optimized for the maximum growth of *Bacillus* and esterase produced from *Bacillus* by calculating the enzyme activity and specific activity.

4.4.1 Effect of Incubation Period on the Growth ad Enzyme Production by *Bacillus sp*.

The effect of incubation period on the growth and enzyme production by *Bacillus sp* is depicted in Figure 2a and 2b respectively.

FIGURE 2a

Effect of Various Incubation Periods on Growth of *Bacillus sp*



From figure 2a, it is clear that the highest growth of the bacteria was obtained at 24 hours of incubation and thereby decreased gradually.

FIGURE 2b

Effect of Various Incubation Periods on Enzyme Production

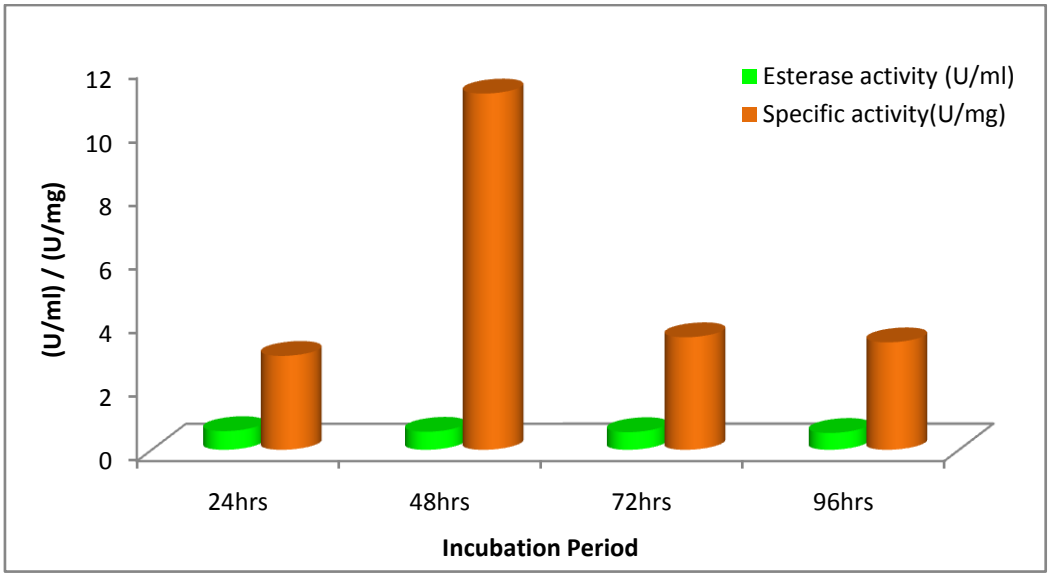


Figure 2b shows the enzyme production of *Bacillus* and the maximum production was found to be at 24 hours of incubation period, whereas the specific activity was found to be higher at 48 hours of incubation period.

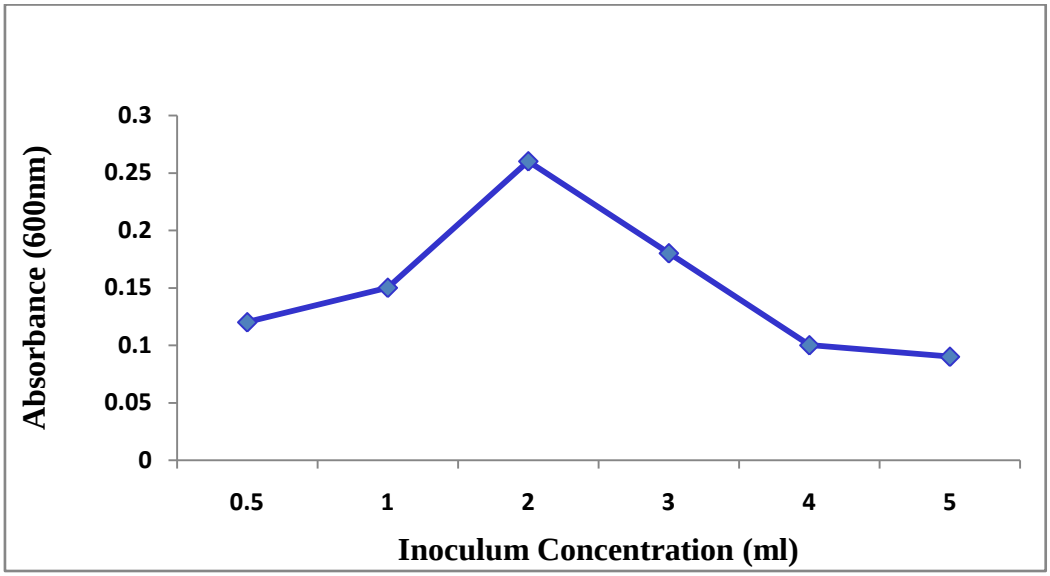
The highest enzyme activity at 24 hours was supported by Lambrechts and Galzy (2014) who had reported that high esterase activity was observed in the production media after 24 hours of incubation period in *Bravibacterium sp*. R312. Kumar *et al.* (2012) also reported that 24 hours culture of *Bacillus sp.* strain DVL2 exhibited highest esterase activity.

4.4.2 Effect of Inoculum Concentration on the Growth and Enzyme Production by *Bacillus sp*.

The effect of inoculum concentration on the growth and the enzyme production by *Bacillus* sp is shown in Figure 3a and 3b respectively.

FIGURE 3a

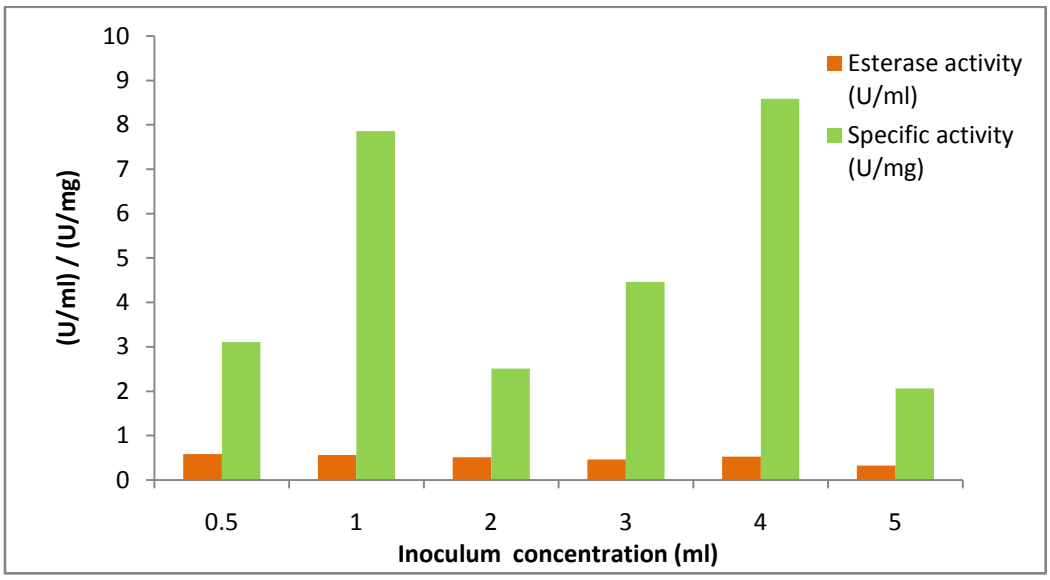
Effect of Inoculum Concentration on the Growth of *Bacillus sp*



