Screening and Partial Purification of Protease Produced by Bacteria Isolated from Termite Soil

R.Gayathri
(14PBC007)

Thesis submitted to
Avinashilingam Institute for Home Science and Higher Education for Women
Coimbatore-641 043

In Partial Fulfillment of the Requirement for the Degree of Master of Science in Biochemistry

April, 2016

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1.0 Introduction

Soil is the richest source of microorganism. The commonly observed microorganism in soil includes species like *Bacillus, Klebsiella, Pseudomonas, Xanthomonas* etc. These microorganisms have an important role in determining the structure and fertility of the soil. Soil microorganisms also act as a source for natural products like antibiotics, enzymes and other bioactive compounds (Sreeremya *et al.,* 2016). Each single strain of microorganism produce different types of enzymes such as hydrolytic, oxidizing or reducing enzymes.

Enzymes are the type of globular protein having specific active site for its substrate. Each enzyme have specific group of substrate and catalyzes reactions in which its specific substrates are involved. It catalyzes the reaction by transforming the molecules and energy (Rao and Kavya, 2014). The enzymes isolated from microbes were used in many industrial processes and new applications were identified continuously. These enzymes have been used as alternate to chemicals for reasons such as to improve the properties and characteristics of products (Anabela *et al.,* 2013).

The microbial enzymes have applications in industries like food, textile, leather, pharmaceuticals, cosmetics, biomaterials and detergent industries (Duza and Mastan, 2013). Some of the commercially important enzymes produced by soil bacteria are amylase, lipase, xylanase, laccase, cellulase and protease. Different enzymes have been used in different industries as follows

- Textile industry – Amylase, Cellulase
- Detergent – Protease, Lipase, Cellulase, Amylase
- Food industry – Protease, Cellulase
- Paper industry – Xylanase, Lipase
- Leather industry – Protease, Lipase (Mukunda *et al.,* 2012)

Cellulase

Cellulose is homopolysaccharide made of glucose units linked by β-1,4 glycosidic bond in linear manner. The cellulose is degraded by the enzyme called cellulase and it is of three types namely endoglucanase, exoglucanse and β-glucosidase (Ferbiyanto *et al.,* 2016). Cellulose is a crystalline insoluble structure present in plants. The conversion of lignocellulosic plant waste materials into useful products like biofuels depends on the activity of cellulase enzyme. Thus requirement of enzyme become increased which results in identifying the better source for the enzyme (Irfan *et al.,* 2012). Major cellulase producing bacteria are *Bacillus* species, *Pseudomonas* species and *Micrococcus* species (Patagundi *et al.,* 2014).

Amylase

Amylase is a type of hydrolytic enzyme having starch as its substrate. It specifically break α – 1, 4 glycosidic linkage and convert starch into simple sugars. It is present in plants and bacteria. Amylase is also present in human saliva, so it is also called as digestive enzymes. The food industry uses amylase in large scale for various processes like making different types of syrups, to improve shelf-life of fruit juices (Karnwal and Nigam, 2013). The other areas where the amylase is used in wider range includes pharmaceuticals, paper industries, clinical research, starch analytical chemistry and medical chemistry (Kaur *et al.,* 2012). *Pseudomonas fluorescens, Bacillus amyloliquefaciens* and *Bacillus licheniformis a*re the three important amylase producing bacteria which produce amylase in large amount. The other species of bacteria which produce amylase in appreciable amount are *E.coli, Micrococcus, Proteus, Candida* and *Serratia* (Karnwal and Nigam, 2013).

Lipase

Lipase hydrolyses the glycerol esters present in acylglycerols to liberate fatty acids and glycerol. Lipase converts long chain triglycerides into diglycerides, monoglycerides, fatty acids and glycerol (Veerapagu *et al.,* 2013). Lipases are

involved in both synthesis and degradation of esters in glycerol and fatty acids. It is present in plants, animals and microbes (Daouadji *et al.,* 2014). Lipases have wide range of applications in food, cosmetic and pharmaceutical industries. Microbial lipases were more effective than other sources because of its stability and specificity. Lipase producing microorganisms were mainly present in the areas contaminated with oilseeds, industrial wastes, decaying food and waste from dairy factories (Qamsari *et al.,* 2011).

Protease

Proteases are the type of hydrolase enzyme which breaks the polypeptide chain that connects the aminoacids present in the proteins (Singh *et al.,* 2015). Proteases have important position in the commercial industries (Chudasama *et al.,* 2015). Proteases were excreted by plants, animals and microbes. The commercially available protease is of microbial origin than the plants and animals because of the following reasons – high productivity from microbes, require limited space for cultivation, genetic manipulation can be done easily (Singhal *et al.,* 2012). The predominant producer of protease are bacteria especially *Bacillus* species which excretes alkaline and neutral protease (Shahzad *et al.,* 2015).

Among these enzymes, proteases are more important because of its wide biotechnological applications (Vikramathithan and Dhandapani 2014). The important applications of protease enzymes are in detergent preparation, pharmaceutical product formation, dehairing process in leather industry, flavoring agent in brewing and cheese making industry and also in laundry and waste processing industries (Aqel, 2012). The proteases have the capacity to be stable and active along with ingredients used in the detergent preparation. It also improves the luster and softness of the fiber by modifying the structure of fibers. Thus protease has wide use in textile industries. The medicinal applications of protease are it is used as healing agent in skin ulcer management (Asker *et al.,* 2013).

The industrially important microbial enzymes – cellulase, amylase, lipase and protease are isolated from different sources like marine soil, marine water, hot springs, dairy effluent water, soil contaminated with vegetable oil and food industry waste.

Termites are the type of polymorphic group of social insects that live in the specially built nest called termataria or termite mounds. The important function of these termite mounds are to protect the insects and to provide optimum environment to the insects. Termite mounds are made of soil particles along with different types of minerals mixed with saliva or the feces of the termites depending on its species.

The composition of termite soil varies with the surrounding soil and its composition depends on type of soil used to build nest and type of termite species. Termite soil is slightly acidic with more amount of total organic matter. The minerals present in termite soil are magnesium, chloride, phosphate and iron in higher amount and calcium, potassium and aluminium in lower concentration than the surrounding soil (Kamalu and Okolie, 2013). The studies conducted by Makonde *et al.* (2015), Manjula *et al.* (2014) and Fall *et al.* (2007) reported that termite mound soil have variety of microorganisms. Therefore the termite soil can be used to isolate microorganisms which produce industrially important enzymes.

The present study entitled **"Screening and Partial Purification of Protease Produced by Bacteria Isolated from Termite Soil"** was carried out with the following objectives

- To isolate and identify the bacteria from termite soil
- To screen the presence of various enzymes
- To extract and purify the protease produced by the isolated bacteria

2.0 Review of literature

The current scenario in biotechnology related industries is to synthesize eco-friendly products from natural resources and to replace the chemicals by biological compounds like enzymes. Replacing chemicals with enzymes reduce environmental pollution. The industries like leather industry, food industry and textile industry requires more amounts of enzymes in order to produce products at cost effective and to reduce the effluents which cause environmental pollution. Thus, the enzymes with novel properties have great demand in industries (Bizuye *et al.,* 2014).

Enzymes are a type of protein molecule and are necessary for all form of organisms (Das and Prasad, 2010). Enzymes catalyze specific biochemical reactions depending on its nature (Duza and Mastan, 2013). Enzymes are present in plants, animals and microbes. But microbial enzymes have great demand in industries because of its diversity in biochemical activity, specificity, stability, effectiveness at various pH and temperature, rapid growth and easy cultivation (Umayaparvathi *et al.,* 2013).

The review of literature related to the present study entitled **"Screening and Partial Purification of Protease Produced by Bacteria Isolated from Termite Soil"** was discussed under the following headings

2.1. Microbial enzymes

2.2. Industrially important microbial enzymes

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2.6. Purification of enzymes

2.1. Microbial enzymes

Microorganisms play a vital role in the production of different types of enzymes which have high demands in industries for various purposes (Pant *et al.,* 2015). The microorganisms which synthesize economically important enzymes are bacteria, fungi and yeast. The enzymes which are commonly isolated from microorganisms are protease, keratinase, cellulase, laccase, xylanase, amylase and lipase (Nigam, 2013).

2.2. Industrially important microbial enzymes

2.2.1. Cellulase

Cellulase undergo cellulolytic process i.e. bioconversion of cellulose. Cellulase is composed of three enzymes namely 1,4 β-endoglucanase, 1,4 β-exoglucanase and β-glucosidase. These enzymes convert cellulose into glucose. Cellulase is used in biopolishing of fabrics and improves softness and brightness of fabrics. It is also used in biomass fermentation to produce biofuels (Saptarini and Indriyati, 2014).

Cellulase is naturally produced by fungi and bacteria in large amount. Cellulase producing bacteria includes *Clostridium themocellum, Pseudomonas fluorescens, Ruminococcus albus* and *Streptomyces.* The fungi which produce cellulase are *Penicillium notatum, Sporotrichum pulverulentum, Aspergillus niger, Fusarium solani, Tricoderma viridae and Penicillium funmiculosum* (Atala *et al.,* 2014).

2.2.2. Amylase

Amylase breaks the glycosidic bonds in starch to form glucose, maltose, maltotriose and dextrin (Kaur *et al.,* 2012). Amylase was the first enzyme to be produced industrially from fungus in the year 1894 for treating digestive disorders. Though amylase was present in plants, animals and microorganisms, microbial amylase have been extensively used in industries for microbiological processes due to its stability. Amylase producing bacteria are mainly present in biogas plant and soil contaminated with kitchen waste and domestic waste water (Patel *et al.,* 2014).

In food industry, amylases are used to produce glucose syrup, high fructose corn syrup, maltose syrup and to reduce viscosity of sugar syrup. It is also used to improve shelf-life and solubility of starch in the brewing industry. In paper industry, it is used to reduce starch viscosity to obtain appropriate paper coating and also used to prepare sizing agents and digestive aids (Karnwal and Nigam, 2013).

2.2.3. Lipase

Lipases are the water soluble hydrolytic enzymes involved in fat digestion by converting triglycerides into lipid molecules. The digestion of lipids catalysed by the lipase occurs in the interface between aqueous and oil phase due to polarity. Bacterial lipase cleaves different types of molecules like carboxy ester, water soluble ester and triglycerol (Thomas and Kavitha, 2015). Commonly available products which induce lipase synthesis are wheat bran, rice bran, dextrins, sugarcane bagasse, coconut cake, olive oil cake and gingelly oil cake (Ramesh *et al.,* 2014).

