EXPLORING THE GENOMES AND METAGENOME OF PHAGWARA SOIL FOR AMYLASES

A Dissertation

Submitted

By

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IN

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Under the guidance of

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CERTIFICATE

This is to certify that PRADIPKUMAR G has completed objective formulation of his Dissertation report titled, "**EXPLORING THE GENOMES AND METAGENOME OF PHAGWARA SOIL FOR AMYLASES**" under my guidance and supervision. To the best of my knowledge, the present work is the result of his original study and research. No part of the project has ever been submitted for any other degree at any University.

The project is fine for the submission and fulfillment of the conditions for the award of degree Master of Technology in Biotechnology.

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DECLARATION

I hereby declare that the Dissertation report entitled "**EXPLORING THE GENOMES AND METAGENOME OF PHAGWARA SOIL FOR AMYLASES**" is an authentic record of my own work carried out as the requirements for the award of degree of Master of Technology in Biotechnology at Lovely Professional University, Jalandhar under the guidance of Mr. Chirag Chopra, Assistant Professor, Department of Biotechnology.

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It is certified that the above statement is correct to the best of my knowledge and belief.

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I am entirely indebted to Mr.CHIRAG CHOPRA (Mentor) for his valuable and constant assistance, cooperation, guidance, listening to my queries and correcting the various documents with attention and care during the entire period. It is due to his immense knowledge the expertise that has encouraged me to start with a challenging and prestigious project.

Without all these people it would not have possible for me to successfully complete my work.

PRADIPKUMAR G

ABSTRACT

In the present-day, amylases are one of the most applicable enzymes in most of the industries. The objective of this study was to produce and characterize the amylases produced by an isolate of an amylase producing bacterial strain from Phargwara, Punjab (India). Among the few isolated amylase producing strains on starch agar medium, the strain Ssta2 showed significant amylase activity on the plate. Characterization of crude amylase showed its higher activity at pH 4.0 and temperature 40°C, with a thermostability of 100% for 1 hour. Enzyme activity was significantly activated by $MgCl_2$ and $CaCl_2$ salts. $HgCl_2$, $CuSO_4$, $FeCl_3$, $CoCl_2$ drastically reduced the enzyme activity. $ZnCl_2$ and $MnCl_2$ did not influence the enzyme activity much at 2mM concentration and decreased the enzyme activity at 5mM concentration. The enzyme showed high stability and activity with SDS, CTAB, T-80 at 2% concentration and decreased the activity at 5% concentration. T-X-100, PMSF, Urea and G-HCl have higher inhibitory effect on enzyme activity. The 16S rDNA sequence of the strain Ssta2 was sequenced and the amylase gene specific sequencing was done and the amylase gene sequence present in the isolated soil metagenomic DNA was explored.

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LIST OF ABBREVIATIONS

IUBMB	International Union of Biochemistry and Molecular Biology
EC	Enzyme Classification
DNS	3,5-Dinitrosalicylic acid
SDS	Sodium Dodecyl Sulfate
CTAB	Cetyl Trimethyl Ammonium Bromide
PEG	Poly Ethylene Glycol
EDTA	Ethylene Diamine Tetraacetic Acid
TE	Tris-EDTA
T-X-100	Triton X 100
T-20	Tween 20
T-80	Tween 80
PMSF	Phenyl Methyl Sulfonyl Fluoride
G-HCl	Guanidine Hydrochloride
cfu	Cell Free Extract
PCR	Polymerase Chain Reaction
NaCl	Sodium Chloride
$MgSO_4.7H_2O$	Magnesium Chloride hepta hydrate
$FeSO_4.7H_2O$	Ferrous Sulfate hepta hydrate
Hg_2Cl_2	Mercury Chloride
$CuSO_4$	Copper Sulfate
$CaCl_2$	Calcium Chloride
$FeCl_3$	Ferric Chloride

$MnCl_2$	Manganese Chloride
$MgCl_2$	Magnesium Chloride
$CoCl_2$	Cobalt Chloride
$ZnCl_2$	Zinc Chloride
H_2O_2	Hydrogen Peroxide
I_2	Iodide
KI	Potassium Iodide
mL	Millilitre
g/L	Gram per Litre
mM	Millimolar
M	Molar
gm	Gram
µmol	Micromoles
µL	Microlitre

CHAPTER 1

INTRODUCTION

Amylases are enzymes that hydrolyze starch and related starchy substrates and give products such as maltose, maltotriose, glucose and dextrins. In a broad sense, the term "amylases" (or amylolytic enzymes) refers to a variety of starch hydrolases and other related enzymes that are active in the hydrolysis, transglycosylation and isomerization of starch molecules with respect to the α-glucosidic bonds present in them and other related polysaccharides and oligosaccharides (Stefan Janecek *et al.,* 2014). Amylases have been derived from several sources including fungi, bacteria and yeasts. Among these, amylases produced by bacterial and fungal sources have dominant industrial applications. Amylases have board applications in starch industries, detergent industries, textile industries, paper industries, pharmaceutical industries and food & beverage industries.

