EXPLORING THE GENOMES AND METAGENOME OF PHAGWARA SOILS FOR PROTEASES

DISSERTATION-2

Submitted in partial fulfillment of the requirement for the award of the degree of engineering

MASTER OF TECHNOLOGY
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By

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CERTIFICATE

This is to certify that **AMIT SITOKE** bearing registration no. **11300936** have completed DISSERTATION-II project report (BTY 731), titled **"EXPLORING THE GENOMES AND METAGENOME OF PHAGWARA SOILS FOR PROTEASES"** under my guidance and supervision. No part of the report has ever been submitted for any other degree at any university.

This report is fit for submission and the partial fulfillment of the conditions for the award of **M.Tech- Biotechnology**.

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DECLARATION

I, hereby declare that this project report entitled "**EXPLORING THE GENOMES AND METAGENOME OF PHAGWARA SOILS FOR PROTEASES**" is carried out by me under the supervision of "Er. Chirag Chopra" for the partial fulfillment of Degree of M. Tech. Biotechnology, Lovely Professional University, Phagwara (Punjab).

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Abstract

The study was done in order to obtained the protease enzyme from soil sample from dairy industry waste site at Phagwara Punjab by the metagenomics approaches and its genomic analysis also performed. A soil DNA i.e metagenome was extracted by the combination of several standard methods. PCR amplification was performed and through 16s rRNA sequence was obtained and also the identification of a protease producing organism executed. The localization of enzyme was achieved through standard assay method and it was found to be intracellular in our case. The primary and secondary screening confirms the presence of gene coding the protease in the soil sample. The culture enrichment was performed to ensure the enhancement of protease producing microorganism. The biochemical characterization reveals the effect of metal ions and inhibitor on enzyme activity, determines the optimum and stability temperature along with optimum pH.

The metagenomic method analyse the entire genome of sample taken-up from the environment and thus microbial population which were untouched and uncultured were explored because the present standard method of microbial cultivation are able to explore only 1% of microbial population in a particular sample and rest are gone wasted thus metagenomics made it possible to bring up the unknown population into the limelight and thus many biological molecules like commercially usefull enzymes, proteins, and amino acids were produced without zero percent of environmental hazards.

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ABBREVIATIONS

ATP: Adenosine Triphosphate

BRENDA: Braunschweig Enzyme Database

BSA: Bovine Serum Albumin

BLASTN: Basic Local Alignment Search Tool

CTAB: Cetyl Trimethyl Ammonium Bromide

DNA: Deoxyribo Nucleic Acid

dNTP: Deoxy Nucleotide Tri Phosphate

EC: Enzyme Classification

ELISA: Enzyme Linked Immunosorbent Assay

ETBR: Ethidium Bromide

EDTA: Ethylene Diamine Tetra Acetic Acid

G-HCL: Guanidine Hydrochloride

H_2O_2: Hydrogenperoxide

IUBMB: International Union of Biochemistry and Molecular Biology

LA: Luria Agar

LB: Luria Broth

LBA: Luria Broth Agar

NCBI: National Center for Biotechnology Information

O.D: Optical Density

PCR: Polymerase Chain Reaction

PMSF: Phenyl Methane Sulfonyl Fluoride

RNA: Ribo Nucleic Acid

Rpm: Revolution Per Minute

rRNA: Ribosomal Ribo Nucleic Acid

TAE: Tris-Acetate-EDTA buffer

TE: Tris-EDTA buffer

TCA: Tri Chloro Acetic acid

CHAPTER – 1

INTRODUCTION

The study of genomic material obtained directly from the environment instead from culture is termed as Metagenomics. Metagenomics is targeted on bacterial DNA, yeast and also of moulds DNA, though the technologies like 16sRNA genes pyrosequencing,enables the high-through put sequence data analysis in lesser time but the intrinsic rate of pyrosequencing decreases the detection of enormous microbes DNA and gives only the genetic material information of single species and thus a large amount of DNA of unculturable microbes get wasted which might be useful in various aspects (1). Metagenomic is the major invention in the field of science, it is a culture independent technique in which genetic material is isolated directly from environmental sample, to determine the taxonomic composition the direct sequencing of metagenomic DNA is favourable, the amplification of phylogenetic marker genes introduces the biasing and avoidance of this bias is the advantage of this method(2). About 210 metagenomic DNA have been isolated from different samples collected from different environments like oceans, soils, gut of human being (3).

The estimation of prokaryotic cells in one gram of forest soil is about 4×10^7 (4), whereas same amount of cultivated soilsand have 2×10^9 p rokaryotic cells(5) , DNA isolated from various soil samples, the number of prokaryotic genomes ranges from 2,000 to 18,000 genomes per gram of soil(6), thus this species diversity within the microbial communities proving the major hurdle for microbiologist.). On the basis of microbial communities that are present in the samples taken from the environment there are different approaches defining metagenomics.These approaches are better to previous strategies,which involves the culture growth and organism removal from its habitat. Metagenomics the focuse of metagenomics is on the usage of DNA sequencing for the prediction of the features of microorganisms and to explore the traits genetic basis.

The work going to present,the analysis of genomes of some culturable organisms and the soil metagenome of Phagwara industrial site soil samples for the production of Protease via both culturable approach and metagenomic approach. Proteases obtained so far by microbial enrichment techniques with microbes which grows only under the laboratory conditions and thus the small pool of the microbial gene is being utilised available in nature. The estimation has been made that only 1% of the microorganisms of the environment can be cultivated standard laboratory techniques (7), the library screening of metagenomic DNA is now the attractive alternate. The cultivation of microorganisms is not needed and the stored genetic information in the genomes of the microorganisms present in an environmental sample forms its basis(8).

A number of new enzymes have been produced using metagenomic DNA isolation in the past years (9). Proteases are ubiquitous enzymes (10) and are important for the various industrial product like in the waste management industries, leather, pharmaceutical, and food industry and are also used widely in the diagnosis of various diseases or illness and thus it constitutes two third of the total of enzymes used in industry (11). In addition, the screenings for novel enzymes including proteases used the cultivation-dependent approach. The number of important enzymes has been produced by cultivable microorganisms; however, the screening rate of the novel nzymes is decreased significantly when the methods of standard cultivation are applied for a high rediscovery (12).

CHAPTER - 2

Terminology

Metagenomics: The study of genomic material obtained directly from the environment instead from culture is termed as Metagenomics.

Enrichment: The soil was enriched for maximum growth of protease producing organism in tryptone-casein and nutrient-casein medium.

Unit definition: One unit of protease defines that release one micromole of tyrosine per minute under standard assay condition.

Standard curve: The tyrosine standard curve relates the activity of enzyme under different physiological conditions and on the basis of standard curve biochemical characterization analysis executed. Effect of temperature, metal ions, inhibitor and pH on enzyme activity was determined

CHAPTER-3

SCOPE OF THE STUDY

The motive of the study was to explore the metagenomes and genomes of the Phagwara industrial soil for protease activity. The accessing of the soil organism involves the various methods. The focus of the experiment performed is the exploration and exploitation of the soil microbial diversity and the cultivation approaches for the same may be direct or indirect. The standard methods of cultivation is applicable only to culture the 1.0% of the soil bacteria , thus vast diversity of microbes of the soil remains unexplored.

The microbial genes cultured through standard cultivation methods, it would not reached the large family of microbes residing in the soil. But now the methods have developed based on the indirect molecular approaches for the analysis and for the isolation of metagenomic DNA from environment i.e soil, water, waste garbage, industrial waste etc without microorganism cultivation . The DNA isolated is the indegenious soil microbial DNA and it is the collective DNA of soil sample termed as soil metagenome. The study involves the soil DNA isolation from the direct cell lysis method and further analysis for protease activity.

CHAPTER-4

Aims and Objectives

1. Collection of soil sample from the Phagwara industrial soil
2. Isolation of soil DNA
3. Functional screening to obtain the proteolytic activity clone from the soil sample
4. Biochemical characterization and analysis of the protease.

CHAPTER-5

Review of literature

5.1 Enzymes

Enzymes are biological molecules and have the ability to catalyse the biological reactions without altering the reaction equilibrium and hence they are called biological catalyst. They accelerate the rate of biological reaction without changing its state and cause no change in reaction equilibrium. The database called BRENDA (Braunschweig Enzyme Database) contains the literature and data on all classes of enzymes that are classified according to the IUBMB (International Union of Biochemistry and Molecular Biology) (13).

Enzymes are readily involved in the biological reactions in-vivo where the physiological parameters like temperature and pH are not sufficient for life process like nerve impulses generation and muscular activity and many more (14). The enzymes are catalytic agents and remain unchanged during reaction on substrate. They are biological molecule proteinacious in nature, some exceptions are there like ribozymes.

They can be monomeric *i.e.* made up of single polypeptide chain or oligomeric *i.e.* consist of two or more than two polypeptide chains. Enzymes are substrate specific and in some cases their activity increases when conjugated with co-factors and co-enzymes. The important characteristics of enzymes includes they can be operate under mild conditions, sterospecificity, specific to the substrate, reusable, natural, eco-friendly and most important they remain unchanged through out the reaction.