Bacillus, Pseudomonas and Burkholderia are some important genus of bacteria which produce lipase in appreciable amount (Paul *et al.,* 2015). Various industrial sectors which use lipase for different purposes are fats and oil processing, food processing, pharmaceutical synthesis, paper manufacturing, cosmetic synthesis, bakery, brewing and leather processing (Bhavani *et al.,* 2012).

2.2.4. Protease

Protease is an enzyme which cleaves the peptide bond present in the proteins (Sathiya, 2013) and the products obtained by the action of protease were peptides and free amino acids. It is a complex group of enzymes with difference in properties like specificity over substrate, pH, temperature, active site and catalytic mechanism (Naik *et al.,* 2013). It helps in cell growth and differentiation (Agarwal *et al.,* 2012). Protease has degradative and synthetic properties. Thus it has importance in maintaining physiological condition of the organisms and also in industries (Garg and Singh, 2015).

2.3. Protease

Protease belongs to the class of hydrolase enzymes. It is a mixture of different types of enzymes such as proteinases, peptidases and amidases (Mukhtar and Haq, 2009). Proteases have resistance to temperature thus it will be active in extreme high or low temperature conditions (Shahzad *et al.,* 2015).

Proteases have molecular weight ranging from 18-90 kDa (Muthulakshmi *et al.,* 2011).

Protease exists as intracellular or extracellular enzyme. The functions of intracellular protease were to maintain cellular and metabolic process such as protein turnover, enzyme maturation and maintaining cellular protein pool where as the extracellular protease are involved in hydrolysis of proteins present in cell free environment (Sneha *et al*., 2014). Naturally protease exists in the inactive form called zymogens. Under favourable environmental conditions, zymogens undergo conformational changes, which result in binding of small peptides to the zymogen. The binding of small peptides convert the zymogen into active protease (Li *et al.,* 2013).

2.3.1. Classification of protease

Proteases are classified into many types based on different categories.

(i) Based on action – Exopeptidase and endopeptidase

(ii) Based on functional group – Endopeptidase is classified into serine proteases, aspartic proteases, cysteine proteases and metallo proteases.

(iii) Based on pH – acidic proteases, alkaline proteases and neutral proteases.

(i) Based on action

Exopeptidase

Exopeptidase cleaves peptide bonds present at terminal region. Based on terminal, the exopeptidase which cleaves carboxy terminal is called carboxypeptidase and one which cleaves amino terminal is called aminopeptidase (Sawant and Nagendran, 2014).

Endopeptidase

Endopeptidase cleaves the bonds which are present internally in the peptide sequence.

(ii) Based on Functional group

a. Serine protease

Serine protease is also known as alkaline protease. It has serine in its active site. The molecular mass of serine protease range from 18-35 kDa and its optimum pH varies from 7-11. This type of protease specifically cleaves the carboxy terminal of the aminoacids arginine and lysine. Trypsin, chymotrypsin, elastase are serine proteases present in animals and subtilisin is present only in bacteria.

b. Metallo protease

Metals like zinc, cobalt or nickel is present in the active site of metallo protease enzyme. Both animals and bacteria synthesize different types of metallo protease. e.g. Collagenase in higher organisms, toxins in snake venom and thermolysins in bacteria (Sriket, 2014).

c. Aspartic protease

Protease having aspartate in its active site is known as aspartic protease and it is acidic in nature. Aspartic protease is present mainly in viruses and not in bacteria or fungi. Since it is acidic in nature, active only at low pH ranges 3-4.

d. Cysteine protease

Cysteine protease has cysteine in its active site. It is neutral or weak alkaline in nature. e.g. Cathepsins and calpins present in mammals (Mienda *et al.,* 2014).

2.3.2. Sources of protease

Protease is present in all kinds of living organisms. Thus, protease can be obtained from different sources like plants, animals and microorganism.

Plant protease

In plants, protease has many functions and one such function is to maintain overall protein turnover at different stages of plants life. Different types of proteases were active during seed development and help in plant growth (Panicker *et al.,* 2009). Proteases which were commonly obtained from plants

are papain, ficin, bromelain etc. Plant based proteases have properties like anti-tumor, anticancer and immunomodulatory properties. Papain is one of the important plant protease used for different purposes like cheese making, as food additive and emulsifier (Baskaran *et al.,* 2014).

Animal protease

Animals also produce different types of proteases. Pancreatic trypsin, chymotrypsin, pepsin and rennin were the most common animal proteases (Sawant and Nagendran, 2014). Trypsin is an example for serine protease. It is used in preparing media for bacteria. Chymotrypsin is used only in diagnostic purposes. The important application of chymotrypsin is to deallergenizing the milk protein hydrosylates. Rennin is a type of protease used in dairy industry to make good flavored curd (Rao *et al.,* 1998).

Microbial protease

Two – third percent of commercially available proteases were obtained from microorganisms (Sathiya, 2013). Microorganisms have the ability to produce both intracellular and extracellular protease (Rani *et al.,* 2012). Both bacteria and fungi synthesize protease enzyme. The type of enzyme produced by the microorganism depends on its species and strain.

Fungal protease

Generally, the fungi produce alkaline protease. The enzyme produced by fungi was easily isolated by solid state fermentation method. Major fungal species which produce alkaline protease are *Aspergillus candidus, A.flavus, A.niger, Fusarium eumartii, Cephalosporium, Rhizopus oligosporus* etc, (Kuberan *et al.,* 2010).

Bacterial protease

Bacteria also synthesize alkaline protease and the proteases produced by bacteria are thermostable and active upto 70˚C. Major types of bacteria which synthesize protease are *Alteromonas sp., Bacillus sp., Lactobacillus helveticus, Pseudomonas aeruginosa, Staphylothermus marinus, Sterptomyces microflavus, Sterptomyces rectus, Thermoactinomyces* sp., *Torulathermophila, Xanthomonas maltophila* (Jisha *et al.,* 2013).

2.3.3. Applications of Microbial protease

Proteases obtained from microbes have a wide range of applications in different industries like food industry, leather industry, pharmaceutical industry and detergent industry.

Detergent industry

Protease is one of the major ingredients used to prepare different kind of detergents like household laundary, reagents used to clean contact lenses and other commercially available detergents (Mienda *et al.,* 2014). BIO-40 was the first detergent developed using bacterial enzyme in 1957. In 1960, Novo introduced the detergent with commercial name BIOTEX using alcalase enzyme produced by *Bacillus licheniformis* (Rao *et al.,* 1998). Among different types of proteases, the serine protease produced by *Bacillus* strains and alkaline protease by fungi are currently used in detergent making (Mienda *et al.,* 2014).

Leather industry

In leather industry, different steps such as soaking, dehairing and tanning are carried out during leather processing and in all these steps various hazardous chemicals are used. One among those chemical is sodium sulfide. The use of chemicals creates problems during effluent disposal and treatment and also results in environmental pollution. Thus to overcome these problems, now-a-days the leather industries prefer enzymes especially protease from microbes than the chemicals. Proteases used in leather processing are mainly

obtained from species like *Aspergillus flavus, Streptomyces, Bacillus amyloliquefacians* and *Bacillus subtilis* (Jisha *et al.,* 2013).

Food and dairy industry

Various processes in food industry which use protease are cheese making, meat tenderization, baking and preparation of protein hydrolysates (Rao *et al.,* 1998).

Silver recovery

The X-ray films used in different purposes have silver impregnated with gelatin. The traditional method used to remove this silver is to burn x-ray film but it leads to environmental pollution. Thus to avoid pollution, the alternate method was developed. In this new method, the alkaline protease having the ability to hydrolyse gelatin was used to remove silver, which is ecofriendly because the usage of protease doesn't cause any harm to the environment (Jisha *et al.,* 2013).

Pharmaceutical industry

Proteases are also used as therapeutic agents due to its diversity and specificity nature. Protease from different microbes is used to treat various diseases. Subtilisin is a type of protease used to treat burns and wounds. The other proteases used for medical purpose were protease from *Aspergillus oryzae* to cure lytic enzyme deficiency syndrome and asparginase from *E.coli* to treat lymphocytic leukemia (Rao *et al.,* 1998). Proteases are used in preparation of wound healing ointments and contact lens enzyme cleaners (Sawant and Nagendran, 2014).

Textile industry

In textile industry, protease is used to remove impurities which are protein in nature. e.g. removal of sericine from silk fiber. The sericine form stiff and dull gum layer on the raw silk fiber. The removal of sericine improves the luster and

softness of the fiber. Protease is also used to impart new and unique finishes to wool and silk fibers by modifying its surface (Josephine *et al.,* 2012).

Research purposes

Proteases cleaves the peptide bond selectively, thus it is used to identify structure function relationship, peptide synthesis and in protein sequencing. It is also used to digest proteins during tissue dissociation, cell isolation and culturing of cells (Sawant and Nagendran 2014).

2.4. Extraction of industrially important enzymes from termite soil

The preferred source for the production of industrially important enzymes is from microbes because microbes can grow rapidly and it is possible to manipulate the microorganisms in order to produce new enzymes with altered or improved properties which will increase the demand for the requirement of the protease in industries for various applications (Josephine *et al.,* 2012). According to Makonde *et al.* (2015) and Manuja *et al.* (2014), it is clear that termite soil have unique variety of microorganisms. Thus, termite soil has been chosen to isolate enzyme producing bacteria.

2.4.1. Termites

Termites are the group of insects which belongs to the group Isoptera. It act as soil engineers and modify the properties of soil and using these soil it builds different form of nest, mound and termitarium. Different types of termite species build different types of mounds. Example – Soil feeding termites build mounds using inorganic particles and fecal matter where as fungus growing termites build using soil, clay and saliva (Makonde *et al.,* 2015). Termites are the source of enzymes which convert lignocellulose into biofuels (Manjula *et al.,* 2011).

2.4.2. Ecological benefits of termites

Generally termites help in maintaining microbial communities in the soil. Thus the different structures formed by termites and the surrounding soil were rich in nutrients and microorganisms. Termites play important role in changing the properties of soil and convert them into fertile soil. It has been reported that removal of termites may lead to disappearance of perennial grass from the vegetation and also reported that trees surrounded by termite mounds became grown well throughout the year but the growth of the shrubs get decreased due to termites.

2.4.3. Termite soil

The earth's surface is covered by layers of soil. The soil is rich in organic matter, nutrients, minerals and microorganisms. The content of the soil varies from place to place. The growth of microorganism on the soil depends on organic matter, aeration, moisture and temperature (Magnet *et al.,* 2013). Termites are the group of insects which alter the properties of soil when it build its mound, termatarium or nest. Thus the properties of soil formed by termites are greatly varied with normal soil (Dhembare, 2013).