Soil is one of the major habitats for vast range of prokaryotes. These prokaryotes build up the decomposition system of soil to decompose the plant and animal wastes deposited over billions of years (Whitman *et al.,* 1998). Although soil contains a huge microbial diversity, less than 1% of bacteria and archaea are identified using the artificial culture techniques (Hugenholz *et al.,* 1998). The genomic analysis of these identified prokaryotes may lead to the discovery of improved varieties of enzymes but there is a significant trade-off involved here with the novelty of these enzymes. Agricultural and Starch industrial soils are considered good sources for valuable improved and novel types of amylases (Poddar, A., Jana, S.C., 2014).

The demand for the improvement and development of the new techniques and processes in modern industries leads to the search for improved and novel enzymes. This demand to seek novel enzymes invokes the need to explore the dark area i.e., the unidentified prokaryotes. Metagenomic study is the only way to explore these unidentified prokaryotes. Metagenomic study has unlocked the new possibilities to identify and amplify the unidentified genes which code for novel enzymes which are commercially effective.

There are various obstacles in the direct isolation of metagenomic DNA. The initial one is that metagenomics do not target only a certain kind of microbes (like bacteria, fungi,

actinomycetes) because soil contains a pool of diverse microbes. The nature of the outer protective layer (cell wall) of these diverse microbes is diverse and also it will be hard to disrupt them in their natural environment. Hence different types of cell disruption techniques should be combined to disrupt a wide range of cells.

In this study, the genomes and metagenome of the microbes present in the soils of Phagwara, Punjab is examined. Industrial soil sample from starch industry is collected for the work. For effective cell disruption, enzymatic (lysozyme), detergent (SDS, CTAB) and mechanical (shaking) methods are used. This study aims at the isolation and examination of amylases in search of novel properties like temperature stability, pH stability, enzyme activity and the effect of inhibitors on the enzyme.

CHAPTER 2

REVIEWS OF LITERATURES

2.1 Enzymes

Enzymes are any proteins that are produced by living organisms to function as a specific catalyst in biochemical reactions.

2.1.1 Enzyme Classification

Enzymes have been classified into six different classes based upon the reactions they catalyze. The procedures for assigning the names to enzymes and to place them in a specific class or a subclass have been approved by the IUBMB Nomenclature Committee and in 1992 this committee has published those procedures in "Enzyme Nomenclature".

2.1.1.1 Class 1: Oxidoreductases

This class holds the enzymes catalyzing redox (oxidation and reduction) reactions.

2.1.1.2 Class 2: Transferases

This class has the enzymes which transfer a group from the donor (one of the substrate) to the acceptor (another substrate).

2.1.1.3 Class 3: Hydrolases

This class consists of the enzymes catalyzing the hydrolytic cleavage of bonds including C–O, C–N and C–C bonds.

2.1.1.4 Class 4: Lyases

This class classifies the enzymes catalyzing the cleavage of C-O, C-N and C-C bonds by means other than oxidation or hydrolysis.

2.1.1.5 Class 5: Isomerases

This class contains the enzymes catalyzing the structural or geometric changes occurring in a single molecule.

2.1.1.6 Class 6: Ligases

This class classifies the enzymes which catalyze the ligation (joining) of two large molecules by formation of a new bond between them.

2.2 Amylases

Amylases belong to the class "Hydrolases" and the subclass "Glycoside hydrolases" (EC 3.2.1). Amylases are the group of enzymes that break down starch molecules into lower sugar units. Diastase (Amylase) is the first enzyme to be reported and isolated in the history of enzymes. All amylases mainly act on the α-1, 4-glycosidic bonds. Amylases hydrolyze these bonds and cleave starch into lower sugars. Amylases randomly act on glycosidic bonds at different locations of the starch molecule so amylolytic hydrolysis of starch results in different kinds of products such as glucose, maltose, maltotriose and dextrin.

Enzyme activity of amylase can be determined by the amount the reducing sugars (glucose) produced by the enzyme (Vidya *et al.*, 2011). The amount of reducing sugars produced by the enzyme can be estimated using the dinitrosalicylic acid method (Miller, 1959).

In 2008, Wang, S. and his colleagues defined that one unit of α-amylase was the amount of enzyme that liberated 1 μmol of sugars equivalent to glucose in 1 minute under assay conditions.