It is clear from the Figure 3a that higher growth of *Bacillus sp* was observed in 2 ml of inoculum concentration. From 0.5 ml to 2 ml of inoculum concentration the biomass was increasing and when the inoculum concentration was increased above 2 ml the biomass was found to decrease gradually.

FIGURE 3b

Effect of Inoculum Concentration on Enzyme Production



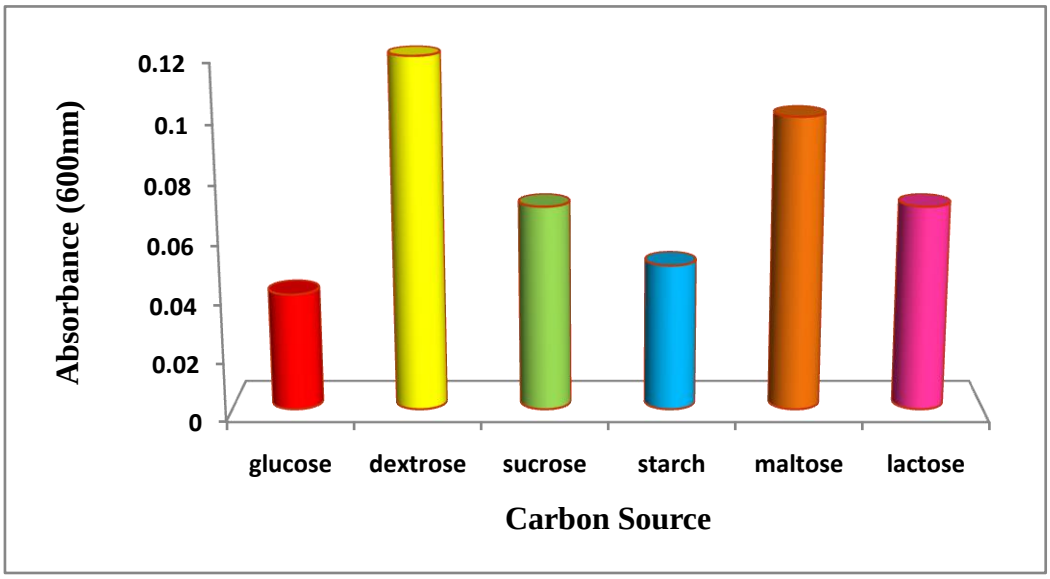
From Figure 3b it was observed that the esterase activity was maximum when the inoculum concentration used was 0.5 ml. Specific activity was found to be higher when 4.0 ml inoculum concentration was used. Studies with the inoculum concentration for esterase activity has not been reported, where for other enzymes like amylase, Baysel *et al.* (2003) has reported that 2% of inoculum concentration was appropriate for amylase production.

4.4.3 Effect of Carbon Source on the Growth and Enzyme Production by *Bacillus* sp.

The effect of carbon source on the growth and enzyme production of *Bacillus* sp was depicted in Figure 4a and 4b respectively.

FIGURE 4a

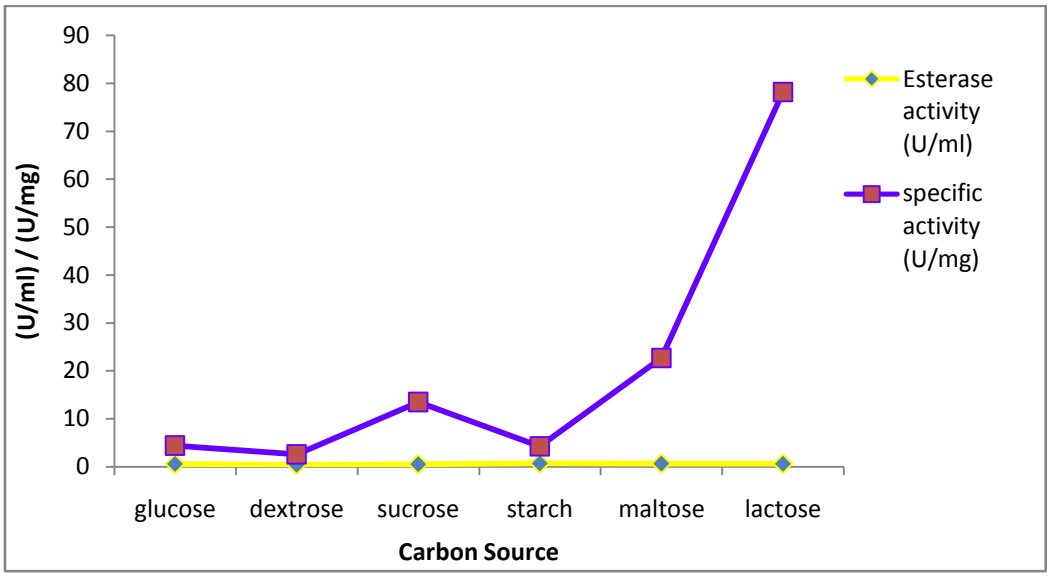
Effect of Various Carbon Source on Growth of *Bacillus* sp



From figure 4a, it is understood that maximum growth occurred when glucose was used as the carbon source, which was followed by dextrose and by maltose.

FIGURE 4b

Effect of Carbon Source on Enzyme Production



Maximum esterase activity was obtained when the medium was supplemented with starch. In addition maltose and lactose were better source for esterase production. Specific activity was high on using lactose.