Recently, consciousness among the global community regarding the environment has motivated the usage of enzymes because of their unmatched selectivity and ability to operate under all physiological conditions and hence time-honored chemical processes have been replaced by enzymes. The major development in the fields like molecular biology, genomics, proteomics, recombinant DNA technology, mutagenesis have made great revolution in the field of enzymology. The usage of enzymes not only reduces the production of wastes and contaminants but also helps in the development of technologies that are sustainable and require less amount of energy. Enzymes became more effective catalyst as the technology developed and hence also enhances the enzyme production in the selective host system (15).

5.2 Classification

Enzymes are produced by all living things and are also ubiquitious in nature and they are intracellularly as well as extracellularly localized. There are many more enzymes which are membrane bound and some are localized inside the cell depending upon their catalytic activity. Intracellular enzymes are effectively involve in the catabolic, synthesis and metabolic activities of cell whereas extracellular are majorly participate in the nutrient catabolism and accelerate their uptake into the cell. The enzymes are named by adding suffix–ase to the substrate name they hydrolyse for example β-galactosidase hydrolyses the terminal β-D-galactose residues in β-galactosides such as lactose, L-asparaginase hydrolyses L-asparagine, amylase hydrolyses amylose (16). Some uniformative names were also given to enzymes like catalase and some enzymes nomenclature do not match with this system like renin, trypsin. The rational system of classification divided the enzymes into six classes based on the reaction type they catalyse and the six major classes are:

1. Oxidoreductase: these are the enzymes which catalyse the oxidation-reduction reactions, examples of this class of enzymes are alcohol dehydrogenase, cytochrome oxidase etc.
2. Transferases: this class includes the enzymes that catalyse the transfer of a group from one compound to another, examples are hexokinase, transaminases, transmethylases, phosphoyrlase.
3. Hydrolases: the enzymes which catalyse hydrolysis reactions comes under this category and examples are protease, lipase, choline esterase, alkaline phosphatase, urease.
4. Lyases: the binding of a group across the double bond are catalyzed by the enzymes that are fall under this class and examples are aldolase, fumarase and histidase.
5. Isomerase: the intra-molecular rearrangements in the reaction and interconversion of isomers are catalyzed by the enzymes of this class and some of them are triose phosphate isomerase, retinine isomerase, glucose phosphate isomerase.
6. Ligases: catalyse the formation of bond between the two compounds by utilizing the energy produced from a pyrophosphate bond cleavage such as is found in ATP and some of the names of enzymes of this class are glutamine synthase, acetyl coA carboxylase, succinate thiokinase.

5.3 Application

5.3.1 Global market of enzymes

Enzymes usage are in fashion in the industries from longer period mainly in paper and pulp industries, detergents and pharmaceutical and biotechnology industries. The market for industrial enzymes in 2010 reached about $3.3 billion and by 2015 the overall capital is of 4.4 billion [17]. The usage of enzymes in the production of leather and bioethanol contributed the highest sales figures, maximum sales are expected in the biofuels (bioethanol) market [18]. Enzymes utilization in beverages and food industry is $ 1.3 billion by 2015.

Proteases have more than 60% of the global market for enzymes because of extensively usage of different types of enzymes for the industrial products like in agrochemical, leather, pharma, food pharmaceuticals, foods, silk and detergent industries. Enzymes in the laundry have 25% of the total sales of different types of enzymes through out the world. A bacterial protease called biotex was used for the preparation of first detergent manufactured by Novo Industry (now Novozymes) in 1956, it has an alcalase which was produced by *Bacillus licheniformis*, in 1994 Lipolase^{TM}, this was the first commercially utilized enzyme in detergent industry manufactured through recombinant technology introduced by Novo Nordisk in 1994 after cloning the *Humicola lanuginose* lipase into the *A.oryzae* genome. The recently added enzyme in detergents used for the removal of guar gum of food stains is *Bacillus* mannanase [19].

Enzymes like cellulases and laccases are extensively used for the denim finishing and decolorization of textile effluents and textile bleaching and the usage of pectate lyase is the alternative enzymatic process in the manufacturing of cotton and thus greatly reduces the company's cost of production [20]. The process is performed at much lower temperatures and uses less water than the classical method. The usage of enzymes minimizes the raw material cost and also reduces the waste material production. *Candida rugosa,* lipase is used by Nippon Paper Industries to remove up to 90% of the waste compounds like hydrophobic components of wood, triglycerides and waxes and also increase the yield. Alkaline lipases from *Bacillus* strains are used in combination with alkaline or neutral proteases as alternatives of chemicals for the production of leather of high quality. This enzyme grows under highly alkaline conditions and hence they are extensively used for the industrial production, the enzymes mediated process produced high quality product with minimum hazards to environment. Phenolic and non-phenolic lignin-related compounds are oxidized by laccases and the environmental pollutants are also degraded by this enzyme [21]. The detoxification of industrial effluents from the paper and pulp, textile, and petrochemical industries has been carried out with the help of these enzyme, and

they are also used as a tool in diagnostic industry. The bioremediation of herbicides, pesticides, and cleaning of water purifier has been performed and are also used as explosives in soil, as a catalyst in drug manufacture and as cosmetic ingredients.

5.3.2 Enzymes in the Industries

Enzymes increases the nutrients digestibility and increases the efficiency in feed utilization and thus degrades the feed components which are not needed and hence increases the efficiency in feed utilization. Feed enzymes like α-galactosidases, xylanases, α-amylases commercially available are phytases, proteases, glucanases, and polygalacturonases, mainly used for swine and poultry [22].

5.3.3 Enzymes in Food Processing

The recombinant fungal lipases in the food industry are *Fusarium oxysporum, Thermomyces lanuginosus* and *Rhizomucor miehi*, produced in *A.oryzae* [23]. The lipases are used in the food industry to maximize the fragrance and flavor. The enzymes utilization in the food industries ranges from the fruit juices product to baked and vegetable fermentation product and dairy product. The fats, oils and other related compounds are mainly treated by this enzyme. The lipases are used as emulsifiers in cosmetics, food and pharmaceutical industry as they mediated the esterification of carbohydrate esters of fatty acids and offers a potential market for the same. Proteases are used for the cheese production in dairy industry, proteases produced by microorganisms like *Endothia parasitica, Mucor pusillus Lindt, Mucor miehei, Bacillus subtilis*, are replacing the calf rennin which has been preferably used in the making of cheese because of its high specificity. The hydrolysis of peptide bond between Phe105-Met106 that produce para-k-casen and macropeptides are the primary functions of these microbial proteases [24].

5.3.4 Enzymes in Chemical and Pharmaceutical Processes

Chemicals industries mainly depend on cost competitiveness with established chemical methods and also the success of the enzymetic processes. The main advantages that biotechnological processes have over the established chemical processes are the lower demand of energy, reduced volumes of byproducts and waste and increased the volume of titeration product. It is estimated that about 150 biocatalytic processes are currently extensively applicable in the industry [25] [26].

5.4 Hydrolases

The enzymes are natural catalyst and are widely used in many areas including leather, food, chemical, detergent, cosmetics industries and have wide range of application in scientific research. The hydrolases is one of the class of enzyme classification with EC number 3. These enzymes are widely used as they are involved in the catalysis of reactions which are associated with cleavage of bond between water molecules. They either added or remove the water molecules and catalyze the cleavage of C-N, C-C, C-O bonds and apart from hydrolysis they also catalyse other reactions like alcoholysis, condensation, lysosomal degradation, transport of secretory proteins across membranes and in association with several co-enzymes and co-factors they are also involve in homeostatic processes. Hydrolases are class of enzyme having number of enzymes which are pretty much important for the commercial usage globally.

5.5 Proteases

Proteases are one of the enzymes of hydrolases group and as per enzyme nomenclature their enzyme classification number is EC 3.4 which indicates subclass of peptidases i.e peptide bond hydrolases. These enzymes are highly specific to their substrate and site of cleavage and cuts the long chain of amino acids into shorter fragments and amino acids component with spectacular site and substrate specificity and hence they are called nature's swiss army knives [27]. The proteases are the enzymes residing in the living forms and they hydrolysis the peptide bonds and are involved in various metabolic processes [28]. The various industries like food, pharmaceutical, leather and textile, detergent and diagnostics industries are being extensively using the proteases enzymes world-wide and about 40% out of the total sale is estimated of protease only. In plants and animals also proteases are present, that accounts the two-third share in the commercially available proteases are microbial proteases [29].

Proteases facilitate the peptide hydrolysis and significantly regulate the cell survival and metabolism at various levels and hence also influence the synthesis, turnover, composition, shape, size and destruction of proteins. Though they are from taxonomic kingdom, almost all biological system produces proteases, it is well known about peptide bond hydrolytic property of protease they are also played decisive regulatory role in maturation, conception, aging, growth, birth and death in all organisms [27]. These enzymes are effectively participates in blood clotting, matrix remodeling, cell growth and migration and in immune system functioning like inflammation and cell death and are also involve in hormone release, zymogen activation and cancer treatment [30]. In plants proteolysis is significantly involve in many process like circadian rhythm, photosynthesis, photomorphogenesis, programmed cell death [31]. Several studies reveals that proteases are also affects the important functions in micro-organism like bacterial spores formation [32], yeast ascospores, slime molds fruiting bodies [33]. A

study showed that there are 50 human genes that code for proteases and thus they are also used as biomarkers in diagnostics [34].