2.4.4. Termite soil formation

Termites transport only the specific and important particles from the soil to build its nest or mounds. The type of particles being transported by the termites depends on size and shape of mound and nest, species, climate and soil. Termites make its mound very strong by adding more amounts of soil and organic matter to the mound surface in order to protect its structure from rain, wind, human activities and animals (Ali *et al.,* 2013).

2.4.5. Properties of termite soil

The clay content of termite mound soil is significantly higher than normal soil (Dhembare, 2013). The minerals present in termite soil are silver, zinc, cobalt, nickel, copper and iron and the amount of these minerals are higher than

the surrounding soils (Ntukuyoh *et al.,* 2012). Sarcinelli *et al.,* 2008 reported that the amount of carbon, nitrogen and phosphorus in the termite soil were comparatively higher than that of soil adjacent to the termite soil or mound. This high concentration is mainly due to deposition of organic matter on the mound and the waste produced by termites by ingesting organic materials from various sources. The termite soil has low aluminium content compared with other soils.

2.4.6. Microorganisms in termite soil

Termite soil is rich in different kinds of bacteria and fungi. According to Manjula *et al.,* 2014, most dominant microorganisms in termite soil are Proteobacteria-32%, Actinobacteria-20%, Bacteriodetes-7%, Acidobacteria-6% and other group such as Gemmatimonadetes, Nitrospirae, Chlamydiae, Chloroflexi, Tenericutes and Deinococcus are less abundant. Deinococcus-Thermus is a special group of hazard resistant bacteria present only in termite soil and not in termite gut.

2.4.7. Applications of termite soil

The important application of termite soil is it is used as biofertilizers because it is rich in minerals and microorganisms. The presence of bacteria like *Rhizobium*, *Pseudomonas*, *Thiobacillus, Desulfovibrio, Clostridium* and *Enterobacter* enhance the fertility of termite soil (Manuja *et al.,* 2014).

2.5. Extraction of enzyme by fermentation

Fermentation is the process of converting complex substrates into simple compounds using microorganisms. During this conversion, number of products namely peptides, enzymes and antibiotics get released as secondary metabolites. Thus, this method can widely used in producing large amount of enzymes from microorganisms. Fermentation used to extract enzymes is of two type's namely solid state fermentation and submerged fermentation.

Solid state fermentation

Solid state fermentation (SSF) uses solid substances like bran, bagasse and paper pulp as substrates. This method takes place slowly and steadily, thus the products were released in the controlled manner. SSF method was used to extract enzymes from microorganisms which require low moisture content for their growth.

Submerged fermentation

Submerged fermentation (SmF) also known as liquid fermentation requires substrate in the form of liquid (molasses or broth). Substrate utilization is very rapid in this method. Thus constant supplementation of nutrient is required. Microorganism requires high moisture has been used in this method to extract enzymes (Subramaniyam and Vimala, 2012).

SmF is more advantage than SSF which includes easy handling, control of environmental factor pH and temperature will be more convenient (Vidyalakshmi *et al.,* 2009).

2.6. Purification of enzymes

Enzymes isolated from any source have some amount of impurities in the form of salts, ions, products similar to the isolated enzyme. The presence of these impurities may alter the properties and activity of the enzyme which results in inactivation of enzymes. Thus, the purification of enzyme is one of the steps to be followed after isolation. Protein purification was also performed to obtain the single type of molecule from protein mixture. For enzyme purification, different methods were followed. The commonly used purification methods are as follows - Ammonium sulphate precipitation and Dialysis.

2.6.1. Ammonium sulphate precipitation

Precipitation is one of the commonly used less expensive procedure to purify the enzymes. In this method, neutral salt like ammonium sulphate and solvents like ethanol, methanol and acetone are used to precipitate the enzymes.

Mostly ammonium sulphate is used to precipitate enzymes. It precipitates different enzymes at different concentration according to the molecular weight of the enzyme. Enzymes with high molecular weight precipitate at low concentration of ammonium sulphate and low molecular weight enzymes at high concentration (Nadeemullah and Mukhtar, 2013).

2.6.2. Dialysis

Dialysis is mainly performed to remove small molecules like ions from the protein mixture using semi permeable membrane of different pore size. The commonly used semi permeable membrane is made of cellulose. The pore size of the membrane to be used, was depends on the size of the enzyme to be purified. During dialysis, molecules with smaller size than the pore will move out whereas the large sized molecules retain on the membrane (Bonner, 2007).

3.0 Experimental procedure

The new trend in biotechnology is the conversion of wastes into useful biomass by enhancing the microorganism growth and thereby producing large amount of commercially important enzymes from it (Pant *et al.,* 2015).

Enzymes are the biocatalysts which enhance biochemical reactions occur in living organisms. All enzymes are protein in nature and is necessary for the life (Dam *et al.,* 2013). Enzymes are of different types and each have role in different types of industries like pharmaceutical, leather, textile, food and detergent industries. The commercial synthesis of different types of enzymes gets increased due to the advent of biotechnology. Proteases are one among these enzymes because of its potential purpose in industries (Umayaparvathi *et al.*, 2013).

Protease is the enzyme having the property of degrading proteins into peptides by cleaving the peptide bonds. Thus it is known as proteolytic enzymes. Proteases are present in different sources like plants, animals and microorganisms (Devi *et al.,* 2014). Among these, microbial proteases have great demand among the industries because its characters are suitable for the biotechnology processes. Thus, more research works are now going on in order to obtain the proteases with great commercial purposes from different sources mainly from the microorganism present in waste materials (Kamran *et al.,* 2014).

Termites are the social insects which play an important role in soil formation. Termites build different structures like mounds, nest, termatarium, etc., using soil. These structures are rich in soil organic matter and fine particles and the soil near to these structures are also rich in nutrients (Ali *et al.,* 2013). The organic matter in the termite soil enhances the growth of bacteria and fungi. The most important genera of bacteria in termite soil are *Rhizobium, Thiobacillus, Pseudomonas, Bacillus, Sterptomyces*, etc., (Manjula *et al.,* 2014). Since, termite soil is rich in microbial diversity, this can be used as a source for isolating the

protease producing bacteria and thereby producing large quantity of protease enzyme.

The present study entitled **"Screening and Partial Purification of Protease Produced by Bacteria Isolated from Termite Soil"** was carried out with the following experimental design:

3.1. Collection of sample

3.2. Isolation and maintenance of bacteria

3.3. Characterization of bacteria

3.3.1. Staining

3.3.2. Biochemical test

3.4. Screening of enzymes

3.4.1. Cellulase

3.4.2. Amylase

3.4.3. Lipase

3.4.4. Protease

3.5. Preparation of crude enzyme extract

3.6. Determination of enzyme activity

3.6.1. Cellulase

3.6.2. Amylase

3.6.3. Lipase

3.6.4. Protease

3.7. Purification of protease

3.7.1. Ammonium sulphate precipitation

3.7.2. Dialysis

3.8. Purification profile

3.8.1. Protease assay

3.8.2. Estimation of Protein content

3.8.3. Specific activity

3.8.4. Recovery percentage

3.8.5. Purification fold

3.1. Collection of sample

The termite soil formed on the surface of the dried leaves was collected from the local area near to Coimbatore, Tamilnadu. The collected soil sample was stored in clean dry polythene bag, brought to the laboratory and kept at room temperature.

3.2. Isolation and maintenance of bacteria

A serial dilution method was used to isolate the bacteria from the termite soil. The soil sample was dissolved in water to make soil suspension. The soil suspension obtained from serial dilution was used to culture the bacteria on the two types of media namely Nutrient agar medium by streak plate method. The individual colonies obtained were sub cultured, stored at 4˚C and used for further characterization. The detailed procedure was explained in Appendix I.

3.3. Characterization of bacteria

The bacteria isolated from the soil sample were characterized by performing staining and biochemical tests.

3.3.1. Staining - Gram staining was done to identify the morphology and to differentiate Gram positive and Gram negative bacteria (Sundarajan, 1995). The procedure was given in Appendix II.

3.3.2. Biochemical test - The various biochemical tests such as Indole, Methyl red, Voges Proskauer, Citrate utilization, Catalase, Starch hydrolysis, Triple sugar agar and Urea hydrolysis test were performed to identify the biochemical characteristics of the isolated bacteria (Kannan, 1996). The tests were carried out as per the procedure in Appendix III.

3.4. Screening of enzymes

The bacterial culture was screened for different types of enzymes

3.4.1. Screening of cellulase

The presence of cellulase producing bacteria was identified by the method given by Basavaraj *et al.,* (2014) and the procedure was described in Appendix IV.

3.4.2. Screening of amylase

Amylase screening was done by the Method of Rao *et al.* (2013) as shown in Appendix V.

3.4.3. Screening of lipase

Lipase screening was done by the Method of Ranjitha *et* al. (2009) as shown in Appendix VI.

3.4.4. Screening of protease

Screening of protease was carried out by the Method of Sayali *et al*. (2014) as given in Appendix VII.

3.5. Preparation of crude enzyme extract

The screened bacterial isolates were inoculated on the medium containing glucose, peptone, yeast extract, $CaCl_2$, K_2HPO_4 and $MgSO_4$ and incubated at 37°C for 24 h in shaking incubator for the production of large amount of crude enzyme. The crude enzyme obtained was collected in the form of clear supernatant by centrifuging the medium (Dam *et al.,* 2013). The procedure was explained in Appendix VIII.

3.6. Determination of enzyme activity

The crude enzyme extract obtained from above procedure was used to determine the activity of different enzymes.

3.6.1. Cellulase – The activity of cellulase enzyme was determined based on Denison and Koehn, 1977 method. The procedure was explained in Appendix IX.

3.6.2. Amylase – Amylase activity was identified using starch as substrate according to the method given by Bernfield, 1955 and the procedure was shown in Appendix X.

3.6.3. Lipase – The activity of lipase in the crude extract was identified as per the procedure in the Appendix XI (Selvam *et al.,* 2011).

3.6.4. Protease – Protease activity for the enzyme extract was carried out using casein as substrate with L-Tyrosine as standard (Dam *et al.,* 2013). The detailed procedure was explained in Appendix XII.

3.7. Purification of protease

The enzyme extracted from the bacterial culture having high activity was partially purified by performing different methods as follows – Ammonium sulphate precipitation and Dialysis.

3.7.1. Ammonium sulphate precipitation

The crude extract was primarily purified by ammonium sulphate precipitation method, also known as salting out method. In this, the crude extract was subjected to precipitating with different concentrations of ammonium sulphate such as 0-20, 20-40, 40-60, 60-80 and 80-100 percent. The percent of ammonium sulphate which shows highest activity of the enzyme was used for further purification (Simpson 2004). The procedure was explained in Appendix XIII.