2.2.1 Types of Amylases

Amylases are generally classified into three types such as α-amylase, β-amylase and γ-amylase. These three types differ in how they hydrolyze the polysaccharide bonds. α-amylase is the most common type of amylases found in both the prokaryotes and eukaryotes. Neither β-amylase nor the γ -amylase is found in animal tissue (Jason & Matthew, 2008).

2.2.1.1 Alpha-amylases [EC 3.2.1.1]

Alpha-amylase hydrolyses the α-1,4-glycosidic linkages at random locations of large alpha-linked polysaccharides (starch and glycogen) and yields glucose, amylopectin, maltotriose and limit dextrin . Alpha-amylase is widely found in all types of cells. It is a calcium dependent metalloenzyme (Jason & Matthew, 2008). It is also termed as 1,4-α-D-glucan glucanohydrolase.

2.2.1.2 Beta-amylases [EC 3.2.1.2]

Beta-amylase hydrolyses the α-1,4-glycosidic linkages of starch or glycogen molecule from the non-reducing end yielding maltose (Jason & Matthew, 2008). In the case of eukaryotes, it is commonly present in the ripening fruit and the seed before germination. It is also termed as saccharogen amylase or 1,4-α-D-glucan maltohydrolase.

2.2.1.3 Gamma-amylases [EC 3.2.1.3]

Gamma-amylase hydrolyses the α-1,4-glycosidic linkages at the non-reducing side of amylopectin and amylose produced by the α-amylase and yields glucose (Jason & Matthew, 2008). The special feature of γ-amylase is that it also cleaves the α-1,6-glycosidic linkages. It is the one and only type of amylase which is most efficient in acidic environments. It is also termed as amyloglucosidase or 1,4-α-D-glucan glucosidase.

2.2.2 Mechanism of action

J. F. Robyt in 1962 reported, in his thesis, a hypothesis on the action pattern of exo- and endo-amylases on amylose and amylose fragments. Amylases have three patterns of action on their substrates: 1) single chain, 2) multi-chain and 3) multiple attack patterns (Figure 1).

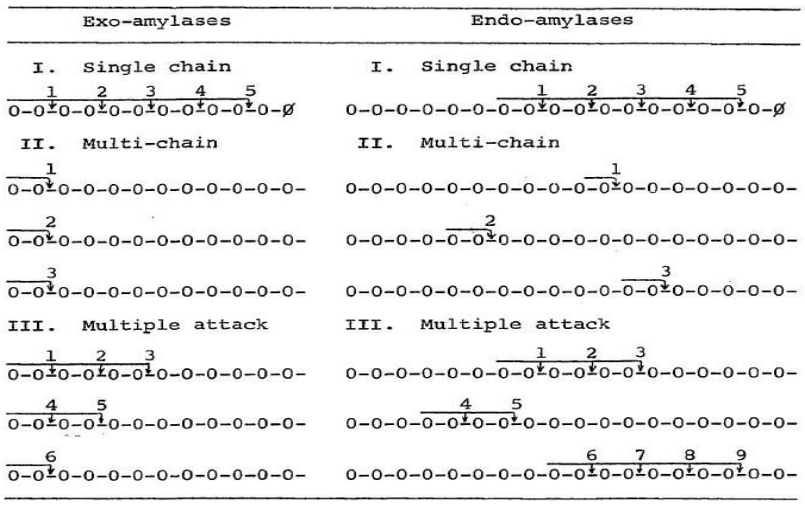

Figure 1: Action pattern of exo-amylases and endo-amylases on amylose and amylose fragments

(Source: Robyt, J. F., 1962)

In Figure 1,

- Arrows represent the sequence of hydrolytic cleavages of glycosidic bonds
- "O" represents D-glucose unit
- "-" represents α-1,4-glycosidic linkage
- "Ø" represents reducing end of a chain

Later Bijttebier *et al.* (2008) confirmed that the hypothesis, developed by J. F. Robyt, on the action patterns of amylases on the starch polymer is true.

2.2.3 Applications

Amylases are applied for process in diverse industries such as starch industry, food industry, biofuel production, detergent industry, paper industry, textile industry and pharmaceutical industry.

2.2.3.1 Starch Industry

Amylases play their major role in the starch industry for starch hydrolysis. In the starch liquefaction process, it converts starch into fructose and glucose syrups (Nielson and

Borchert, 2000). Amylases produced by *Bacillus* species have a special interest in large-scale processes because of their significant thermostability character (Prakash and Jaiswal, 2010).

2.2.3.2 Food Industry

Amylases play vital roles in baking, brewing, preparation of digestive aids, fruit juices and starch syrups. For baking process, a thermostable amylase from *Bacillus stearothermophilus* is more commercially used (Van der Maarel *et al.,* 2002). Amylases are also used for the clarification of beer or fruit juices, or for the pretreatment of animal feed to improve the digestibility of fiber (Ghorai *et al.,* 2009).