5.5.1 Classification of Proteases

Proteases are the enzymes which are useful for living organism and its physiological activity made it a necessary component for all living life from humans to viruses and these are the enzymes which hydrolyze the peptide bond and thus they are termed as peptidases, proteases and proteinases [35] and on the basis of their origin the proteolytic enzymes are classified as: microbial, plant, animal, sand human bacterial, fungal and viral enzymes. Proteases are the members of the hydrolase family of enzymes and are classified under the sub-family known as peptide hydrolases or peptidases. Based on the site of action proteases are divided into endopeptidases or the exopeptidases. The exopeptidases, aminopeptidases, and the endo peptidases shows different pattern of peptide bonds cleavage and the gives the respective dipeptides from the polypeptide chain cleavage. The peptide bonds hydrolysis is catalyzed by the exopeptidases near the N- or C-terminal ends of the substrate. Aminopeptidases catalyzed reactions gives dipeptides which are single amino acids and can gives the tripeptides form the substrate N-terminal, peptidyl dipeptidases gives dipeptides from the C-terminal end of a polypeptide chain, while the endopeptidases breaks the peptide bonds within and distant from the ends of a polypeptide chain [36].

The protease enzyme is also classified on the basis of their mechanism of catalytic activity and also on the basis of their active site amino acid residues. The glutamic proteases, metalloproteases, aspartic proteases, metalloproteases, threonine proteases, serine proteases, asparagine proteases, and proteases whose catalytic mechanism is not known are some different kind of proteases based on the above criteria. The proteases are also classified on the basis of similarities of sequences and this classification of protease gives the structured based classification and the homologus families are arranged and grouped into clans [37].

5.5.2 Review on proteases isolated by metagenomic technique

The proteases are obtained by using metagenomic approaches from various sources like goat skin surface, deserts and from various types of soil from different region and glaciers. The researches has been done revealing that a wide range of microbial genes and their product are not discovered and characterized which might be having plenty of biomolecules that are commercially important. The various types of enzymes were obtained through metagenomics including almost all the six class of enzymes and proteases of different nature are also obtained like serine proteases, metalloproteases, alkaline proteases, carboxyproteases, aspartic proteases, cysteine proteases and many more. [38]

This technique is a powerful tool of isolation and identification of enzymes from the microbes of different sources because through standard cultivation techniques only 1 percent of microbes were cultivated and rest were destroyed and wasted. It is reported that vast majority of novel biocatalyst were obtained through metagenomics technique which involve both sequence based and functional based approaches of screening to isolate and identify the genes encoding novel enzymes. The biocatalyst obtained from this technique includes the soil DNA isolation from the soil sample and then metagenomics library preparation using suitable vector and further sequence based and functional based method of screening were employed for positive clones [39]. The biochemical characterization and quantitative and qualitative assays were performed to obtain the temperature stability, pH optimum, effect of metal ions and inhibitors on enzyme activity.

The enzymes obtained from natural environment through metagenomics based strategies successfully exploit the whole genome of the sample taken which is not possible through standard cultivation techniques. The biodiversity of the earth have large variety of microbes which are unexplored yet but metagenomics made it possible.

5.5.3 Proteases assays and purification

The estimation of proteolytic activity can be obtained via both approaches quantitatively and qualitatively respectively. The proteolysis products or the residue of protein substrate gives the estimation of protease activity and various methods of assay and detection for proteolytic activity are available having varying range of detection and specificity.

5.5.3.1 Qualitative analysis

These methods are employed for the protease activity screening studies and are rapid in nature. The protein substrates were used as an indicator to detect proteolytic activity on which clear zone formation takes place due to hydrolysis. The methods which were commonly used for this type of analysis are protein agar plate assay, radial immunodiffusion and thin layer enzyme assay [40]. The qualitative analysis has been performed by number of methods available in the literature to analyse the protease activity with suitable substrate. The studies showed the specific substrate for enzyme protease activity analysis such as skim milk, gelatin, keratin [41] and BSA (Bovine Serum Albumin) [42]. The quantitative analysis of proteolytic activity also estimated for radial or zone diffusion assay by punching the holes on specific protein substrate agar plate which produces the clear zone after hydrolysis by enzyme.

Coomassie prestained agarose gel are also used for the protease activity analysis. In 1983 the study developed the method of thin layer enzyme assay to detect the microbes in a mixed sample which are producing protease [43]. All these methods of quantitative analysis of proteolytic activity are pretty much specific and accurate and the observations

revealed from these tool are of much importance to predict the enzyme encoding genes in a mixed population. These are the methods which are widely used in researches of biocatalyst as they are having huge potential for fulfilling the commercial consumption.

5.5.3.2 Quantitative analysis

The quantitative analysis performed to study the extent of proteolytic activity. The quantification of protease activity was obtained by several methods such as spectrophotometry, fluorimetry enzyme linked immunosorbent assay-based assays (ELISA) and radiometry. The protease activity was assayed by using synthetic as well as natural substrates. The quantitative analysis of proteolytic activity were most commonly executed by the peptides estimation of acid-soluble hydrolysed product obtained due to proteolysis of the substrates. The quantification reveals that at what temperature an enzyme is giving the highest activity and upto what extent and optimum pH and temperature at which enzyme is stable for longer period is also assessed. Calorimetric method with folin's reagent and absorption at particular wavelength also used for peptides residues estimation to detect proteolytic activity [44]. In plants, animals and microbial sample the proteolytic activity is analysed spectrophotometrically using substrates of chromogenic or insoluble chromolytic in nature. The natural substrates used for quantitative analysis of proteolytic activity includes collagen, keratin, fibrin, gelatin, elastin etc they are also insoluble in nature and soluble substrates are also employed by making them insoluble by certain modification such as cross linking or entrapment and also synthesized substrates using 3,5-dinitro-salicylic acid making them thermally modified and chromogenic in nature [45].

A chromogenic tripeptide, benzyloxy carbamoylglycyl-1-pyrolyl-1-citrulline-p-nitroanilide, is used to analyse highly alkaline protease, HAP-PB92, from *bacillus* alkaliphilic in nature [46]. The assaying by using chromolytic substrates have certain limitations poor separation of hydrolysed and non hydrolysed reaction component thus magnetic beads with radiolabelled peptides are used. Studies show that substrates with fluorescent peptides also used to determine the protease specificity for amino acids at the site of cleavage (C and N terminal), this assay involves the fluorescent oligopeptide energy transfer [47]. The protease quantification also reported by using assays based on enzyme linked immunosorbent assay [48].

5.6 Proteases purification

The purification of biocatalyst have lots of hurdles and till now it is known that with each step of purification, the enzyme activity decreases. The commercial usage, and characterization and all applications of proteases depends upon the ease with which it is purified. A lots of research has been done to developed such method of purification by which activity loss can minimised. Proteases like alkaline protease from different sources has been purified to homogeneity. Apart from this fact that no general method of purification till date is discovered, several alkaline protease purification involves the culture separation by centrifugation from fermentation broth. The method of ultrafiltration, salting out by ammonium sulphate, solvent extraction by using ethanol and solvent extraction by using acetone are used to concentrate the fermentation broth. Since most of the alkaline protease are extracellular in nature so methods to concentrate these enzymes includes lyophilization, heat treatment of enzyme, use of activated charcoal, poly ethylene glycol or temperature sensitive hydrogel [49]

Chromatographic techniques are used in combination to purify the enzymes like ion exchange chromatography, affinity chromatography, hydrophobic interaction chromatography and gel filteration chromatography. In several researches the alkaline proteases are purified by using the affinity chromatography and the matrices used in this purification includes sephadex-4-phenylbutylamine, immobilized casein glutamic acid, hydroxyapatite, casein agarose, aprotinine agarose and immobilized N-benzoyloxycarbonyl phenylalanine agarose. This method is not widely used even though having high throughput success because enzyme ligands are costly and are of labile nature [50]

CHAPTER-6

EQUIPMENT, MATERIALS AND EXPERIMENTAL SETUP

6.1. EQUIPMENTS

Equipments and instruments:

S.No.	Materials	Company
1.	Autoclave	NSW Pvt. Ltd.,India
2.	Face Mask	Smart Care
3.	Glass wares	Borosil Glass
4.	Hot air oven	NSW Pvt. Ltd., India
5.	Incubator	Yorco Incubator Bacteriological
6.	Laminar air flow	Rescholar Equipment
7.	Microwave	INALSA
8.	Microscope	Magnus
9.	Micropipette	P'Fact A
10.	Microtips	TARSONS
11.	Orbital shaker	REMI
12.	pH meter	Hanna
13.	Plastic wares	Poly lab
14.	Refrigerator	LG
15.	Weighing balance	Adventurer, DHAVS
16.	Centrifuge	REMI

6.2 MATERIALS

Medias and Chemicals:

S.No.	Chemicals/Medias	Company
1.	Skimmed milk agar	Himedia LaboratoriesPvtLtd.
2.	Casein	Himedia LaboratoriesPvtLtd.
3.	Ethanol	ChangshuYangyuan Chemical
4.	Tryptone	Himedia LaboratoriesPvtLtd.
5.	Nutrient agar	Himedia Laboratories Pvt Ltd.
6.	Agarose	Titan Biotech Ltd.
7.	Luria broth	Central Drrug HousePvtLtd.
8.	Lysozyme, Proteinase and Rnase	Himedia laboratoriesPvtLtd
9.	Chloroform	Central Drug HousePvtLtd.
10.	Isoamyl alcohol	Titan Biotech Ltd.
11.	Calcium chloride	Himedia LaboratoriesPvtLtd.
12.	Sodium Dodecyl Sulphate	Himedia Laboratories Pvt Ltd.
13.	Polyethylene glycerol	Himedia Laboratories Pvt Ltd.