3.7.2. Dialysis

The precipitate showing highest enzyme activity obtained from ammonium sulphate precipitation method was further purified by dialysis in order to remove salts or other small molecules which is present along with protease enzyme. Tris HCl is used as buffer, based on the procedure followed by Roe (2001) and the procedure was depicted in the Appendix XIV.

3.8. Purification profile

3.8.1. Protease assay

The activity of protease was determined in the enzyme sample purified by ammonium sulphate precipitation and dialysis as by the method explained in Appendix XII.

3.8.2. Estimation of protein content

The amount protein present in the crude extract, ammonium sulphate precipitated and dialyzed protease sample was calculated by Lowry's method using Bovine serum albumin as standard for which the procedure is explained in Appendix XV.

3.8.3. Specific activity

The specific activity for the crude extract, ammonium sulphate precipitated and dialyzed sample was calculated using the formula as follows

$$\text{Specific activity} = \frac{\text{Total activity (Units/ml)}}{\text{Total protein (mg/ml)}}$$

3.8.4. Recovery percentage

Recovery percentage was calculated from the percentage of total protease activity of the sample and the crude extract using the formula

$$\text{Recovery percentage} = \frac{\text{Protease activity of the sample}}{\text{Protease activity of the}} \text{ X } 100$$

3.8.5. Purification fold

Purification fold was calculated for ammonium sulphate precipitated sample and dialyzed sample using specific activity of the sample and the crude extract. The formula used was as follows

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

4.0 Results and Discussion

Enzymes are important biomolecule present in plants, animals and microorganisms. The production of industrially important enzymes from various sources has been increased tremendously in the last three decades. Protease is one among the industrially important enzymes to be produced in large quantity (Rebecca *et al.,* 2012).

Proteases are the proteolytic enzymes which hydrolyze the peptide bonds present in proteins. It is present in wide variety of organisms including plants, animals, bacteria and fungi. Proteases are classified as acidic, neutral and alkaline based on its activity at different pH (Sharmila *et al.,* 2012). 65% of the total industrial enzyme market use protease enzyme for its variety applications in different industries like food, pharmaceutical, leather and detergent. Commercially, alkaline proteases are used in detergent industry due to its compatibility and stability with all commonly used detergent components such as surfactants and oxidizing agents (Mrudala and Shyam, 2012). Thus, the aim of the present study is to isolate and purify the bacterial protease from termite soil.

The results of the present study entitled **"Screening and Partial Purification of Protease Produced by Bacteria Isolated from Termite Soil"** was discussed under the following headings

4.1. Isolation and identification of bacteria from termite soil

4.1.1. Isolation of bacteria

4.1.2. Characteristics of isolated bacteria

4.2. Enzymes produced by isolated bacteria

4.2.1. Qualitative screening for enzymes

4.2.2. Quantitative assay of enzymes

4.3. Isolation and purification of protease

4.3.1. Isolation of crude protease

4.3.2. Purification by Ammonium sulphate precipitation

4.3.3. Purification by Dialysis

4.3.4. Comparison of purification profile

4.1. Isolation and identification of bacteria from termite soil

4.1.1. Isolation of bacteria

The bacteria was isolated from the termite soil by serial dilution method and cultured in nutrient agar medium by streak plate method. The colonies formed were noticed and the clearly formed colony was selected for the further use. The selected colony was subcultured and stored at 4˚C. The colonies selected for the study was showed in the Plate 1.

Plate 1

Bacteria isolated from termite soil

The presence of bacteria in the termite soil was reported by Sreeremya *et al.,* 2016. The other studies which prove the presence of bacteria in the termite soil were reported by Fall *et al.,* 2007 and Manjula *et al.,* 2014. Singh *et al.,* 2015 and Patil *et al.,* 2015 isolated the bacterial from the soil samples.

4.1.2. Characteristics of isolated bacteria

The morphology and characteristics of the selected colony was identified by staining and biochemical test. The biochemical test performed to characterize the isolated bacteria were Indole test, Methyl red test, Voges proskauer test, Citrate utilization test, Starch hydrolysis test, Triple sugar iron agar test, Urease test and Catalase test.

Staining result showed that the isolated bacterium was rod in shape and it appeared purple in colour. This indicates the isolated bacterium was Gram positive in nature. The results of the biochemical test were showed in the Plate 2 and Plate 3. The characterization of the isolated bacteria was summarized in the Table 1.

Plate 2

Biochemical tests

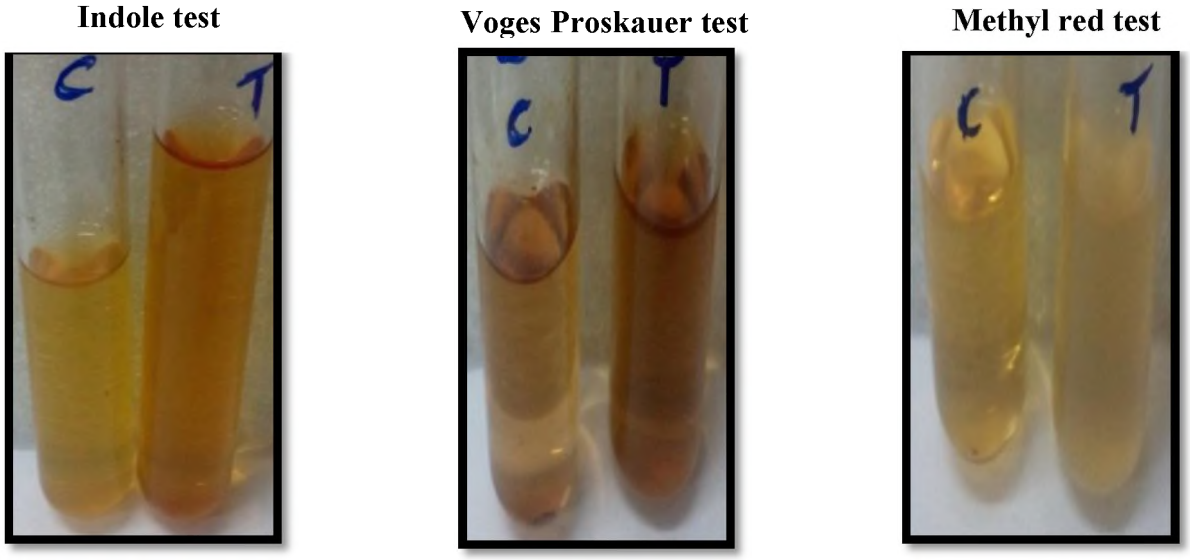


Plate 3

Biochemical tests

Citrate utilization test

Triple sugar iron agar

Urease test

Starch hydrolysis

Table 1

Biochemical characterization

Biochemical test	Observation	Result
Indole Test	No colour change	Negative
Methyl red Test	No colour change	Negative
Voges Proskauer Test	No colour change	Negative
Citrate Utilization Test	Conversion of green to blue colour of the agar	Positive
Starch Hydrolysis Test	Formation of zone of inhibition	Positive
Triple Sugar Iron Test	Colour change in agar from red to yellow, Hydrogen sulphide formation	Positive
Catalase Test	Formation of bubble	Positive
Urease Test	Conversion of yellow coloured agar into pink colour	Positive

From the table 1, it was clear that the isolated bacteria had the following characteristics – It has the ability to use citrate as carbon source and produce acetate and alkaline carbonate and the synthesis of catalase, urease and amylase by isolated bacteria. The negative result for the indole test indicates that the isolated bacteria did not have the ability to convert tryptophan to indole due to the absence of tryptophanase. Result of Methyl red test indicates that the bacterial culture produce less amount of acid during glucose fermentation. And also it does not produce acetyl methyl carbinol during glucose metabolism. Triple sugar iron agar test indicates that bacteria undergo glucose, lactose or sucrose fermentation with hydrogen sulphide formation.

Based on the results obtained from staining and biochemical tests, the isolated bacterium has been identified as *Bacillus* sp. It is similar to the result reported by Duza and Mastan, (2013) and Vikramathithan and Dhandapani, (2014).

4.2. Enzymes produced by isolated bacteria

Qualitative and quantitative tests were carried out to identify the enzymes produced by the isolated bacteria.

4.2.1. Qualitative screening for enzymes

The isolated bacterial culture was used to screen the presence of four enzymes namely cellulase, amylase, lipase and protease were carried out and the results were depicted in the Plate 4.

Cellulase

The ability of the isolated bacterial culture to produce cellulase enzyme was confirmed by qualitative screening using carboxy methyl cellulose agar as the medium. The presence of zone of inhibition around the bacterial culture in Plate 4a indicates the synthesis of cellulase. The presence of cellulase producing bacteria in the termite was reported by Saptarini and Indriyati, 2014.

Amylase

The production of amylase by the bacteria isolated from termite soil was identified using starch agar as the specific agar. The zone of inhibition formed in the agar shows the amylase production and the zone was clearly viewed using iodine solution. The presence of halo zone was showed in Plate 4b. The amylase isolated from agricultural soil also shows the clear zone of inhibition as reported by Madhav *et al.,* 2011 and Karnwal and Nigam, 2013.

Lipase

Rhodamine B and olive oil were used to identify the lipolytic activity of the isolated bacteria. Orange fluorescence in Plate 4c confirms the synthesis of lipase by the bacteria isolated from termite soil. Similar result was observed for the bacteria isolated from soil by Rabbani *et al.,* 2015 and from waste vegetable oil contaminated soil by Lechuga *et al.,* 2016 and Thomas, 2015 from slaughter house soil.