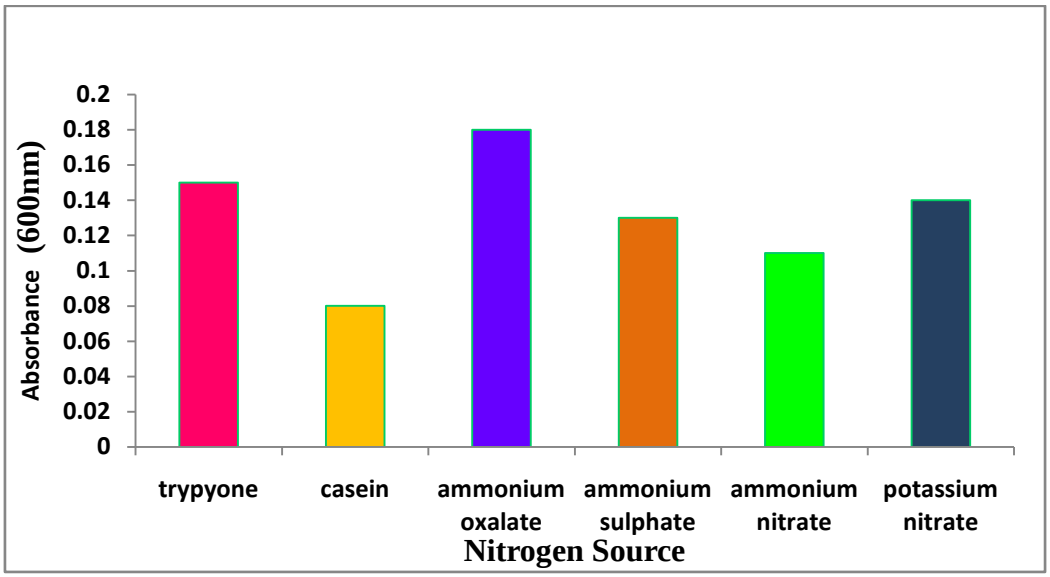
Esakkiraj *et al.* (2012) had shown that *Bacillus altitudinis* AP-MSU showed the highest esterase production was found in fructose. Other carbon sources such as maltose and lactose were supported maximum esterase production. Kdemi *et al.* (1998) stated that fructose showed highest influence in esterase production by the bacterium *Bacillus circulans* MAS2. In addition maltose, lactose also better source for esterase production. Instead of fructose, the results of maltose and lactose are in agreement with the results of these two authors.

4.4.4 Effect of Nitrogen Source on the Growth and Enzyme Production by *Bacillus* sp.

Figure 5a and 5b describes the effect of nitrogen sources for maximum growth and enzyme production of *Bacillus* sp.

FIGURE 5a

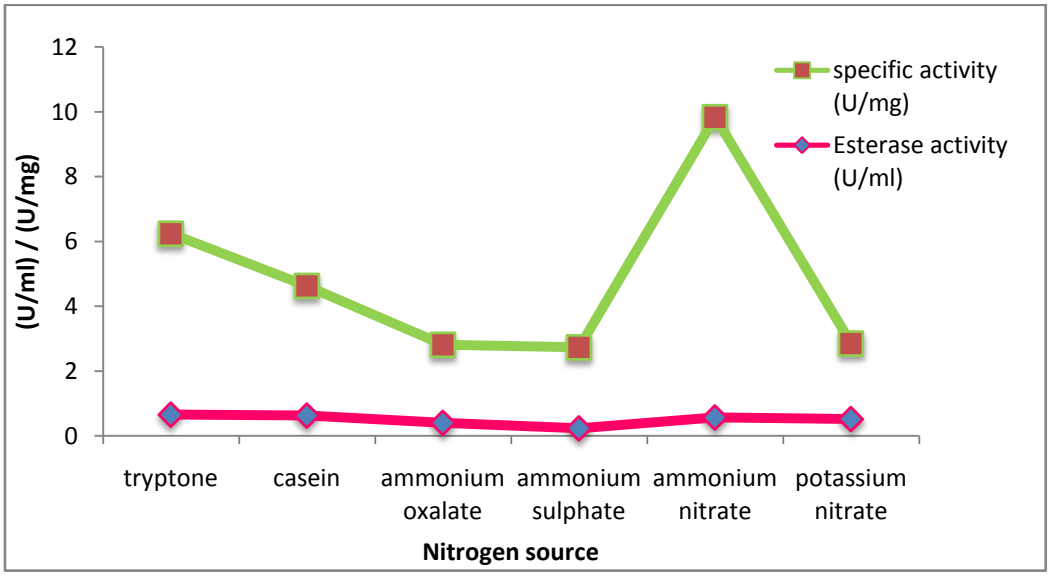
Effect of Various Nitrogen Source on Growth of *Bacillus* sp



From the above figure, it is clearly understood that the growth was highest when ammonium oxalate was used as the nitrogen source.

FIGURE 5b

Effect of Various Nitrogen Source on Enzyme Production



Tryptone was found to be the best among the nitrogen sources supplied for the production of esterase. In addition, casein was better in enzyme production. The specific activity was high in ammonium nitrate.

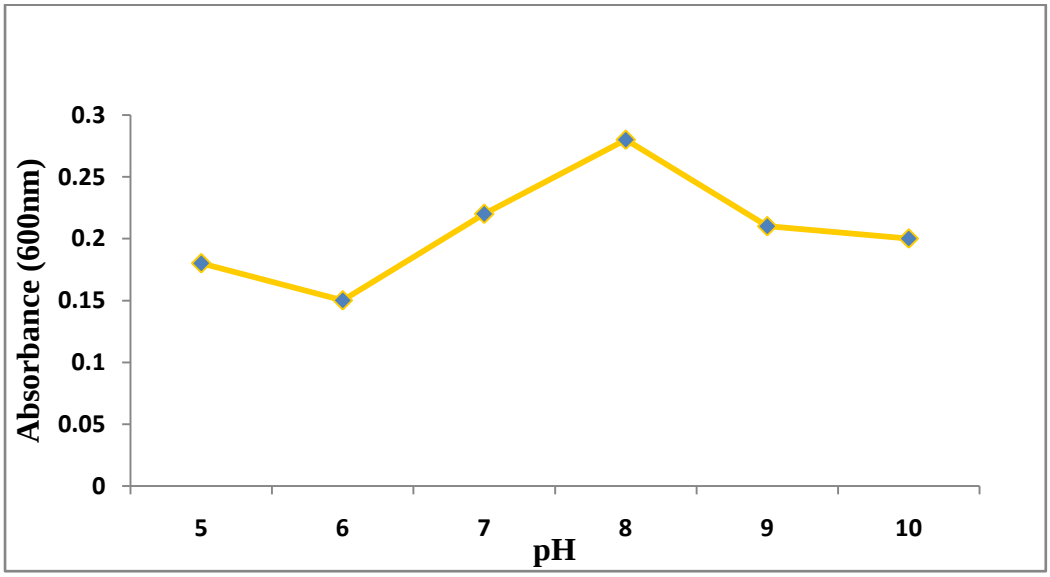
Previous findings showed that casein medium has highest esterase production in *A. pullulans* (Kudanga *et al.,* 2007). Kademi *et al*. (1998) stated that showed the highest esterase *Bacillus circulans MAS2* production in yeast extract medium.