6.3 EXPERIMENTAL SETUP:

In order to obtained the maximum amount of protease enzyme from the soil sample dna the removal of the humic substances is the major problem so the analytical methods had been adopted to remove humic substances without much loss of sample DNA. PCR amplification to obtain multiple copies has been performed. The detection of metagenome has been carried out by agarose gel electrophoresis, after preparing the gel the loading of metagenomic DNA with dye into the wells with proper precaution and tackling has been performed. The whole apparatus must be in the highly sterile condition, wearing of gloves and mask while loading the DNA is necessary because of the carcinogenic nature of ethidium bromide.

The agarose gel was dissolved in the TAE buffer and heated in the microwave. After kepting it for few minutes at room temperature added the ETBR and then poured it into the gel tank placed the comb and remove the comb when gel solidified. The loading of metagenome, by well mixed it with loading dye, into the gel wells. The apparatus must be connected with correct electrodes negative and positive terminals and loading of DNA must be done at negative terminal. The sample of soil collection and enrichment of soil has been done to obtain the desired colonies for metagenome, and genomic DNA.

The preparation of plates with tryptone and casein as substrates for the desired colonies which gives the clear zone on streaking it with culture having protease activity.

CHAPTER-7

Research Methodology

7.1 Sample collection: soil samples were collected aseptically into sterile polythene zip lock bags using sterilized tools. The soil was collected from Phagwara region of Punjab state in India. The soil was dried and stored at room temperature for subsequent use.

7.2 Isolation of micro-organisms: 0.5g of soil sample was dissolved in sterile water for serial dilution upto 10^{-3} and 200µl of these dilutions was spread on LBA plates and incubated at 37^0C overnight along with 200µl of undiluted suspension. On the basis of morphology different colonies were picked up and streaked on LA plate and incubated for 12 hours at 30^0C.

7.3 Storage and maintenance of microbial culture: The plates were stored at 4^0C for maximum 1 week and were maintained in active state by progressive sub-culturing onto LBA plates.

7.4 Isolation of protease producers: Enrichment technique was employed for the isolation of microbes producing protease. In 100ml of enrichment media 1g of soil was inoculated and incubated overnight at 37^0C. The overnight culture was then spread plate on LB Agar plate and grown overnight at 37^0C. On the basis of colony morphology single colonies were identified and 5 strains were isolated.

7.5 Primary screening for protease producing microbes: The cells from the previous plates were streaked onto skimmed-milk agar plates and were grown at 37^oC overnight. The positive protease producers were identified by formation of zone of clearance. The best protease positive strain was freshly sub-cultured and taken for further studies. These strains were named as sstp1, sstp2, sstp3, sstp4 and sstp5

7.6 Preparation and storage of crude extract: The overnight grown culture of strain sstp2 on LB medium at 30^0C and 180 rpm was centrifuge at 8000Xg for 7-8 min, the supernatant (culture media) was saved for further analysis and pellet was lysed by dissolving it in the 200µl of lysis buffer which was already diluted with distilled water in 1:1 ratio. At 18000 rpm for 10 minutes the cell lysate was centrifuged and supernatant (cell free extract) thus obtained was stored in fresh eppendroffs at 20^0C for further analysis.

7.7 Secondary screening and localization: the supernatant and cell free extract were used for ascertaining the localization of our protease whether intracellular or extracellular. This was done using the standard agar-diffusion assay. On skimmed milk agar plate four holes were bored. Culture medium, cell-free extract, lysis buffer (control)

and sterile LB broth (control) were added to the holes and the plates were incubated at 37^0C for 6 hours. The localization was determined on the basis of clear zone formation in the respective fractions.

7.8 Biochemical characterization: Biochemical analysis was performed to determine the optimum temperature, pH and stability at different temperatures and pH values. The analysis of effect of metal ions on enzyme activity and inhibitors influence on enzyme activity was also determined.

7.8.1 Effect of Temperature on SSTP2 activity: Effect of temperature on SSTP2 activity was studied using the tyrosine standard curve using 1% Casein as substrate. Table 7.1 shows the substrate was incubated with the enzyme at different temperatures for 30 minutes. The reaction was stopped by adding 10% TCA and incubating for 10 minutes. Next, the reactions were centrifuged, 1mL of supernatant was taken, 1mL of 1N NaOH was added followed by 1mL of Folin's Reagent. The O.D. was taken at 660nm.

S.No.	Temperature ^{0}C	Substrate µl	Buffer µl	Enzyme µl	TCA µl	1N NaOH ml	Folin's reagent ml
1	10	500	450	50	500	1	1
2	20	500	450	50	500	1	1
3	30	500	450	50	500	1	1
4	40	500	450	50	500	1	1
5	50	500	450	50	500	1	1
6	60	500	450	50	500	1	1
7	70	500	450	50	500	1	1
8	80	500	450	50	500	1	1
9	90	500	450	50	500	1	1

Table-7.1: Enzyme incubation at different temperature for 30 minutes.

7.8.2 Analysis for enzyme stable temperature: For determining the most stable temperature for SSTP2, the enzyme was pre-incubated at different temperatures (10 ^{0}C to 90 ^{0}C) for one hour. Next, substrate was added and the reaction was incubated at 50 ^{0}C for 30 minutes. The reaction was stopped by adding 10% TCA and incubating for 10 minutes table 7.2 shows the whole preparation. Next, the reactions were centrifuged, 1mL of supernatant was taken, 1mL of 1N NaOH was added followed by 1mL of Folin's Reagent. The O.D. was taken at 660nm.

S.No.	Substrate µl(1% casein)	Buffer µl	Enzyme µl	TCA µl	1N NaOH ml	Folin's reagent ml	Temperature ^{0}C
1	500	450	50	500	1	1	10
2	500	450	50	500	1	1	20
3	500	450	50	500	1	1	30
4	500	450	50	500	1	1	40
5	500	450	50	500	1	1	50
6	500	450	50	500	1	1	60
7	500	450	50	500	1	1	70
8	500	450	50	500	1	1	80
9	500	450	50	500	1	1	90

Table-7.2: Enzyme incubation at 50^0C for 30 minutes.

7.8.3 Optimum pH analysis: the optimum pH analysis was performed in the following way that firstly lysis buffer of different pH ranges from 2-12 was prepared and then sample and control was prepared for analysis. The eleven sample tubes contain substrate, enzyme and buffer of different pH and equal number of control tubes only have substrate and buffer of different pH. The all twenty two tubes were incubated at 50^0C for 30 minutes then after appropriate amount of 10% TCA was added in each tube to stop the reaction. The incubation of 10 minutes at room temperature after adding TCA was performed and then centrifuged at 12000 rpm for 10 minutes, after centrifugation 1ml of supernatant from each tube was collected in fresh glass test tube and NaOH and folin's reagent were added in equal amount for further O.D analysis. The whole preparation showed in table 7.3.

S.No.	pH	Buffer (µl)	Substrate (µl)	Enzyme (µl)	TCA (µl)	1N NaOH (ml)	Folin's reagent (ml)
1	2	450	500	50	500	1	1
2	3	450	500	50	500	1	1
3	4	450	500	50	500	1	1
4	5	450	500	50	500	1	1
5	6	450	500	50	500	1	1
6	7	450	500	50	500	1	1
7	8	450	500	50	500	1	1
8	9	450	500	50	500	1	1
9	10	450	500	50	500	1	1
10	11	450	500	50	500	1	1
11	12	450	500	50	500	1	1

Table-7.3: enzyme incubation at different pH for 30 minutes.