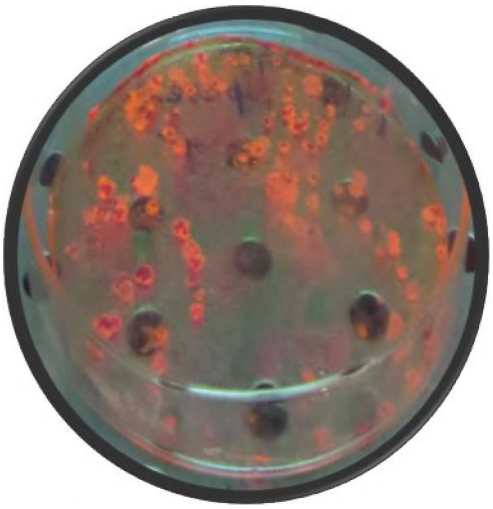
Plate 4

Screening of enzymes

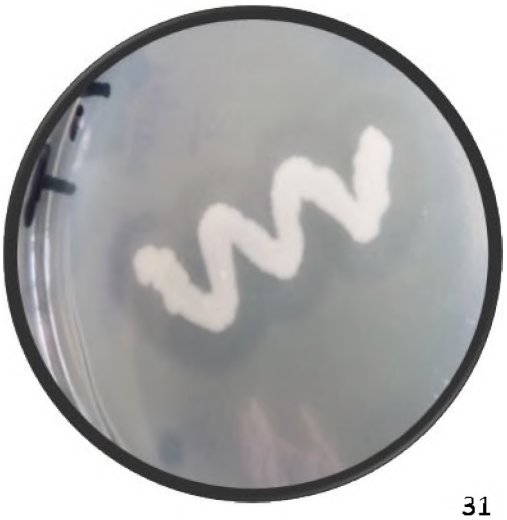
4a. Cellulase

4b. Amylase

4c. Lipase



4d. Protease



Protease

The synthesis of protease by the bacterial culture was identified using peptone gelatin agar medium. The isolated bacteria inoculated on the peptone gelatin agar show the clear zone of inhibition which indicates the production of protease. The zone formed was displayed on the Plate 4d. The bacterium isolated from the soil also shows the similar zone of inhibition (Bhavani *et al.,* 2013 and Josphine *et al*., 2012).

4.2.2. Quantitative assay of enzymes

The crude enzyme was extracted from the isolated bacterial culture and used as enzyme source to determine activity of cellulase, amylase, lipase and protease. The total activity of the four enzymes was shown in Figure 1 and the specific activities for the above enzymes were tabulated in the Table 2.

Determination of cellulase

The cellulase activity in the crude extract was determined spectrophotometrically using glucose as the standard. The total activity was calculated by constructing the standard graph and the value found was 2.82 U/ml. The protein content was also determined for the crude extract and the value obtained for protein content (2.58 mg/ml) was used to calculate specific activity. Cellulase showed 1.09 U/mg as its specific activity. According to Shanmugapriya *et al.* (2012), the cellulase isolated from the cow dung shows the highest activity of about 4.32 U/mg.

Determination of amylase

The amount of amylase in the crude extract was quantitatively estimated using maltose as its standard. The crude extract has total amylase activity of 2.71 U/ml and the specific activity calculated for the amylase was 1.05 U/mg. Kaur *et al.,* 2012, reported that the total amylase activity in *Bacillus amyloliquefaciens* isolated from soil in the potato field was found to be 0.0053U/ml.

Determination of lipase

The lipolytic activity in the crude extract was determined titrimetrically again sodium hydroxide. Total lipase activity was found to 1U/ml and specific activity to be 0.38 U/mg in the crude extract. The bacteria isolated from the termite soil shows low lipase activity where as the bacteria from hill soil shows high activity of 4.280 U/ml (Rajeshkumar *et al.,* 2013).

Determination of Protease

The protease activity was estimated using casein as substrate and L-tyrosine as standard. 3.70 U/ml was the calculated protease activity in the crude extract and its specific activity was found to be 1.43 U/mg. The protease isolated from different sources shows variation in total activity as follows – protease isolated from alkaline hot spring shows 1.2 U/ml (Wilson and Remigio, 2012).

Figure 1

Activity of different enzymes in crude extract

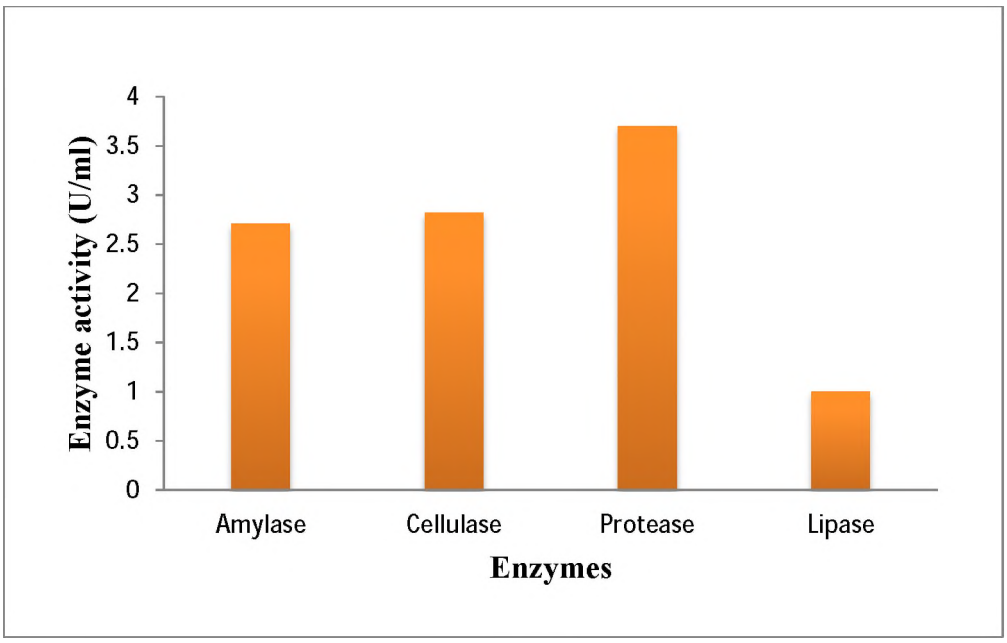


Table 2

Specific activity of the enzymes

Enzyme	Specific activity (U/mg)
Cellulase	1.09
Amylase	1.05
Lipase	0.38
Protease	1.43

From the Figure 1 and Table 2, it was clear that the isolated bacterial culture has the ability to produce different enzymes at varying concentration. It produces protease in higher amount and lipase in very minute quantity. The activity of protease was found to be higher in the crude extract and it has been used for the further purification. The results are similar to the studies carried out by Duza and Mastan (2013), which shows that the *Bacillus* sp produced very small amount of lipase when compared with other species.

4.3. Isolation and Purification of protease

4.3.1. Isolation of crude protease

The isolated bacterial culture having higher proteolytic activity was further increased by inoculated on the specific growth medium. The cell free extract was obtained from the growth medium by centrifugation and has been used as crude protease. The crude protease showed total activity of 3.70U/ml and 1.43U/mg of specific activity. Table 3 shows the purification profile of crude protease. According to result reported by Aqel (2012), the *Bacillus* strain HUTBS71 showed enzyme activity of 3.2U/ml. To increase the specific activity of protease, the crude extract was subjected to purification by ammonium sulphate precipitation and dialysis.

Table 3

Purification profile of crude protease

Parameter	Crude extract
Total activity (U/ml)	3.70
Protein content (mg/ml)	2.58
Specific activity (U/mg)	1.43
Recovery percentage (%)	100
Purification fold	1.00

4.3.2. Purification by ammonium sulphate precipitation

The crude protease extract isolated from the bacterial culture was primarily purified by ammonium sulphate precipitation method. Different concentration of ammonium sulphate ranging from 0-20, 20-40, 40-60, 60-80 and 80-100 percent were used and among these various concentrations, 60-80% concentration shows increased total activity and specific activity when compared with the crude extract. The other fractions did not show significant enzyme activity.

Aqel, 2012 reported that protease isolated from the *Bacillus* sp have maximum activity at 75-80% ammonium sulphate saturation. Protease isolated from *Penicillium janthinellum* and *Neurospora crassa* also show highest total protease activity at 70% ammonium sulphate concentration (Abirami *et al.,* 2011).

The purification profile for the protease purified by ammonium sulphate precipitation at different concentration was summarized in the Table 4.

Table 4

Purification profile of ammonium sulphate precipitated protease

Sample	Total Activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg)	Recovery Percentage (%)	Purification fold
0-20%	0.33	1.78	0.19	11.80	0.10
20-40%	0.34	1.31	0.26	12.14	0.20
40-60%	0.56	0.92	0.60	20.00	0.50
60-80%	0.78	0.37	2.10	27.90	1.92
80-100%	0.30	0.35	0.80	10.71	0.73

4.3.3. Purification by dialysis

The precipitate obtained from 60-80% ammonium sulphate concentration showed highest enzyme activity (0.78 U/ml) was used for further purification by dialysis. Total activity, specific activity, protein content, recovery percentage and purification fold for the dialyzed sample was showed in the Table 5. After dialysis, the specific activity of the enzyme increased from 2.10 U/mg (60-80%) to 2.90 U/mg with 2.60 purification fold.

Increase in specific activity proves that purification of protease by dialysis method efficiently purifies the enzyme by removing impurities present in the precipitate obtained from ammonium sulphate precipitation. Table 5 summarizes the purification profile for dialyzed sample.

The similar result was reported for the protease isolated from *B. cereus* having purification fold of 3.05 after dialysis (Umayaparvathi *et al.,* 2013) and by Muthulakshmi *et al.,* 2011 for the protease isolated from *A.flavus* with purification fold of 2.53.

Table 5

Purification profile of dialyzed sample

Parameter	Dialysis
Total activity (U)	0.87
Protein content (mg)	0.30
Specific activity (U/mg)	2.90
Recovery percentage (%)	31.07
Purification fold	2.60

Summary of Purification profile

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	3.70	2.58	1.43	100	1.00
Ammonium sulphate precipitation (60-80%)	0.78	0.37	2.10	27.90	1.92
Dialysis	0.87	0.30	2.90	31.07	2.60

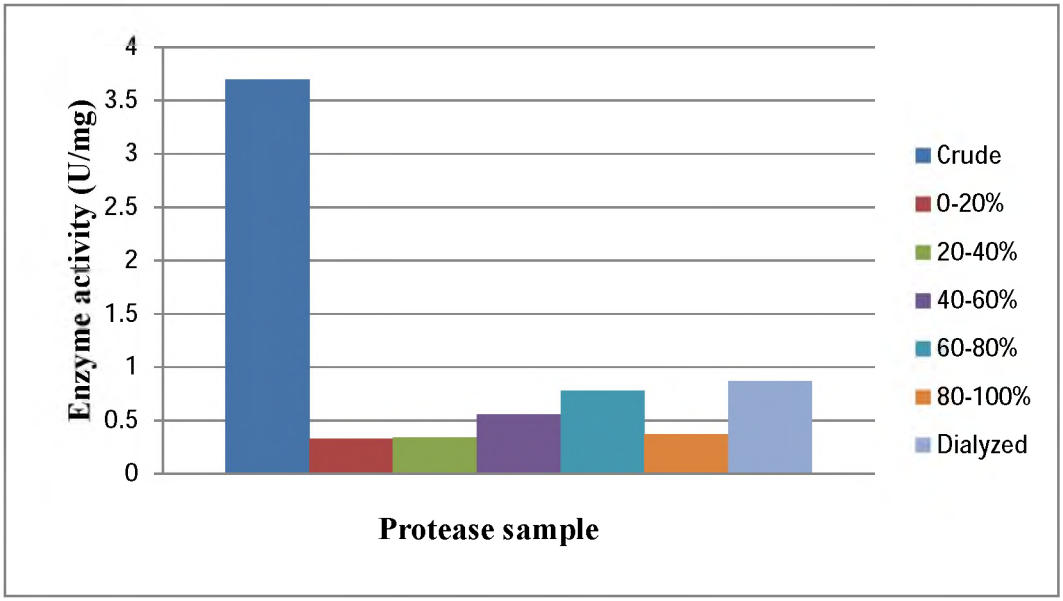
4.3.4. Comparison of purification profile

Total protease activity

The crude extract showed highest protease activity when compared with ammonium sulphate precipitate and dialyzed sample. Among various concentration of ammonium sulphate, 60-80% showed the highest protease activity and 80-100% showed the lowest protease activity. This indicates that after 80% ammonium sulphate attains saturation and does not precipitate the enzyme. Protease activity in dialyzed sample was found to be high when compared with ammonium sulphate precipitation. Total protease activity for the crude and purified protease was graphically explained in Figure 2.