4.4.5 Effect of pH on the Growth and Enzyme Production by *Bacillus* sp.

Various pH concentrations were used to analyze the growth and enzyme production. Figure 6a and 6b shows the effect of pH on the growth and enzyme production by *Bacillus* sp.

FIGURE 6a

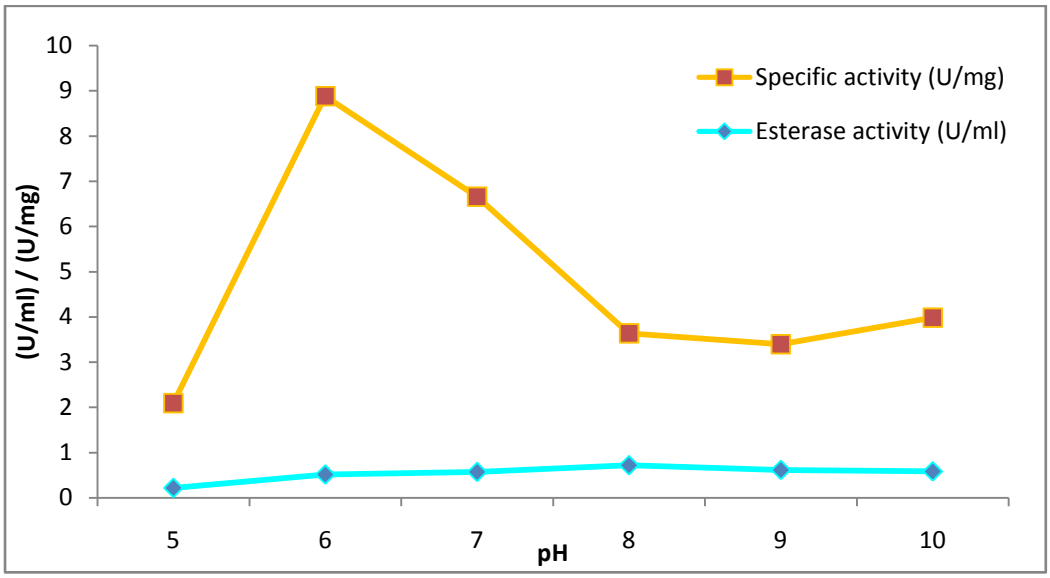
Effect of pH on the Growth of *Bacillus sp*



It is evident from the above figure that the best pH for the growth of *Bacillus* sp was pH 8.

FIGURE 6b

Effect of pH on Enzyme Production



From Figure 6b it was observed that, the enzyme activity was found to be higher in pH 8. The specific activity was higher in pH 6 followed by pH 7.

The results are in agreement with the results of Kademi *et al.* (1998) who showed that esterase activity was high at pH 8.1 by *Bacillus circulans.* MAS2. In the effect of pH, the optimum pH range 7 – 8 was also found in some species. For example, *A.pullulans* showed high esterase activity at pH 8.0 (Kudanga *et al.,* 2007) and Esakkiraj *et al.* (2012), who showed that the maximum amount of esterase was produced at pH 7 – 8.

4.4.6 Effect of Metal Salts and Metal Ions on the Growth and Enzyme Production by *Bacillus sp*.

Figure 7a and 7b shows the effect of metal salts and metal ions on the growth and enzyme production.

In various concentrations of sodium chloride used, maximum growth was observed in production media containing 5 % NaCl. Among the metal used lead acetate produced maximum biomass. It was showed in figure 7a.

FIGURE 7a

Effect of Various Metal Salts and Metal Ions on Growth of *Bacillus sp*

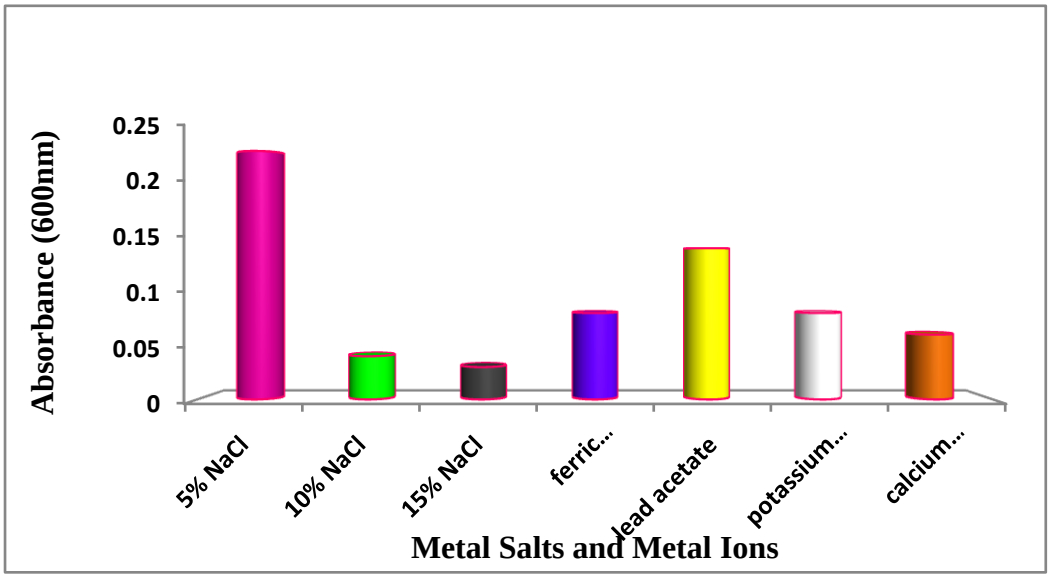
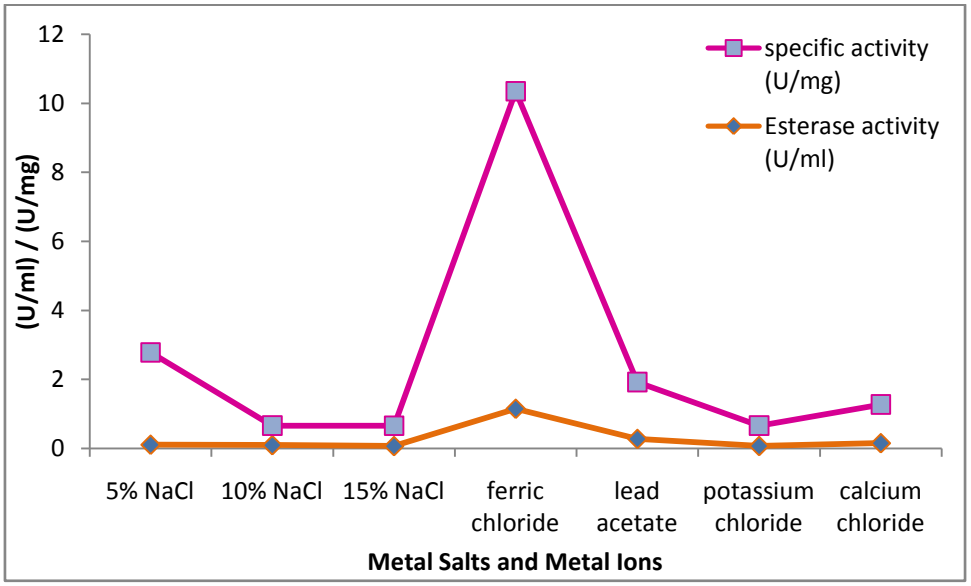