7.8.4 Effect of inhibitors on enzyme activity: the enzyme activity was affected by several inhibitors so the alteration in enzyme activity upon interacting with different inhibitor was analysed in this section. This time only one control was prepared in which buffer, substrate and enzyme was added in appropriate amount and sample also contain buffer, substrate, enzyme along with appropriate amount of different metal ions which were taken in with different concentrations. The further procedure was similar as discussed above incubation at 50^0C for 30 minutes then TCA was added to stop the reaction then incubation at room temperature for 10 minutes and centrifuged and 1ml of supernatant was collected and NaOH and folin's reagent were added in equal amount for further O.D analysis. Table below shows the whole set up of this analysis with different concentrations of metal ions:

S.No.	Inhibitor	Concentration	Enzyme µl	Substrate µl	Buffer µl	TCA µL	1N NaOH ml	Folin's reagent Ml
1	Gn-HCL	8M	50	500	450	500	1	1
2	PMSF	1mM, 5mM	50	500	450	500	1	1
3	Tween-80	20%	50	500	450	500	1	1
4	SDS	20%	50	500	450	500	1	1
5	Triton-X-100	20%	50	500	450	500	1	1
6	UREA	8M	50	500	450	500	1	1
7	H_2O_2	30%	50	500	450	500	1	1
8	C-TAB	20%	50	500	450	500	1	1
9	Tween-20	20%	50	500	450	500	1	1

Table-7.4: Enzyme incubation with different concentration of inhibitor.

7.8.5 Effect of metal ions on enzyme activity: Effect of metal ions on SSTP2 activity was studied by addition of pre-specified concentration of respective metal ions to the standard protease assay reaction. The details of the same have been mentioned in the table below.

S.No.	Metal ion	Concentration	Enzyme µl	Buffer µl	Substrate µl	TCA µl	1N NaOH ml	Folin's reagent ml
1	$FeCl_2$	2mM	50	450	500	500	1	1
		5mM	50	450	500	500	1	1
2	$CuSo_4$	2mM	50	450	500	500	1	1
		5mM	50	450	500	500	1	1
3	$CaCl_2$	2mM	50	450	500	500	1	1
		5mM	50	450	500	500	1	1
4	$FeCl_3$	2mM	50	450	500	500	1	1
		5mM	50	450	500	500	1	1
5	$MnCl_2$	2mM	50	450	500	500	1	1
		5mM	50	450	500	500	1	1
6	$MgCl_2$	2mM	50	450	500	500	1	1
		5mM	50	450	500	500	1	1
7	$CoCl_2$	2mM	50	450	500	500	1	1
		5mM	50	450	500	500	1	1
8	$ZnCl_2$	2mM	50	450	500	500	1	1
		5mM	50	450	500	500	1	1

Table-7.5: Enzyme incubation with different concentration of metal ion.

7.9 Optimization and Isolation of Metagenomic DNA from soil: The metagenomic DNA isolation has been performed by the modification of several methods. Here in this study we applied the method of metagenomic DNA isolation with humic acid removal [51] [52] [53]. Soil was mixed with DNA extraction buffer [100 Mm Tris-HCl [pH 8.0], 100 mM sodium EDTA [pH8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1% CTAB] and lysozyme, proteinase K (10 mg/ml) and RNase were added and incubated at 37°C. Then, 20% SDS was added and the samples were incubated at 65°C for 2hour with continuous inverting for every 5mins. The supernatants were collected after centrifugation at 6,000g for 10 min and transferred into fresh centrifuge tubes. The crude DNA was extracted using chloroform isoamyl alcohol and precipitated with PEG and NaCl. In the end, crude DNA was washed with 70% ethanol, dried and dissolved in TE buffer. The humic impurity was removed from the Calcium chloride treatment at lower concentration. Then the DNA was reprecipitated using chilled ethanol and sodium acetate treatment. In the end, pure DNA was washed with 70% ethanol, dried and dissolved in TE buffer.

This method was optimized in comparision with the original method [51]. The results showed that extraction of DNA without humic impurities by the proposed was better than the proposed original method.

7.9.1 Optimization and Isolation of Genomic DNA: The genomic DNA isolation was performed for 16s rRNA analysis to identify the species of protease producing microorganism. The DNA isolation was performed as followed procedure, 1.5 ml of overnight culture was centrifuged in an autoclaved eppendroffs at 8000 rpm for 10 minutes. Supernatant was discarded and pellet was resuspended in 400µl of lysis buffer and 10µl of proteinase k was added and incubated at 37^0C for 30 minutes. 30µl of 10% SDS added and incubated at 65^0C for 30 minute with every 5 minutes invertion. After this 30 minute incubation, centrifuged at 10000 rpm for 10 minute supernatant was then collected and equal volume of chloroform isoamyl alcohol was added and centrifuged at 12000 rpm for 10 minute. Aqueous layer was collected and double volume of absolute icecold ethanol and 1/10th volume of 3M sodium acetate was added and centrifuge it. Supernatant was discarded and 70% ethanol was added to pellet and centrifuge it at maximum speed for 10 minute then discarding the supernatant drying of pellet was performed and resuspend it in TE buffer.

7.9.2 Identification of isolated Microbial strain by 16s rDNA Sequencing:

The first step in the identification of bacteria by 16s rDNA is the amplification of the gene. The forward primer, 5’ CAGCAGCCGCGGTAATAC 3’ and the reverse primer 5' ACGAGCTGACGACAGCCATG 3’ were sued to amplify a fragment of a gene. The PCR conditions used were initial denaturation at 94°C, for 3 min followed by 30 Cycles of 94°C for 15 s; 60°C for 15 s; 72°C for 30 s and a final extension of 72°C for 5 min.

The 20µl reaction mixture was used contained 12µl biological water, 2µl 10X buffer, 1µl forward primer, 1µl reverse primer, 1µl dNTP mix, 2µl of template DNA and 1µl of Taq DNA polymerase. The PCR amplified product was then visualize on agarose gel electrophoresis and processed for sequencing. Either the forward or reverse primer can be used in sequencing PCR reaction. The sequence thus obtained was edited and aligned with the sequences in the Public domain (GenBank) by BLASTN programme [54]. DNA sample of culture SSTP2 were amplified, sequenced and locally aligned on the basis of the sequence similarity with the 16S ribosomal genes of known organism and the organism was given a genus and a species.

CHAPTER-8

Results and discussion

8.1 Metagenomic DNA analysis result

The metagenomic DNA has been isolated from the soil sample collected from the industrial site of Phagwara.

Fig-8.1 Metagenomic DNA with humic acid

After $CaCl_2$ treatment the humic acid contamination was precipitated in the pellet.

Fig-8.2 The DNA is suspended in the TE buffer

The metagenomic DNA is finally extracted and suspended in the TE buffer for further analysis in the experiment performed. The DNA isolation is carried out by using the method in

combination and modification with several other method to avoid the humic acid presence(30)(31) as in metagenomics analysis the major hurdle in DNA isolation is the humic acid existence and this may reduce the quantity and quality of the desired gene isolation.

Fig-8.3 0.8% Agarose Gel showing Metagenomic DNA

The gel electrophoresis confirms the metagenomic isolation, the bands are showing the DNA from the sample collected.

Spectrophotometer analysis of extracted DNA from the soil at different wavelength with respect to 1X TE buffer as blank.

Wavelength	O.D.
230.0	0.046
260.0	0.055
280.0	0.056

Concentration of DNA= A_{260}*50*100

=0.055*50*100

=**275µg/ml**

Ratio of DNA concentration with respect to protein

A_{260}/A_{280} =0.055/0.056

=**0.098**

Ratio of DNA concentration with respect to RNA

A_{260}/A_{230} =0.055/0.046

=**1.23**

8.2 Primary screening

It confirms that soil sample has the protease encoding gene because the streak shows the clear zone in the skimmed milk plate.

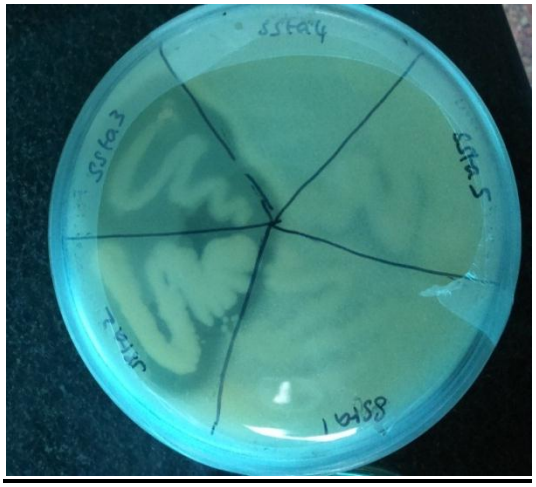

Fig-8.4 Skimmed Milk agar plate showing the isolated cultures of protease producer

The clear zone in the strains SSTP2 and SSTP3 in the above plates containing skimmed milk agar, the protease enzyme encoding genes have degraded the skimmed milk as it has the property to degrade the skimmed milk agar and hence shows that the sample has protease encoding genes.

8.2.1 Secondary screening and localization: it was made confirms that protease encoding genes are present in the soil sample and our enzyme was intracellular as the clear zone formation in bore 3 contains cell free extract.