Figure 2

Total activity of crude and purified protease

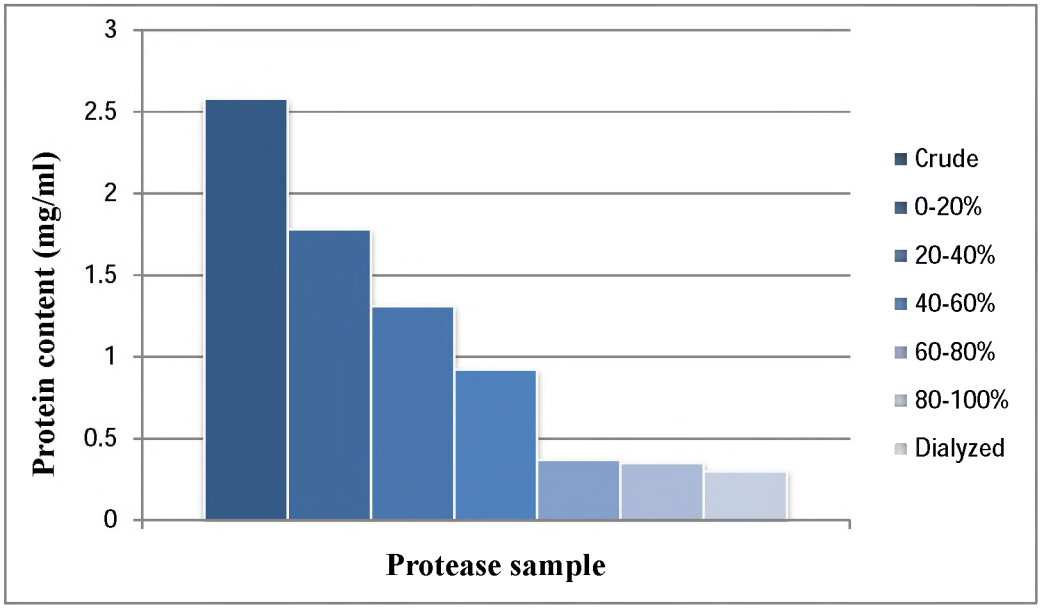


Protein content

The amount of protein present in crude, ammonium sulphate precipitate and dialyzed sample was estimated. Protein content was found to be higher in crude extract (2.58 mg/ml) and all other samples shows low protein content than the crude extract. Among the purified samples, 0-20% ammonium sulphate precipitate showed high protein content (1.78 mg/ml) and the dialyzed sample had low protein content (0.30 mg/ml) compared to that of other samples. Pant *et al.,* 2015 reported that protein content in the crude extract obtained using *Bacillus subtilis* was found to be 2.31mg/ml. Figure 3 represents the total protein content in different types of protease sample.

Figure 3

Protein content for different types of protease samples

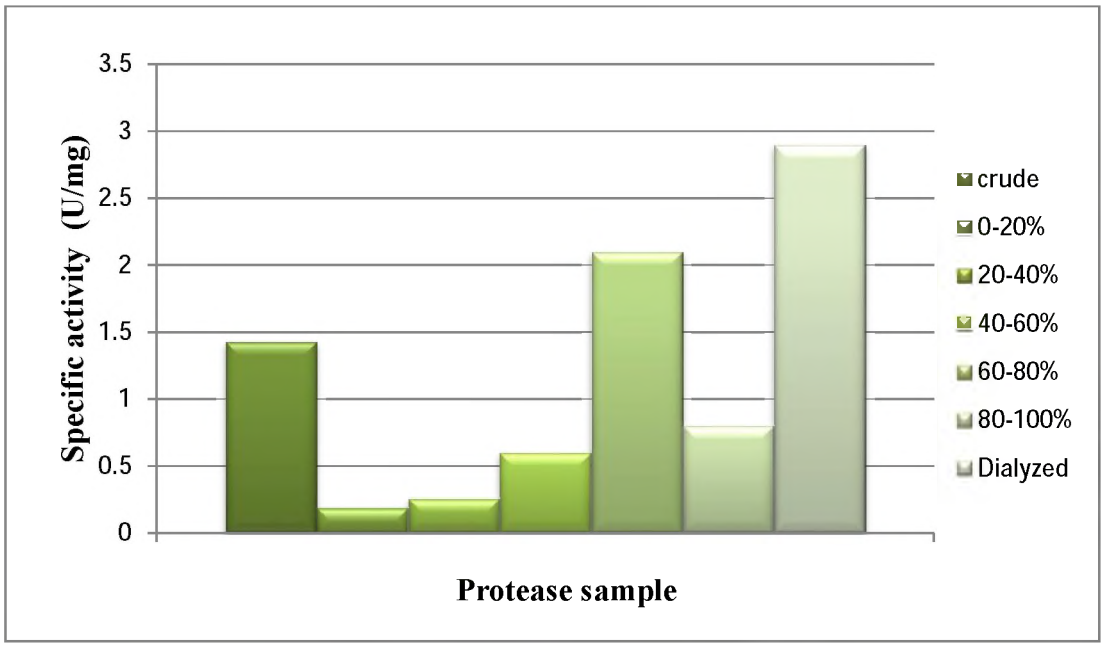


Specific activity

Specific activity refers to enzyme purity. The enzyme with more purity shows the highest specific activity. Dialyzed sample have the more specific activity value (2.90 U/mg) than 60-80% ammonium sulphate precipitate (2.10 U/mg) and the crude (1.43 U/mg). This indicates that the purification of protease by dialysis shows the purity level. Specific activity of crude protease, ammonium sulphate precipitate and dialysis were showed in the figure 4.

Figure 4

Comparison of specific activity of crude extract with purified protease

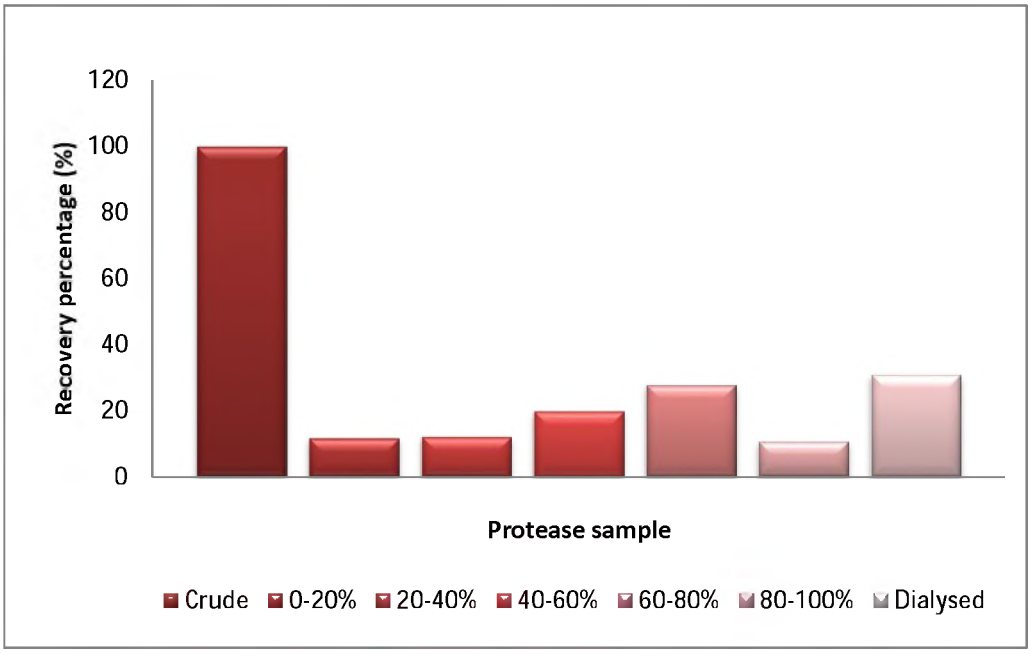


Recovery percentage

The recovery percentage of crude protease was found to be higher than the purified protease. Among different precipitates obtained from ammonium sulphate precipitation, 60-80% ammonium sulphate precipitated fraction showed 27.90% of recovery and the dialyzed sample shows 31.07% recovery. Recovery percentage for crude and purified protease sample were represented in Figure 5.

Figure 5

Recovery percentage for the crude, precipitated and dialyzed protease

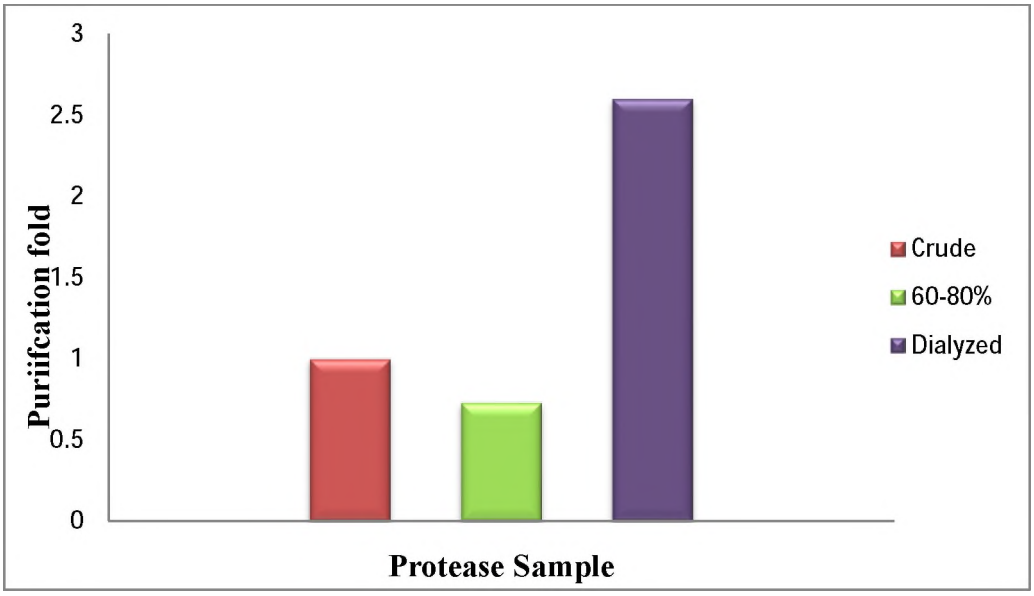


Purification fold

Purification fold for the isolated protease was found to be higher in dialyzed sample and 60-80% ammonium sulphate precipitate. The other precipitates (0-20%, 20-40%, 40-60% and 80-100%) showed lesser value than the crude extract. Dialyzed sample showed the highest value (2.60) than ammonium sulphate precipitation sample, indicating the purity of the enzyme to be more in the dialyzed sample. Figure 6 showed the purification of crude, 60-80% ammonium sulphate precipitate and dialyzed protease sample.