FIGURE 7b

Effect of Various Metal Salts and Metal Ions on Enzyme Production



Esterase activity and the specific activity were found to be highest in the medium containing 5 % NaCl when compared to 10 % and 15 % of NaCl. Both esterase activity and specific activity was higher in the medium supplemented with ferric chloride followed by lead acetate and calcium chloride supplemented medium.

The result of NaCl concentration was agreement with Esakkiraj *et al*., (2012). He reported that based on different percentage of NaCl, maximum growth was obtained at 5% NaCl and zinc sulphate enhanced the esterase production followed by zinc chloride and calcium chloride. Takeda *et al.* (2006) who also reported that esterase activity was high in zinc chloride, barium chloride and calcium chloride by *Burkholderia cepacia*.

SUMMARY AND CONCLUSION

5.0 SUMMARY AND CONCLUSION

Every day, large amounts of fish waste are produced all over the world. Deposition of these wastes produces environmental pollution problems. However these wastes can be used for the production of cosmetics, fertilizers, food packaging and enzyme isolation.

Many of the life processes that biotechnology can make use, are chemical reactions that are controlled by enzymes which are proteins. Some biotechnological process use whole organisms, while others use enzymes that have been purified from the organisms that make them. Enzymes are used in various large scale industries like food, detergent, textile, leather industries and so on.

Microbial sources are mainly used to produce industrially important enzymes. Microbes such as fungi and bacteria are always the targets of commercial enzyme production. To improve enzyme production, optimization of growth conditions is expected. In this study, the effect of different conditions on growth and esterase production by the isolated organism was investigated.

Thus, salient findings of present study entitled **''Isolation of Microorganisms and Production of Microbial Enzymes from the Waste of *Catla catla* Fish''** was summarized as follows:

Fish intestine was collected from the market and 1g of intestine was homogenized using 0.9% NaCl solution. This was serially diluted in sterile distilled water from 10^{-1} to 10^{-7} dilutions. 1 ml of the sample of each dilution was poured in to tryptone soy agar medium and incubated at 37˚C for 24 hours. From this, 7 individual colonies were sub cultured onto nutrient agar media. The colonies were analyzed on different agar pates with selective media. From this it was analyzed that the organisms has the ability to produce protease, amylase, lipase and esterase.

The isolated colonies were sub cultured on nutrient agar slants. The cultured organisms were identified by Gram staining technique, colony morphology and biochemical characteristics. Based on the above tests the

isolated microorganisms were identified as *Bacillus sp, Neisseria meningitides,* and *Staphylococcus* and these were stored at 4˚C for further study.

The organisms *Bacillus sp, Neisseria meningitides* and *Staphylococcus* were found to secrete protease, amylase, lipase and esterase. The enzymes secreted by these organisms were estimated and specific activity was calculated. It was found that among the secreted enzymes by these 3 organisms, the concentration of esterase from *Bacillus sp* was high.

The conditions needed for maximum growth and esterase enzyme production was optimized. The incubation period for high biomass and maximum enzyme production was found to be 24 hours whereas the specific activity was found to be at 48 hours of incubation period.

Inoculum concentration greatly influences the growth and enzyme production. The growth was found to be highest when the inoculum concentration was 2 ml. But the enzyme production was maximum at 0.5 ml and the specific activity was high at 4 ml.

In case of carbon source, the growth was higher in the presence of dextrose followed by maltose. However, it was observed that highest amount of esterase was produced in the medium supplemented with starch followed by maltose and lactose. The specific activity was high on lactose.

Ammonium oxalate was found to be the best nitrogen source for the growth of *Bacillus sp*. The amount of enzyme produced was estimated to be higher in the medium supplemented with tryptone. The specific activity was high in ammonium nitrate.

pH is an important parameter which influence the microbial growth and enzyme production. The optimum pH for growth was found to be 8.0, whereas at pH 9.0 maximum enzyme production was observed and the specific activity was high at pH 6.0 followed by 7.0.

Metal salts and metal ions were also added to the medium and the growth and esterase activity was estimated. It was found that among various concentration of sodium chloride, 5 % sodium chloride supported the maximum growth, enzyme production and specific activity.

In the presence of lead acetate the growth was found to be maximum. The esterase activity and specific activity was found to be highest in the presence of ferric chloride followed by lead acetate and calcium chloride.

Thus the present study concluded on the fact that the huge amount of fish waste disposed in the environment, causing pollution problems can be effectively used for the enzyme production which has immense industrial applications.

Suggestions for Future Studies:

- Enzymes producing microorganisms can be isolated from other fishes also.
- Enzymes like cellulase, chitinase and collagenase from fish waste can be isolated and completely studied.
- Apart from intestine, wastes from the other parts of fish can be used for the enzyme production.
- Genetic manipulation of microorganisms for higher enzyme production.