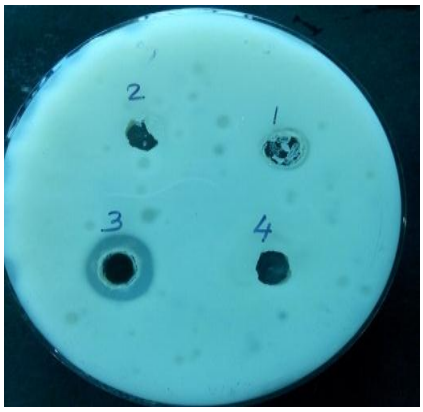

Fig-8.5 skimmed Milk agar plate showing the zone of clearance in bore 3

8.3 Biochemical characterization

8.3.1 Tyrosine standard curve: all the analysis of enzyme activity like optimum temperature, stability temperature, optimum pH and effect of metal ions and inhibitor were assayed in comparison with tyrosine standard curve.

S.No.	Tyrosine Concentration uM	O.D Nm
Blank 1	0	0
S1	110	0.041
S2	220	0.201
S3	330	0.313
S4	440	0.543
S5	550	0.696
S6	660	1.032
S7	770	1.118
S8	880	1.271
S9	990	1.517
S10	1100	1.644

Table-8.1: O.D table for tyrosine.

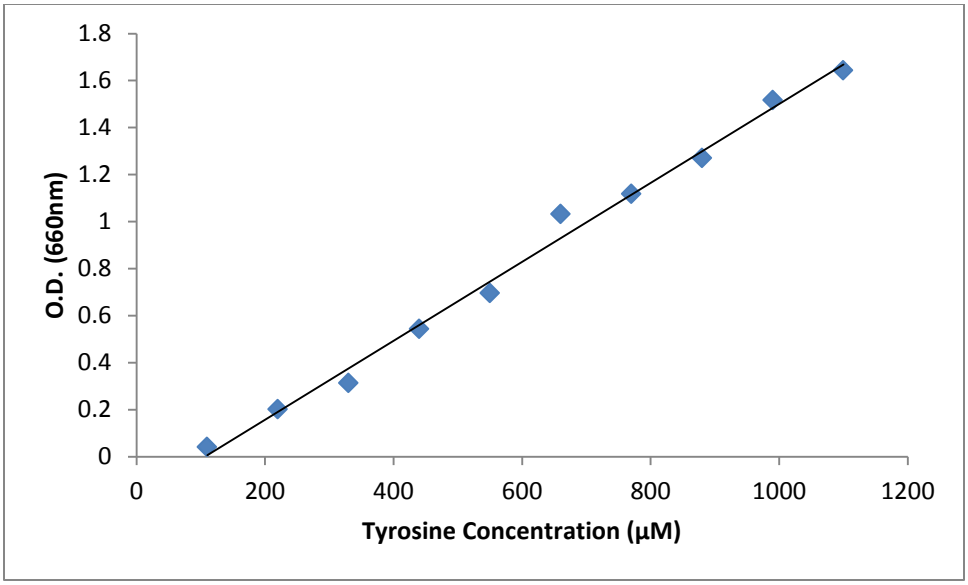

Fig-8.6 tyrosine standard curve

8.3.2 Temperature optimum and thermal stability: the analysis showed that in this study protease obtained has optimum temperature of 50^0C when incubated at different temperature ranges from 10^0 to 90^0C for one hour and it is also stable at 50^0C showing its maximum activity stably. The activity was measured on the basis of standard tyrosine curve. On the basis of previous work on protease produce from other microorganism like *Pseudomonas thermarium* the optimum temperature recorded was 60^0C and activity was stable between 40^0C to 70 ^{0}C [55].

Temperature	Final O.D	Activity (IU/mL)
10	0.012	6.482505
20	0.106	57.26212
30	0.110	59.42296
40	0.113	61.04359
50	0.126	68.0663
60	0.104	56.18171
70	0.016	8.643339
80	0.012	6.482505
90	0.011	5.942296

Table-8.2: O.D table for optimum temperature and activity.

It was confirmed from the graph below that enzyme optimum temperature of our enzyme was 50^0C as maximum activity was obtained at this temperature. The black marker in graph shows the maximum activity at 50^0C.

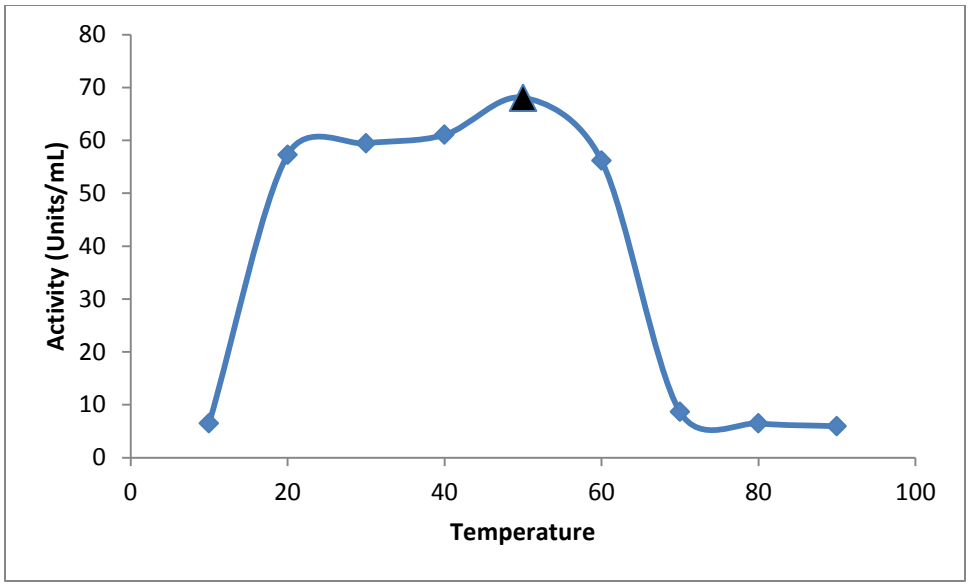

Fig-8.7: effect of temperature on protease activity

Temperature	Final O.D	Activity (1U/mL)
10	0.117	63.20442
20	0.132	71.30755
30	0.134	72.38797
40	0.251	135.5924
50	0.363	196.0958
60	0.130	70.22713
70	0.163	88.05402
80	0.137	74.00859
90	0.127	68.60651

Table-8.3: O.D table for stability temperature and activity.

It was confirmed from the graph below that stability temperature of our enzyme was 50^0C as maximum activity was obtained at this temperature. The black marker in graph shows the maximum activity at 50^0C.

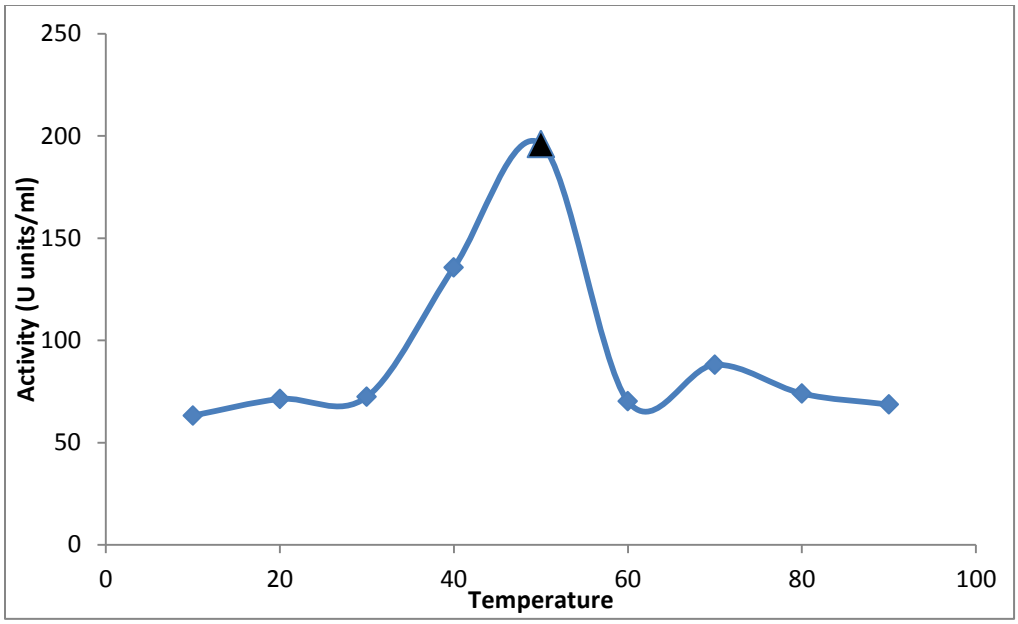

Fig-8.8: effect of temperature on stability

8.3.3 pH optimum: enzyme activity was determined at different pH ranges from 2-12 and optimum pH was found at 10.0 as maximum activity obtained at this Ph. Earlier studies shows that protease activity was maximum at alkaline pH ranging from 3-11 [56].

pH	O.D	ACTIVITY
2	0.070	37.81461
3	0.080	43.2167
4	0.115	62.124
5	0.129	69.68692
6	0.137	74.00859
7	0.144	77.79006
8	0.151	81.57152
9	0.173	93.45611
10	0.198	106.9613
11	0.163	88.05402
12	0.159	85.89319

Table-8.4: OD table for optimum pH and activity

It was confirmed from the graph below that optimum ph of our enzyme was 10 as maximum activity was obtained at this ph. The black marker in graph shows the maximum activity at optimum ph.