Figure 6

Purification fold of crude and purified protease



5.0 Summary and Conclusion

The present study entitled **"Screening and Partial Purification of Protease Produced by Bacteria Isolated from Termite Soil"** was carried out with the objectives of isolation and identifying the bacteria present in the termite soil, to determine the various enzymes produced by the bacteria and the isolation and purification of protease produced by the isolated bacteria.

The bacteria was isolated from the termite soil and its morphology and characteristics were identified by staining and biochemical test. From staining and biochemical test, it has been found that the isolated bacteria was Gram positive, rod shaped bacteria belonging to *Bacillus* species.

The isolated bacteria was screened for the synthesis of four types of enzymes namely cellulase, amylase, lipase and protease by qualitative and quantitative method. In qualitative screening, it has been identified that the isolated bacteria have the ability to produce all the four enzymes. To estimate the amount of each enzyme produced by the bacteria, the quantitative assay was performed. From the assay results, it was clear that the isolated bacteria have the ability to synthesize protease enzyme in the larger amount. Thus protease was selected for the further purification.

The protease was extracted from the isolated bacteria and it was purified by ammonium sulphate precipitation and dialysis. Out of five fractions used, 60-80% ammonium sulphate precipitates the large amount of protease from the crude extract and it has been used for the further purification by dialysis. After dialysis, the specific activity and purification fold was increased to that of ammonium sulphate precipitates and crude extract. Increase in specific activity and purification fold indicates that the dialyzed sample was purified to better extent from ammonium sulphate precipitation and crude extract.

Thus the result of the present study concluded that the bacteria belongs to the *Bacillus* species was present in the termite soil and it has the ability to produce protease in larger quantity than the other enzymes namely cellulase, amylase and lipase. The methods used to purify the protease (Ammonium sulphate precipitation and Dialysis) increased the purity of the enzyme by increasing the specific activity.

Recommendations for future study

- Purification of protease by Ion exchange chromatography and Gel filtration chromatography
- Determination of molecular weight
- Optimization of pH, temperature, media composition and substrate concentration to increase the protease production

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Appendices

Appendix I

Isolation of bacteria by serial dilution method

(Dam *et al.,* 2013)

Reagents

- Sterile Distilled water

Procedure

1. 1 gm of the soil was dissolved in 10 ml of sterile distilled water to make soil suspension.
2. The soil suspension was diluted to 10 times using distilled water by transferring 1 ml of the suspension to the 9ml of sterile distilled water. This represents 10^{-1} dilution.
3. From 10^{-1} dilution, 1ml was transferred to 9ml of sterile distilled water (10^{-2} dilution).
4. Dilution step was repeated till 10^{-7} dilution.
5. From 10^{-2} to 10^{-7}, 1 ml of the suspension was inoculated on the nutrient agar medium and the plates were incubated at 37°C for 24 to 48h.

Appendix II

Gram's staining

(Sundarajan, 1995)

Principle

The application of solution of iodine to cells previously stained with crystal violet produces purple coloured iodine dye complexes in the cytoplasm of the bacteria. The cells that are previously stained with crystal violet and iodine are next treated with a decolorizing agent such as 95% ethanol or a mixture of acetone and alcohol. The difference between Gram positive and Gram negative bacteria is in the permeability of the cell wall to these "purple coloured iodine dye complexes" when treated with the decolourizing solvent. While Gram positive bacteria retain purple iodine – dye complexes after the

treatment with the decolourizing agent, Gram negative bacteria do not retain complexes when decolourized. To visualize decolourized Gram negative bacteria, a red colored stain such as safranine is used after decolourization.

Materials

1. Crystal violet
2. Solution A – 2g of Crystal violet was dissolved in 20ml of ethanol
3. Solution B – Dissolve 0.8g of ammonium oxalate in 80ml distilled water
4. Mix solution A and B
5. Gram's Iodine - 2g potassium iodide, 1g iodine, 300 ml distilled water
6. 95% Ethanol
7. Safranine - 0.25g safranine in 10 ml ethanol and 90 ml water

Procedure

The smear was fixed on the slide and cover it with crystal violet for one minute. It was then rinsed with tap water. A few drops of gram's iodine was added and allowed to react for 30 sec – 1min. Again it was rinsed with water and ethanol was added in drop wise manner to remove excess crystal violet from the smear. Then it was washed with tap water and stained with safranine for 45 sec. Finally, the slide was washed, dried and viewed under microscope.

Appendix III
Biochemical test
(Kannan, 1996)

1. Indole test

Principle

Tryptophan is an essential amino acid which is oxidized by bacteria resulting in the formation of indole, pyruvic acid and ammonia. The indole test was done by inoculating the test organism into tryptophan broth which contains tryptophan. The indole produced was detected by adding Kovac's reagent which produce cherry red colour.

Materials

- Peptone broth
- Kovac's reagent

Procedure

The bacterial isolates were inoculated on the tryptone broth and incubated at 37°C for 48 h. After incubation, 1ml of Kovac's reagent was added and shook gently. The formation of cherry red colour indicates positive result whereas absence of cherry red colour indicates negative result. The positive and negative results were compared with the control (without inoculating bacterial culture).

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 indicators.

Materials

- MR-VP broth
- Methyl red reagent - Dissolved 100 mg of methyl red in 300 ml of 95% ethanol. Added 200ml of distilled water and filtered it.

Procedure

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

3. Voges – Proskauer test

Principle

The bacteria belonging to enterobacteriaceae ferment glucose to produce butylene glycol and acetoin which are more neutral in nature. The end products are detected by Barrit's reagent.

Materials

- MR- VP broth
- Barrit's reagent

  Solution A: Dissolved 5g α- naphthol in absolute ethanol with constant stirring.

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

Procedure

MR-VP broth were sterilized, inoculated with bacterial culture and incubated at 37^0C for 24 h. 40% KOH solution (VP reagent B) and Barrit's α-napthol solution (VP reagent A) was added after incubation period. Gently shook the tubes for 30 sec 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.

Materials

- Simmon's Citrate agar medium

Procedure

The organism was streaked on to Simmon's citrate agar slants. The tubes were incubated, at 37˚C for 24-48 h. Following incubation period the

citrate tubes turns from green to deep Prussian blue colour which indicates positive result. No colour change indicates negative result.

5. Triple sugar iron agar test

Principle

Microbes use carbohydrates as energy source depending on their enzyme components. Major products of carbohydrate catabolism are lactic, formic or acetic acid with production of H_2 or CO_2 as gas.

Materials

- Triple sugar iron agar

Procedure

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

6. Catalase test

Principle

Some organisms posses the enzyme catalase that splits hydrogen peroxide into oxygen and water. Presence of catalase is indicated by bubbles of free oxygen gas.

Materials

- 3% Hydrogen peroxide

Procedure

Picked up a colony of the bacteria from a plate and transferred the colony on a microscope glass slide in a drop of water. Placed a few drops of 3% hydrogen peroxide over the culture. If bubble appears within 20 seconds, the organism shows positive catalase activity. Absence of bubble formed shows negative catalase activity.

7. Urea hydrolysis test

Principle

Urea is a waste nitrogenous material excreted out by animals. Some organism 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 increase the pH of the medium. This can be detected by incorporating a pH indicator in the medium, which changes the colour during alkaline conditions.

Materials

- Urea broth medium

Procedure

Inoculate the urea broth medium with bacterial culture. Incubate the culture at 37°C for 48 h. The phenol red indicator will turn to pink. Urease positive tubes are deep pink in colour. No change indicates negative result.

8. Starch hydrolysis test

Principle

Starch is a soluble polymer of glucose, which act as a source of carbon for microorganism, which has an ability to degrade them. Degrading microorganism transports the degraded form across the membrane of the cell. Some bacteria posses the ability to produce amylase that breaks starch into maltose. The amylase is an extra cellular enzyme, which is released from the cells of microorganism.

Materials

- Starch Agar Medium
- Iodine solution: Dissolved 1g of iodine and 2g of potassium iodide in 300 ml distilled water.

Procedure

Prepared starch agar plates and streaked with suitable culture. Allowed the microbe to grow at 37^0C for 48 h and flooded with iodine solution. A clear zone around the organism indicates positive result. Dark blue colouration of medium with no clear zone formation indicates negative result.

Appendix IV

Screening for cellulase

(Basavaraj *et al.*, 2014)

Materials

- Carboxy methyl cellulose agar medium: Carboxy methyl cellulose – 2.0 g, Beef extract – 5.0 g, Peptone – 5.0 g, NaCl – 5.0 g, Agar – 20 g, Distilled water – 1000 ml, pH – 7.0
- 1% Congo red solution
- 1M NaCl

Procedure

1. For extracellular cellulase, the isolated bacteria were inoculated on Carboxy methyl cellulose agar plates.
2. Incubated CMC agar plates were flooded with 1 % Congo red and allowed to stand for 15 min at room temperature.
3. 1M NaCl was thoroughly used for counterstaining the plates. Clear zones appeared around growing bacterial colonies indicating cellulose hydrolysis.
4. The bacterial colonies having clear zone were selected for identification and cellulase production.

Appendix V

Screening for amylase

(Rao *et al.*, 2013)

Materials

- Starch agar medium : Starch – 20 g , Beef extract – 3.0 g, Peptone – 5.0 g, Agar – 15 g, Distilled water- 1000 ml
- Iodine solution: Dissolve 1g of iodine and 2g of potassium iodide in 300 ml distilled water.