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APPENDICES

APPENDIX I

1. Composition of Nutrient Broth

Composition	Amount
Peptone	10g/l
Beef extract	10g/l
Sodium chloride	5g/l

2. Composition of Nutrient Agar

Composition	Amount
Peptone	10g/l
Beef extract	10g/l
Sodium chloride	5g/l
Agar	15g/

3. Composition of Peptone Gelatin Medium

Composition	Amount
Beef extract	5g/l
Peptone	5g/l
Gelatin	4g/l
Agar	20g/l
pH	7

4. Composition of Tryptone Soy Agar Medium

Composition	Amount
Pancreatic digest of casein	15g/l
Papaic digest of soybean meal	5g/l
NaCl	5g/l
Agar	15g/l
Ph	7

5. Composition of Starch Agar Medium

Composition	Amount
Beef extract	5g/l
Peptone	5g/l
NaCl	5g/l
Starch (soluble)	2g/l
Agar	20g/l
Ph	7

6. Composition of Tributyrin Medium

Composition	Amount
Peptone	5 g/l
Yeast extract	3 g/l
Agar	12 g/l
Tributyrin	10g/l

7. Composition of Basal Medium

Composition	Amount
Disodium hydrogen phosphate	6 g/l
Potassium dihydrogen phosphate	3 g/l
Ammonium chloride	1 g/l
Sodium chloride	0.5 g/l
Yeast extract	0.05 g/l

APPENDIX II

Screening of Enzymes

(Kar and Gosh, *2008,* Kumar *et al., 2012* and Selvam *et al.,* 2011)

AMYLASE

The colonies were inoculated using starch agar plates and incubated at 37˚c for 48 hours. 1% lugol's iodine solution was flooded in the culture plates. Amylase activity was identified by the formation of transparent zone surrounding the colony.

PROTEASE

Using peptone gelatin agar the isolates were inoculated and incubated at 37˚C for 15 hours. 15% $HgCl_2$ was flooded in the culture plate and the formation of clear zone around the colony indicates the proteolytic activity.

LIPASE AND ESTERASE

The isolates were inoculated on tributyrin medium and incubated at 37˚C for 24-48hrs. Formation of zone of clearance indicates the lipase/esterase activity.

APPENDIX III

Gram Staining

(Sundarajan, 1995)

PRINCIPLE

A smear is prepared on the slide, stained with crystal violet and then treated with iodine, which acts as a mordant. The crystal violet-iodine complex (CV-I) imparts purple colour to the cells. The intensely stained cells are then washed with ethanol. This serves as a lipid solvent. The gram positive bacteria contains low lipid content, hence the low amount of lipid is easily dissolved by alcohol. In gram-negative cells, dehydration of cell wall protein does not occur completely. This facilitates the release of the unbound crystal violet complex leaving the cell colorless or unstained. If the smear is counter stained with safranine, the gram-negative cells are easily seen due to absorption of safranine and imparting the cells pink color, while gram-positive cells retain the blue colour of the primary stain.

REAGENTS

Crystal violet

Solution A: 2 g Crystal violet in 20 ml ethanol

Solution B: 0.8 g Ammonium oxalate in 80 ml water. Mix solution

A and B.

1. Grams iodine: 2 g Potassium iodide, 1 g iodine, 300 ml distilled water.

2. Safranine: 0.25 g Safranine in 10 ml ethanol and 90 ml water.

PROCEDURE

Thin smear of culture were made on glass slides, air dried and then heat fixed. Added crystal violet for 30 seconds and washed with distilled water. Now, flooded the smear with grams iodine for 30 seconds and washed

with distilled water. Decolorized the smear with 95% ethanol until no more colour flows from the smear, and counter stained with safranine for 1minute and washed with water, after drying, the slide was examined under oil immersion.

APPENDIX IV

Biochemical Tests

(Kannan, 1996)

1. INDOLE TEST

PRINCIPLE

Tryptophan present in peptones of the culture media is acted upon by the enzyme tryptophanase and converted into indole, pyruvic acid and ammonia. Indole reacts with kovac's reagent (para-dimethyl aminobenzaldehyde) to produce a cherry red colour product.

REAGENTS

1. Medium: 1 % peptone broth

2. Kovac's reagent: Dissolved 5 g of p-dimethyl aminobenzaldehyde in 75 ml of amyl alcohol and added 2.5ml of conc. HCl.

PROCEDURE

Peptone broth was taken in a test tube, sterilized, cooled, inoculated with the bacterial culture and incubated at 37˚C for 24 hrs. After incubation period, added Kovac's reagent. Cherry red ring formation indicates indole positive. Absence of red ring indicates negative result.

2. METHYL RED TEST

PRINCIPLE

Organisms belonging to enterobacteriaceae ferment glucose via pyruvate and produces mixed acids such as acetic acid, lactic acid, succinic acid,

formic acid, ethanol, CO_2 and H_2. Because of the abundant acid production, the final pH of the broth drops to less than 4.5, which can be detected by pH indictors.

REAGENTS

1. Medium: MR-VP broth (Methyl red- Voges proskauer)

2. Methyl red reagent: Dissolved 100mg of methyl red in 300ml of 95% ethanol. Added 200ml of distilled water and filtered.

PROCEDURE

MR-VP broth was inoculated with the bacterial culture and incubated at 37˚C for 24 hrs. Methyl red solution was added after incubation period. Change in colour of the broth from yellow to red indicates a positive result. Absence of red colour indicates negative result.

3. VOGES PROSKAUER TEST

PRINCIPLE

One group of bacteria belonging to Enterobacteriaceae ferment glucose to produce butylenes glycol and acetoin which are more neutral in nature. The end products are detected by VP reagent.

REAGENTS

1. Medium: MR-VP broth

2. Barrit's reagent

Solution A: Dissolved 5 g of alpha naphthol in 95 ml of absolute ethanol with constant stirring.

Solution B: Dissolved 40 g of potassium hydroxide in 75 ml of distilled water and 0.3 mg of creatine was added to the solution and made up to 100 ml with distilled water.

PROCEDURE

MR-VP broth was sterilized, inoculated with bacterial culture and incubated at 37˚C for 24hrs. 40% KOH solution (VP reagent B) and Barrit's alpha napthol solution (VP reagent A) was added after incubation period. Gently shook the tubes for 30 seconds with the caps off to expose the media to oxygen. Change in the colour of broth from yellow to pink indicates the positive result. No colour change indicates negative result.

4. CITRATE UTILIZATION TEST

PRINCIPLE

Certain organisms can utilize citrate as sole carbon source and grow. During the growth, acetate and other alkaline carbonates are produced. This reaction is shown by the change in colour of the indicator (Bromothymol blue)

REAGENT

Medium: Simmon's Citrate agar medium

PROCEDURE

The organism was streaked onto simmon's citrate agar slants. The tubes were incubated at 37˚C for 24-48 hrs. Following incubation period the citrate tubes turn from green to deep Prussian blue colour which indicates positive result. No colour change indicates negative result.

5. TRIPLE SUGAR IRON TEST

PRINCIPLE

Microbes use carbohydrates as energy source depending on their enzyme components. Major products of carbohydrate catabolism are lactic, formic or aetic acid with production of H_2 or CO_2 as gas. Fermentative degradation is carried out in a fermentation broth containing pH indicator under durham's tube for gas collection.