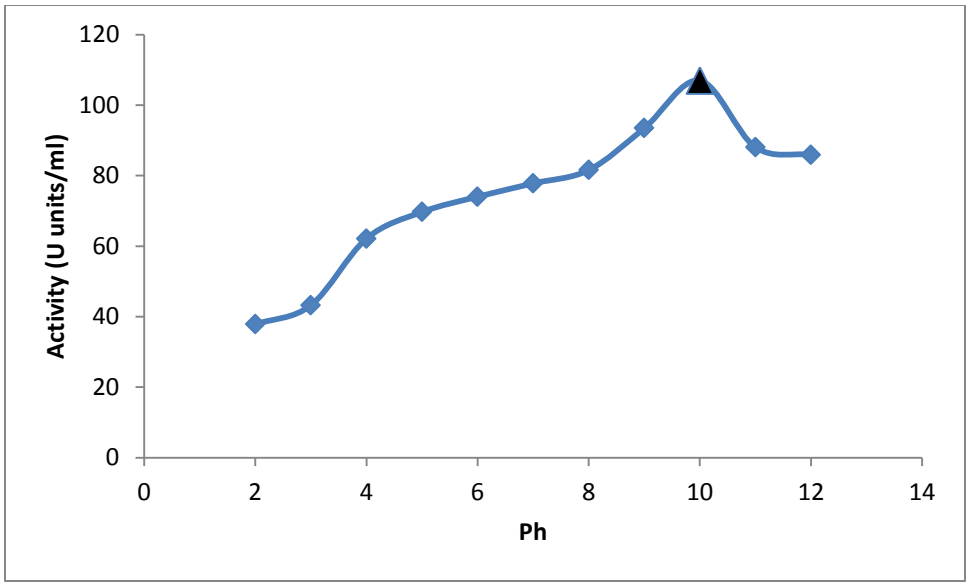

Fig-8.9: effect of pH on enzyme activity

8.3.4 Effect of metal ions: the effect of several mono and divalent cations was analysed and the results were obtained at 2Mm and 5mM concentration.

Metal ion	2mM	5mM	activity	Activity
No metal ion	0.16	0.16	86.43339	86.43339
Fe2+	0.13	1.14	70.22713	615.8379
Cu2+	0.51	0.84	275.5064	453.7753
Ca2+	0.28	0.64	151.2584	345.7336
Fe3+	0.12	1.15	64.82505	621.24
Mn2+	0.136	1.23	73.46839	664.4567
Mg2+	0.21	1.16	113.4438	626.6421
Zn2+	0.272	0.598	146.9368	323.0448
Co2+	0.216	0.554	116.6851	299.2756

Table-8.5: O.D table for metal ion and activity.

The graph below shows that the activity of enzyme thus obtained was significantly promoted by the metal ions Fe^{2+}, Fe^{3+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, the studies done in the past have shown that alkaline proteases activity greatly increased by these metal ions Co^{2+} reduces the enzyme activity. Co^{2+}, Zn^{2+}, and Ca^{2+} reduces the activity of alkaline protease.

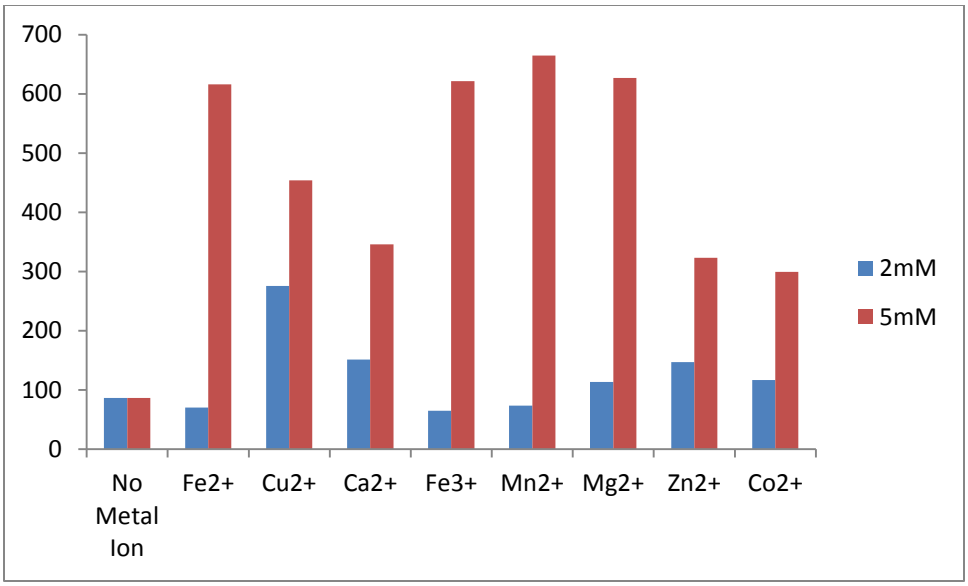

Fig-8.10: effect of metal ions on enzyme activity

8.3.5 Effect of inhibitors: the effect of enzyme inhibitors on our enzyme was also investigated and from this analysis the nature of enzyme was determined. The enzyme was pre-incubated with inhibitors and then it was assayed with casein as substrate.

Inhibitor	O.D.	Activity	Inhibitor	O.D.	Activity
No inhibitor	0.513	277.1271	No inhibitor	0.9513	277.1271
G-HCL(1M)	0.361	195.0153	G-HCL(2M)	0.135	72.92818
PMSF(1mM)	0.175	94.53653	PMSF(5mM)	0.088	47.53837
H_2O_2(2%)	0.505	272.8054	H_2O_2(5%)	0.195	105.3405
Tween-80(1%)	0.560	302.5169	Tween-80(2%)	1.012	546.6912
Tween-20(1%)	0.345	186.372	Tween-20(2%)	0.776	419.202
SDS(1%)	0.388	209.601	SDS(2%)	0.625	337.6304
T-X-100(1%)	0.295	159.3616	T-X-100(2%)	0.330	178.2689
UREA(1M)	0.400	216.0835	UREA(2M)	0.505	272.8054
CTAB(1%)	0.387	209.0608	CTAB(2%)	0.409	220.9454

Table-8.6: O.D table for inhibitor and activity

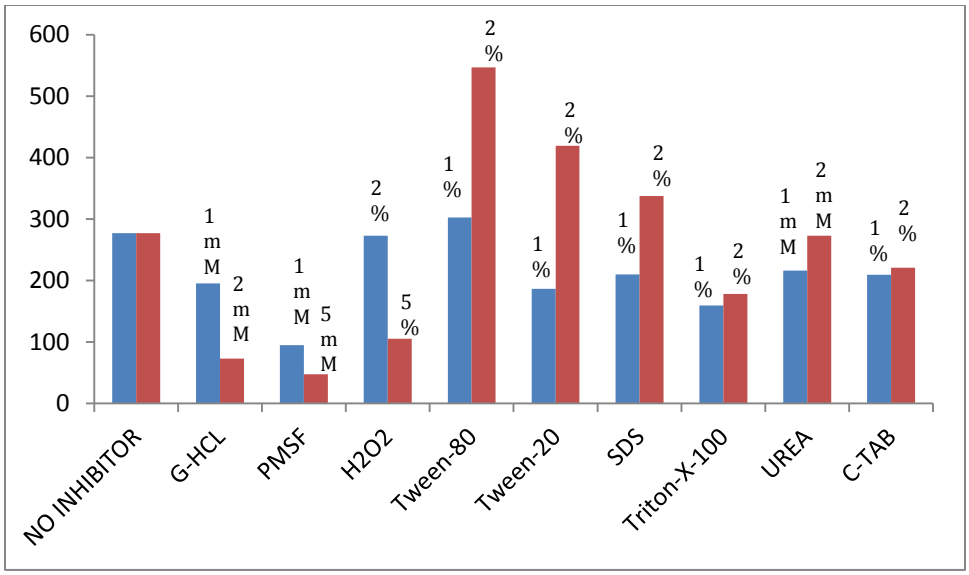

Fig-8.11: effect of inhibitor on enzyme activity

Enzyme activity was found to be increased in 2% concentration of tween 80 and 20 and was found to be decreased significantly by G-HCL and PMSF in 5mM concentration. In the presence of 5mM H_2O_2 the enzyme activity was to be decreased. The enzyme activity was almost stable in SDS, C-TAB, UREA, and Triton-X-100.

8.4 PCR amplification of soil DNA: the method employed for humic acid removal from soil DNA were effective as sharp band was observed on 1% Agarose gel. The DNA from soil culture SSTP2 was amplified using the SPR13 primers.