Procedure

1. Starch agar medium was prepared then poured into the sterile petriplates after sterilization.
2. A loopful of culture was inoculated into Starch agar media and incubated at 37^0C for 24 h.
3. After the incubation period the plates were flooded with iodine solution and checked for the presence of zone of hydrolysis around the bacterial colonies.

Appendix VI

Screening for lipase

(Ranjitha *et al..,* 2009)

Materials

- Olive oil
- Rhodamine B

Procedure

1. This is a plate assay to detect bacterial lipase in a medium containing Olive oil and the fluorescent dye Rhodamine B.
2. Growth medium was adjusted to pH 7.0, autoclaved and cooled to about 60˚C.
3. Then 1% of olive oil, 10 ml of Rhodamine B solution (0.001% w/v) was added with vigorous stirring and emulsified by mixing for 1 min with a homogenizer.

4. 20 ml of medium was poured into each petridish.
5. The overnight culture was spotted in the centre of the plate and incubated for 48 h.
6. The plates were incubated for 16 h at 30^0C.
7. Lipase production was monitored by irradiating plates with UV light at 350 nm. After 16 h of incubation, bacterial colonies began to show an orange fluorescence. With continuing incubation time, orange fluorescent halos were formed around the colonies.

Appendix VII
Screening for protease
(Sayali, 2013)

Materials

- Peptone gelatin agar medium: Beef extract – 3.0 g, Peptone – 5.0 g, Gelatin – 4.0 g, Agar – 20.0 g, Distilled water – 1000 ml, pH - 7.0
- 15% mercuric chloride

Procedure

1. For extracellular protease, the isolates were inoculated on peptone gelatin agar medium and incubated at 37^0C for 24 h.
2. The appearance of clear zone around the colony after flooding the plates with 15% mercuric chloride indicated the presence of proteolytic activity.

Appendix VIII
Preparation of crude enzyme extract
(Dam *et al.,* 2013)

Materials

- Nutrient broth
- Glucose
- Peptone

- Yeast extract
- $Cacl_2$
- K_2HPO_4
- $MgSO_4$

Procedure

1. 10ml of the bacterial culture was inoculated on the 90ml of sterile broth containing glucose, peptone, yeast extract, $CaCl_2$, K_2HPO_4 and $MgSO_4$ and incubated in shaking incubator at 37°C for 24h.
2. After incubation, the cells were harvested at 15000 rpm for 10 min.
3. The clear supernatant obtained was stored at 4°C.

Appendix IX

Determination of cellulase

(Denison and Koehn, 1977)

Principle

The production of reducing sugar (glucose) due to cellulolytic activity is measured by dinitrosalicylic acid method.

Materials

- Filter paper disc: Cut the What man filter paper No.1 with a paper punch to ensure the same surface area of substrate in the reaction tube.
- Dinitrosalicylic acid reagent (DNS)
- 40% Rochelle salt solution (Potassium Sodium tartarate)
- Standard glucose: Dissolved 50 mg glucose in 50 ml distilled water

Procedure

1. Pipetted out 0.5 ml of extract to 32 mg of dry Whatman No.1 filter paper. Incubated the mixture at 50^0C for 1 hour.
2. Immediately after removing the enzyme - substrate mixture from the bath, added 0.5 ml of DNS reagent.
3. Heated the mixture in a boiling water bath for 5 minutes.

4. While the tubes were warm, add 1.0 ml potassium sodium tartarate solution. Cooled to room temperature. Then added water to make 5 ml volume.
5. Measured the absorbance at 540 nm. Prepared a standard graph with glucose in the concentration range 50 µg to 1000 µg/ml.

Appendix X

Determination of amylase

(Bernfield, 1955)

Principle

The reducing sugars produced by the action of α and β amylase react with dinitrosalicylic acid and reduce it to a brown colored product nitroaminosalicylic acid.

Materials

- 0.1M Sodium acetate buffer (pH 4.7)
- 1% Starch solution
- Dinitrosalicylic acid reagent(DNS)
- 40% Rochelle salt solution (Potassium Sodium tartarate)
- Maltose solution Dissolved 50 mg maltose in 50 ml distilled water in a standard flask and store it in a refrigerator.
- Enzyme solution

Procedure

1. Pipetted out 1 ml of starch solution and 1ml of properly diluted enzyme in a test tube.
2. Incubated it at 27^0C for 15 minutes.
3. Stopped the reaction by the addition of 2ml Dinitrosalicylic acid reagent.
4. Heated the solution in a boiling water bath for 5 minutes.
5. While the tubes were warm, add 1ml Potassium Sodium tartarate solution. Then cooled in running tap water.
6. Made up the volume to 10 ml by addition of 6 ml water.

7. Read the absorbance at 560 nm. Terminated the reaction at zero time in the control tubes.
8. Prepared a standard graph with 0-100 µg maltose.

Appendix XI

Determination of lipase

(Selvam *et al.*, 2011)

Materials

- 0.1M Tris-HCl buffer (pH 8.0)
- 95% ethanol
- 0.1M NaOH
- Phenolphthalein

Procedure

1. Lipase activity was determined titrimetrically on the basis of olive oil hydrolysis.
2. 1 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.
3. The reaction mixture was mixed well and incubated at 37°C for 30 min. Both test and blank were performed.
4. 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.
5. Liberated fatty acid was titrated against 0.1M NaOH using phenolphthalein as an indicator. End point is an appearance of pink color.
6. A unit lipase is defined as the amount of enzyme, which releases one micromole fatty acid per minute under specified assay conditions.

Appendix XII

Determination of protease

(Dam *et al.*, 2013)

Principle

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

Materials

- 25mM Tris Hcl buffer, pH 8.0
- 0.65% Casein solution
- 110mM Trichloroacetic acid reagent
- Folin ciocalteau reagent
- 500mM Sodium carbonate
- 1.1mM L-Tyrosine standard

Procedure

1. 5ml of 0.65% casein solution was added to the 1ml of the enzyme sample and incubated at 10 minutes at room temperature. The reaction was stopped by the addition of 5ml of 110mM TCA and incubated at 37°C for 30 minutes.
2. The reaction mixture was filtered using Whatman filter paper and filtrate was collected.
3. To 2ml of the filtrate, 5ml of 500mM Sodium carbonate and 1 ml of Folin ciocalteau reagent was added and incubate at 37°C for 30 minutes. Simultaneously, Tyrosine with different concentration was taken as standard.
4. After 30 minutes, the colour developed was measured at 660nm.

Appendix XIII

Ammonium sulphate precipitation

(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 is brought about either by adding solid substance or by adding solution of known saturation, generally a fully saturated (100%) solution. Once the concentration at which the target protein precipitates is known, fractional precipitation with ammonium sulphate can be used as an early step in the purification protocol.

Materials

- Ammonium sulphate – Add ammonium sulphate to the protein solution as a solid according to the concentration as follows: 0 – 20% - 5.35g/100 ml, 20 – 40% - 11.45g/100ml, 40 – 60% - 36.60g/100ml, 60 – 80% - 52.30g/100 ml, 80 – 100% - 70.70g/100ml.
- Resuspension buffer – Resuspend the concentrated protein with 25 mM Tris HCl after ammonium sulphate precipitation.
- Protein sample – Crude extract

Procedure

1. Use a graduated cylinder to measure the volume of the protein solution to be concentrated. Pour the solution into a beaker with a capacity approximately twice the measured volume of the protein solution. Place the beaker in a bed of ice.
2. Weigh the necessary amount of ammonium sulphate required to give the desired saturation.
3. Place the beaker containing the protein solution (and a large magnetic stir bar) on a large stir plate in the cold room.

4. Slowly add the solid ammonium sulphate to the stirred protein solution 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˚C.
7. Decant the supernatant solution, saving both the protein pellet (0-20%) and the supernatant.
8. Return the supernatant to the cold room and slowly add ammonium sulphate to the supernatant to achieve a concentration of 40%. Allow the mixture to stir for 60 minutes to ensure complete precipitation.
9. Transfer the solution to the appropriate centrifuge tube and centrifuge the solution at 10,000 g for 15 minutes at 4˚C.
10. Decant the supernatant solution, saving both the protein pellet (20-40% fraction) and the supernatant.
11. Repeat steps 8-10, adding ammonium sulphate to concentrations of 60%, 80%, and so on, up to 100% saturation, removing precipitated proteins by centrifugation at each step before increasing the ammonium sulphate concentration.
12. Resuspend the recovered protein pellets (0-20%, 20-40%, 40-60%, 60-80% and 80-100%) in resuspension buffer (25 mM Tris-HCl buffer, pH 8.0).

Appendix XIV
Dialysis
(Roe, 2001)

Principle

Dialysis is commonly used for removing salts from proteins. The presence of salts in proteins interferes in many ways. Special semi-permeable membranes called dialysis tubes have the property to allow compounds with

small molecular weights to pass through them, while those with high molecular weights like protein are held back.

Materials

- Dialysis bag
- Magnetic bar and Stirrer motor
- 2% Sodium bicarbonate
- 0.05% EDTA
- 20% Ethanol
- 0.1% Sodium azide
- 25 mM Tris HCl buffer (pH 8.0)

Procedure

1. Select dialysis tubing of suitable diameter and pore size, cut into suitable lengths to contain the volume required.
2. Submerge in a solution of 2% Sodium bicarbonate and 0.05% EDTA. Boil for 10 minutes.
3. Discard the solution and boil for 10 minutes in distilled water. Repeat once more.
4. Prior to use, rinse the dialysis tubing inside and outside with distilled water and buffer.
5. Seal one end of the tubing with a double knot or dialysis clip. Check for leakage using distilled water.
6. Pour in the solution to be dialyzed (ammonium sulphate precipitated sample) and seal the top end with a double knot or dialysis clip.
7. Place the bag in a large volume of 25 mM Tris HCl buffer and agitate gently with a magnetic bar and stirrer motor.
8. Leave to reach equilibrium, usually ≥ 3 hours, preferably at 4˚C.
9. Small molecules pass freely and get diluted by the large volume of fluid in the external medium. Repeat changes of the buffer every 8 hours.

Appendix XV

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.

Materials

- 2% Sodium Carbonate in 0.1 N in Sodium Hydroxide (Reagent A)
- 0.5 % 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 µg protein.

Procedure

Pipetted out 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard into a series of test tubes. Pipetted out 0.5 ml of the sample in other tubes. Made up the volume to 1 ml in all the test tubes. A tube with 1ml of water served as the blank. Added 5 ml of Reagent C to each tube including the blank. 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. Blue colour developed. Took the readings at 660 nm. Drew a standard graph and calculate the amount of protein in the samples.