REAGENT

Medium: Triple sugar iron agar (pH-7.3).

PROCEDURE

TSI agar was prepared and sterilized and the slants were prepared. The test organism was inoculated. The tubes were incubated for 24 hrs and then examined for acid production, alkaline production, gas production and hydrogen sulphide production.

6. CATALASE TEST

PRINCIPLE

Some organisms possess the enzyme catalase that splits hydrogen peroxide into oxygen and water.

$$2H_2O_2 \xrightarrow{catalase} 2H_2O + O_2$$

Presence of catalase is indicated by bubbles of free oxygen gas.

REAGENT

3% Hydrogen Peroxide (1:10 dilution)

PROCEDURE

To 1ml of bacterial culture, 0.5ml of 3% hydrogen peroxide was added. Immediate liberation of air bubbles indicates that the organism is catalase positive and no liberation of air bubbles indicates that the organism is catalase negative.

7. UREA HYDROLYSIS TEST

PRINCIPLE

Urea is a waste nitrogenous material excreted out by animals. Some degrade the nitrogen and carbon bond in urea to form carbon dioxide in the presence of a hydrolytic enzyme urease. The carbon dioxide reacts to form ammonium carbonate, an alkaline end product, and increases the pH of the

medium. This can be detected by incorporating a pH indicator in the medium, which changes the colour during alkaline conditions.

REAGENT

Medium: Christensen's Urea Agar Medium

PROEDURE

Sterile Christensen's Urea Agar tubes were prepared and the tubes were inoculated with the bacterial culture and incubated at 37˚C for 24-48 hrs. Urease positive tubes are deep pink in colour. No change indicates negative result.

8. STARCH HYDROLYSIS TEST

PRINCIPLE

Starch is an insoluble polymer of glucose, which acts as a source of carbon for microorganisms, which has an ability to degrade them. Degrading microorganism transports the degraded from across the membrane of the cell. Some bacteria possess the ability to produce amylase that breaks starch into maltose. The amylase is an extracellular enzyme, which is released from the cells of microorganism.

REAGENTS

1. Medium: Starch Agar Medium

2. Iodine solution: Dissolved 1g of iodine and 2g of potassium iodine in 300ml of distilled water.

PROCEDURE

Sterile starch agar plates were prepared and the bacterial culture was plated onto the plates. The plates were incubated at 37˚C for 48 hrs and flooded with iodine solution. A clear zone around the organism indicates positive result. Dark blue coloration of medium with no clear zone formation indicates negative result.

9. CARBOHYDRATE FERMENTATION TEST

PRINCIPLE

In bacteria, bio oxidation reactions are very important as they help provide energy either by oxidation of organic substances or by fermentation. In the earlier case, by utilizing organic compounds as electrons donors with oxygen as the ultimate acceptor, bacteria produce co_2 and water. The ability to utilize free oxygen is due to Cytochrome enzyme system and the process is called respiration while fermentative organisms utilize organic compounds but lack a cytochrome system and produce carbon – di – oxide and water besides a complex end product such as alcohol, acid, or aldehyde. The acid, alkali or gas production results in a visible change in the inoculated broth due to the presence of suitable indicator. The fermentation is carried out in a fermentation tube which is a culture tube containing a Durham tube for detection of gas production.

Materials / Reagents

Medium

- Peptone (Trypticase) - 10.g
- Glucose - 5g
- Phenol red - 0.018g
- Distilled water - 1000ml
- pH - 7.3

Procedure

Fermentation broth was prepared with sugar as the carbohydrate source. Added approximately 10ml in the test tubes maintain three tubes for each given culture and one tube as inoculated control. Inverted Durham's tube into all of the test tubes, plugged it with cotton and autoclaved at 15lbs pressure for 20 minutes. Inoculate each culture in their respective tubes except in the control. Incubate all the tubes at 37ºC for 24 – 48 hours. Observe the reactions that develop in the fermentation medium by comparing

with the control i.e change in colour from red to yellow (due to acid production) and / or appearance of bubbles (due to gas production).

10. NITRATE REDUCTION TEST

PRINCIPLE

Certain bacteria use nitrates in the places of oxygen as an external terminal acceptor. Nitrate can easily be reduced to nitrite by nitrate reductase. In aerobic bacteria, oxygen is first used to prevent nitrate reduction and utilize nitrate. The nitrite may further give rise to nitrogen, ammonia an oxide.

PROCEDURE

Nitrate broth was sterilized, inoculated and incubated at 37˚C for 18-24 hrs. After incubation, o.5ml of alpha napthalamine reagent and 0.5ml of sulfanilic acid was added. Red coloration indicates the positive result and absence of red colour indicates the negative result.

11. OXIDASE TEST

PRINCIPLE

To determine the presence of oxidase enzymes, the reagent (impregnated into strips of filter paper), which contains tetramethyl-p-phenylenediamine, serves as an alternate substrate for the cytochrome oxidase reaction. In the reduced state the reagent is colourless but when oxidized it becomes purple.

REAGENTS

1% Tetramethyl-p-phenylenediamine dihydrochloride

PROCEDURE

The filter paper disc impregnated with oxidase reagent was placed aseptically on a clean sterile slide. With the help of sterile glass rod, a small amount of culture was transferred to one disc. The colour change of the disc was examined.

APPENDIX V

Determination of Protease Activity

(Tsuchida *et al., 1986*)

PRINCIPLE

Protease cleaves the substrate. On treatment with Folin Ciocalteau reagent, the tyrosine produced to reduces the phosphomolybdic phosphotungstic components of the reagent to give a blue colored product, which is measured spectrophtometrically at 660nm.

REAGENTS

1. 1% BSA in 20mM borate buffer (pH 9.0) : 1 g of BSA in 100ml of borate buffer (20mM borate buffer = 7.4 g of boric acid and 0.294 g of $CaCl_2$ (2mM) dissolved in 1 litre of water)
2. 0.55 mM Sodium carbonate: 58.58 g Sodium carbonate in 1000 ml of distilled water.
3. 10% TCA
4. Folin Ciocalteau reagent(1:3 dilution)
5. 20mM Tris buffer : 2.4 g of Tris buffer + 0.294 g $CaCl_2$ (2mM) dissolved in 1000 ml of water(pH 8.0)
6. Tyrosine standard: 10 mg of tyrosine was weighed accurately and made up to 10 ml with distilled water. This solution contains 1 mg of tyrosine per ml.
7. Whatman No. 1 filter paper.