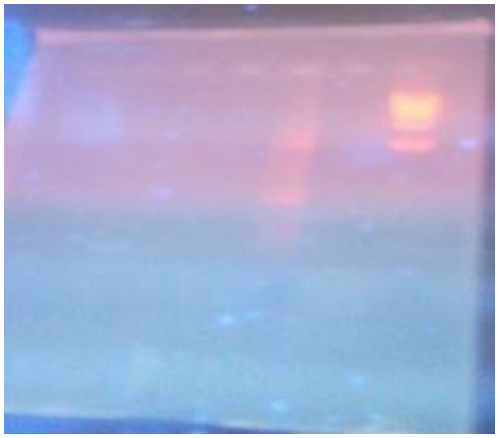

Fig-8.12 Agarose gel showing PCR amplified DNA

8.4.1 Strain identification by 16s rDNA Sequencing:

The amplified 16S rDNA sample was sent for sequencing to Yaazh Xenomics, Chennai. The amplified sequence was 573 bases long and was analysed using BLASTn tool of NCBI. The sequence revealed the organism to be *Bacillus thuringiensis* strain BPN 05/2.

Bacillus thuringiensis strain BPN 05/2 16S ribosomal RNA gene, partial sequence

Sequence ID: gb|KP006647.1| Length: 1463 Number of Matches: 1

Range 1: 50 to 622 GenBank Graphics ▼ Next Match ▲ Previous M

Score	Expect	Identities	Gaps	Strand
1059 bits(573)	0.0	573/573(100%)	0/573(0%)	Plus/Plus

```
Query  1    CTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCCATAAGACTG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  50   CTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCCATAAGACTG  109

Query  61   GGATAACTCCGGGAAACCGGGGCTAATACCGGATAATATTTTGAACTGCATAGTTCGAAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  110  GGATAACTCCGGGAAACCGGGGCTAATACCGGATAATATTTTGAACTGCATAGTTCGAAA  169

Query  121  TTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  170  TTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTG  229

Query  181  AGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  230  AGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACT  289

Query  241  GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  290  GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGG  349

Query  301  ACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  350  ACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCT  409

Query  361  GTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAG  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  410  GTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAG  469

Query  421  AAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCC  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  470  AAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCC  529

Query  481  GGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACG  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  530  GGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACG  589

Query  541  GCTCAACCGTGGAGGGTCATTGGAAACTGGGAG  573
            |||||||||||||||||||||||||||||||
Sbjct  590  GCTCAACCGTGGAGGGTCATTGGAAACTGGGAG  622
```

8.4.2. Amplification and sequencing of the protease gene:

The protease gene was amplified using degenerate primers SPR13 forward (5' GCNGTNATYGACACCGGCGTATA 3') and reverse (5' NGGNGTNGCCATNGATGTACCGCT 3'). The amplicon was approximately 1.2kb long and was sequenced. The following sequence was obtained:

aatgtgttgcataggtaatgtggaacctgcgcaaaattaactggcaggaagcgcgcgatctgaacggctttaacgatgcgagcgaaatta
aagtgggcgtgctggataccggcattgatgcgggccatccggatctgaaagatcaggtggcgggctatatttatgaacatccggatctgcc
gggcgcgagcagcgatcaggatctgattggccatggcacccatgtggcgggcaccattgcggcgaccattaacaacgatgtgggcattaa
cggcattagccgcgcgcgcattcatgcgtggaaaatttttgatgatcgcccggatctgctgacccatccggatggcaccgcggaatttgcgt
attttgtggatccggtgatgtatctgcgcgcgctgctggattgcgtggatcagggcattgatgtgattaacctgagcattggcggcggcggc
gcgccggatccgaccgaaagcgcggcgtttgaagcgctgctggcgaacggcagcaccattgtggcggcgatgggcaacgaacgccgcg
atggcagcccgattagctatccggcggcgatgccgggcgtgattgcggtgggcgcgaccaacctgcaggatcgcattaccaactttagca
accgcggcaaccatattaccattgcggcgccgggcgatgcgatttggagcaccctgccgacctatccgggccagattggctggcgcgcgg
aacgcggcccggatggccattggtggcagggcaaagcggcgattcgcgaaaccgattatgatgcgtggccgggcaccagcatggcggcg
ccgcatgtggcggcggcggcggcgctgtatattgcgaacggcggcaaacgcgatccggcggcgattcgcagcgcgctgaccgcgagcgc
ggataaagtgccggcgatgggcgaacaggattttaccccggattttggctatggccgcctgaacctggaacgcctgattgcgggcattggc
accaacgattgaagtttgattgggacccgtatgcatggcagatca

The sequence was then translated using the online Expasy Translate Tool (http://web.expasy.org/translate/) of the Swiss Institute of Bioinformatics. The translated sequence was as follows:

MWNLRKINWQEARDLNGFNDASEIKVGVLDTGIDAGHPDLKDQVAGYIYEHPD
LPGASSDQDLIGHGTHVAGTIAATINNDVGINGISRARIHAWKIFDDRPDLLTHPD
GTAEFAYFVDPVMYLRALLDCVDQGIDVINLSIGGGGAPDPTESAAFEALLANGS
TIVAAMGNERRDGSPISYPAAMPGVIAVGATNLQDRITNFSNRGNHITIAAPGDAI
WSTLPTYPGQIGWRAERGPDGHWWQGKAAIRETDYDAWPGTSMAAPHVAAAA
ALYIANGGKRDPAAIRSALTASADKVPAMGEQDFTPDFGYGRLNLERLIAGIGTN
D.

The sequence was again analysed using the BLASTp tool of NCBI and the results revealed that the protease belonged to the S8_S53 superfamily of proteases. The sequence showed absolute identity with the bacterium *Sinorhizobacterium meliloti*.

protease [Sinorhizobium meliloti]

Sequence ID: ref|WP_033053688.1| Length: 515 Number of Matches: 1

Range 1: 188 to 515 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
674 bits(1738)	0.0	Compositional matrix adjust.	328/328(100%)	328/328(100%)	0/328(0%)

```
Query  1    MWNLRKINWQEARDLNGFNDASEIKVGVLDTGIDAGHPDLKDQVAGYIYEHPDLPGASSD  60
            MWNLRKINWQEARDLNGFNDASEIKVGVLDTGIDAGHPDLKDQVAGYIYEHPDLPGASSD
Sbjct  188  MWNLRKINWQEARDLNGFNDASEIKVGVLDTGIDAGHPDLKDQVAGYIYEHPDLPGASSD  247

Query  61   QDLIGHGTHVAGTIAATINNDVGINGISRARIHAWKIFDDRPDLLTHPDGTAEFAYFVDP  120
            QDLIGHGTHVAGTIAATINNDVGINGISRARIHAWKIFDDRPDLLTHPDGTAEFAYFVDP
Sbjct  248  QDLIGHGTHVAGTIAATINNDVGINGISRARIHAWKIFDDRPDLLTHPDGTAEFAYFVDP  307

Query  121  VMYLRALLDCVDQGIDVINLSIGGGGAPDPTESAAFEALLANGSTIVAAMGNERRDGSPI  180
            VMYLRALLDCVDQGIDVINLSIGGGGAPDPTESAAFEALLANGSTIVAAMGNERRDGSPI
Sbjct  308  VMYLRALLDCVDQGIDVINLSIGGGGAPDPTESAAFEALLANGSTIVAAMGNERRDGSPI  367

Query  181  SYPAAMPGVIAVGATNLQDRITNFSNRGNHITIAAPGDAIWSTLPTYPGQIGWRAERGPD  240
            SYPAAMPGVIAVGATNLQDRITNFSNRGNHITIAAPGDAIWSTLPTYPGQIGWRAERGPD
Sbjct  368  SYPAAMPGVIAVGATNLQDRITNFSNRGNHITIAAPGDAIWSTLPTYPGQIGWRAERGPD  427

Query  241  GHWWQGKAAIRETDYDAWPGTSMAAPHVAAAAALYIANGGKRDPAAIRSALTASADKVPA  300
            GHWWQGKAAIRETDYDAWPGTSMAAPHVAAAAALYIANGGKRDPAAIRSALTASADKVPA
Sbjct  428  GHWWQGKAAIRETDYDAWPGTSMAAPHVAAAAALYIANGGKRDPAAIRSALTASADKVPA  487

Query  301  MGEQDFTPDFGYGRLNLERLIAGIGTND  328
            MGEQDFTPDFGYGRLNLERLIAGIGTND
Sbjct  488  MGEQDFTPDFGYGRLNLERLIAGIGTND  515
```

CHAPTER- 9

CONCLUSION

The study showed the metagenomic application to obtain the commercially useful biological catalyst from the uncultured microbes which are not been cultured by standard culturable techniques. Only one percent of total microbial population from soil are investigated through standard techniques but metagenomics approaches made it possible to explore the entire genome of environmental sample for protease or any other enzyme activity which are commercially important.

In the present study protease enzyme activity was explored by genomic and metagenomic approaches. The effect of metal ions, inhibitor, temperature were determined and optimum pH and thermal stability were also analysed under standard assay condition and enzyme activity explored on the basis of tyrosine standard curve.

CHAPTER- 10

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