PROCEDURE

Took two test tubes marked as Test and Control. Added 2ml of substrate into both the tubes. 0.5 ml of enzyme was then added to the test and 2.5 ml of 10% TCA to the control. Incubated the tubes for 30 minutes at 45˚C. The enzymatic reaction was stopped by the addition of 2.5 ml of 10% TCA to the test. This was followed by the addition of 0.5 ml of enzyme to the control. Filtered the solution through Whatman No.1 filter paper. Took 1ml of

each of the filtrate and added 5 ml of Folin Ciocalteau reagent (1.3 dilutions). The reaction mixture was left aside for 30 minutes at room temperature. The blue color developed was measured at 660nm in a spectrophotometer. As blank added 1 ml of distilled water, 5 ml of 0.55 mM sodium carbonate and 0.5ml of Folin Ciocalteau reagent.

APPENDIX VI

Determination of Amylase Activity

(Bernfield, 1995)

PRINCIPLE

The reducing sugars produced by the action of α – and / β – amylases react with dinitrosalicyclic acid and reduce it to a brown colored product, nitroaminosalicyclic acid.

REAGENTS

1. Sodium acetate buffer, 0.1M Ph 4.7
2. 1 % starch solution: Prepared a fresh solution by dissolving 1 g starch in 100 ml sodium acetate buffer. Slightly warmed, if necessary.
3. Dinitrosalicyclic acid reagent
   Dissolved by stirring 1g Dinitrosalicyclic acid, 200mg crystalline phenol and 50 mg sodium sulphite in 100 ml of 1 % NaOH. Stored at 4˚C.
4. 40 % Rochelle salt solution (Potassium sodium tartarate)
5. Standard maltose: Dissolved 50mg maltose in 50ml distilled water in a standard flask & stored it in a refrigerator.

PROCEDURE

Pipetted out 1ml of enzyme in a test tube. Incubated it at 27˚C for 15 minutes. Stopped the reaction by the addition of 2ml of Dinitrosalicyclic acid reagent. Heated the solution in a boiling water bath for 5 minutes. While the tubes were warm, added 1ml of potassium sodium tartarate solution. Then cooled it in running tap water. Made up the volume to100ml in all the tubes.

Read the absorbance at 560nm. The reaction was terminated at zero time in the control tubes. Prepared a standard with 50-1000µg maltose.

CALCULATION

One unit of enzymes activity was expressed as milligram of maltose produced per milligram of protein.

APPENDIX VII

Determination of Lipase Activity

(Selvam *et al*., 2011)

PRINCIPLE

Lipase hydrolyses fats to fatty acids and glycerol. The activity of lipase is estimated based on a titrimetric determination of liberated fatty acids. The fatty acids are titrated against 0.05N methanolic sodium hydroxide using phenolphthalein as indicator.

REAGENTS

1. 0.1M Tris-Hcl buffer
2. 0.1M NaOH

PROCEDURE

Lipase activity was determined titrimetrically on the basis of olive oil hydrolysis [24]. One ml of the culture supernatant was added to the reaction mixture containing 1ml of 0.1M Tris-HCl buffer (pH 8.0), 2.5 ml of deionised water and 3 ml of olive oil. The reaction mixture was mixed well and incubated at 37 °C for 30 min. Both test and blank were performed. After 30 minutes the test solution was transferred to a 50 ml Erlenmeyer flask. 3 ml of 95% ethanol was added to stop the reaction. Liberated fatty acid was titrated against 0.1M NaOH using phenolphthalein as an indicator. End point is an appearance of pink color [25]. A unit lipase is defined as the amount of enzyme, which releases one micromole fatty acid per minute under specified assay condition

CALCULATION

lipase activity was expressed as units per gram of dry substrate.

APPENDIX VIII

Determination of Esterase Activity

(Esakkiraj *et al., 2012*)

PRINCIPLE

Esterases catalyze hydrolysis reaction by converting esters into an acid and an alcohol using water as nucleophile. The assay is based on measurement of the change in absorbance at 405nm.

MATERIALS REQUIRED

100mM Tris buffer, pH 7.2

Alpha naphthol: 10mM dissolved in acetonitrile

100mM Alpha naphthyl acetate

PROCDURE

Pipetted out 0.5ml of enzyme extract in a test tube. Add 1ml of Tris buffer, 10.5 ml of Alpha naphthyl acetate. The mixture was incubated for 15 minutes at room temperature. Then kept in ice box for 5 minutes and then the absorbance were measured at 405nm using UV-visible spectrophotometer. The amount of esterase production was determined with a standard graph of p-nitrophenol. One unit of esterase activity is equivalent to one micromoles of p-nitrophenol released under assay condition followed. The esterase activity was presented as units per gram of dry substrate (U/g).

APPENDIX IX
ESTIMATION OF PROTEIN
(Lowry *et al*., 1951)

PRINCIPLE

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

REAGENTS

1. Solution A: 1% copper sulphate
2. Solution B: 2% sodium potassium tartarate
3. Solution C: 2% sodium carbonate in 0.1 N NaOH
4. Solution D: mixed just before use, 1ml of solution A, 1ml of solution B and 100ml of solution C.
5. Solution E: 1N Folin-Ciocalteau reagent (mixed equal volumes of commercially available reagent and distilled water just prior to use). Stored protected from light.
6. Standard BSA: 50mg BSA in 50 ml of 0.1N NaOH. Diluted 1:10 for working standard.

PROCEDURE

Aliquots of standard protein solution (0-1000µg) were taken and the enzyme samples were made up to 1ml with 0.1 N NaOH. Shake well to treat the protein with alkali. Added 1ml of solution D mixed well and incubated at 37˚Cfor 3 minutes. Added 0.1 ml of solution E to each tube mixed well and incubated at 37˚C for 3 minutes. Read the color developed at 670nm against a reagent blank. Fit a linear regression in a scientific calculator and read the protein concentration in the aliquot taken.

APPENDIX X

DETERMINATION OF BIOMASS

PRINCIPLE

Bacterial population (cell mass) or amount of growth can be determined by measuring the turbidity or optical density of a broth culture. The light transmission is less when there is more turbidity. Since turbidity is directly proportional to the number of cells, this property is used as an indicator of bacterial concentration in a sample. Turbidity is quantified with a colorimeter that measures the amount of light energy. The light energy transmitted or absorbed directly through suspension is measured at 600nm as the percentage of transmission (%T) and is directly proportional to the cell mass concentration.

MATERIALS / REAGENTS

i. Nutrient broth

ii. Bacterial culture

PROCEDURE

- Sterile nutrient broth was prepared in conical flasks.
- The flasks were inoculated using bacterial culture.
- The content was incubated at 37ºC for 24 hours.
- After incubation, to determine the biomass, 3ml of the fermented broth was taken and the optical density was read at 600nm.