2.2.3.3 Biofuel Production

For the production of ethanol, the most used liquid biofuel, starch is the commonly used substrate because of its low price and easy availability as raw material (Chi, Z. *et al.,* 2009). In this production, the bioconversion of starch into ethanol involves two steps: i) liquefaction and saccharification by amylases to convert starch into simple fermentable sugar and ii) fermentation of sugars into ethanol by fermenting microorganism, mostly *Saccharomyces cerevisiae* (Öner, 2006). Bacterial amylases having high thermostability or from engineered strains of *Escherichia coli* or *Bacillus subtilis* are used during the saccharification (Sanchez & Cardona, 2008).

2.2.3.4 Detergent Industry

In terms of both volume and value, detergent industries are the major consumers of enzymes to enhance the detergent's ability to remove tough stains and to make the detergent ecofriendly. About 90% of all the liquid detergents in the market contain amylases (Gupta *et al.,* 2003 and Hmidet *et al.,* 2009). Removal of starch is significant in giving whiteness to cloths because starch is an attractant for many particulate soils. Hence amylases are used to degrade the starchy food residues such as potatoes, gravies, custard, chocolate, etc. Since the washing environment is highly oxidative, amylases having activity at lower temperatures are necessary (Chi, M. *et al.,* 2009).

2.2.3.5 Paper Industry

In the pulp and paper industry, amylases are used to modify the starch of coated paper to produce high molecular weight and low-viscosity starch (Van der Maarel *et al.*, 2002 and Gupta *et al.,* 2003). This is to make the surface of paper sufficiently smooth and strong for improving the quality of the paper. The viscosity of the natural starch is very high for paper sizing, so amylases are used to partially degrade the polymer chain in a batch or continuous processes (Gupta *et al.,* 2003).

2.2.3.6 Textile Industry

Starch (sizing agent) is used to yarn for a fast and safe weaving process. During weaving process, this starch should be removed from the woven fabric to prevent the breaking of threads. Amylases are used to selectively remove the starch without affecting the fibres (Gupta *et al.,* 2003 and Ahlawat *et al.,* 2009). Amylases from *Bacillus* stains were commonly used in textile industries for past many years.

2.2.3.7 Pharmaceutical Industry

In 2013, Naidu and Saranraj reported that bacterial amylases are commercially used as digestive acids in pharmaceutical and clinical industries. In case of indigestion condition after meals, amylase/protease/lipase enzyme combination is administered orally to digest the starch, proteins and fats in the food respectively.

2.2.4 Purification of Amylases

In 2010, Souza and Magalhães reviewed that commercially amylases are not required to be purified but for applications, in case of clinical and pharmaceutical industries, require high purity amylases. In laboratory scale, amylases are completely purified by chromatography techniques including ion exchange, gel filtration, hydrophobicity interactions and reverse phase chromatography and by liquid-liquid extraction. Conventionally, amylases are extracted by protocols using organic solvents (ethanol, acetone), ammonium sulfate precipitation and ultrafiltration. These conventional protocols are widely employed because of its low costs, simplicity and ease of scale up. Ahmadia *et al.,* 2010 and Khattak *et al.,* 2013 reported

separately that the Ammonium sulfate precipitation and Acetone precipitation are the simple and low-cost methods to effectively purify amylases from different protein contaminations. Application of both the methods results good purity of amylases for physico-chemical characterizations.

2.2.5 Assay Methods

Like any other enzymes, amylases can also be assayed by both quantitative and qualitative assays

2.2.5.1 Quantitative Assay

Since glucose is the final reducing sugar produced after a complete cleavage of starch polymer (or fragments), amylase activity can be assayed using glucose estimation methods. This is quantitative assay to find the amylase activity. DNS assay is one of the most popular assay methods for measuring the activity of glycoside hydrolases (Gusakov *et al*., 2011). 3,5-dinitrosalicylic acid (DNS) is the main component of DNS reagent. DNS reagent also consists of crystalline phenol and Sodium sulphite. Firstly the substrate (starch) and the enzyme are allowed to react and DNS reagent is added to stop the enzyme- substrate reaction. DNS selectively binds only to the reducing sugars, which are produced by the enzyme-substrate reaction. Next, the mixture is heated by which the enzyme is inactivated and DNS effectively binds to the reducing sugar (glucose) to give absorbance at 540nm.

2.2.5.2 Qualitative Assay

Amylolytic activity is qualitatively assayed by the agar plate method. Enzyme is filled in the small holes made on a agar plate containing only starch. During the incubation, enzyme diffuses through the agar and degrades starch in the medium. Enzyme activity is determined by addition of iodine solution (Fossi *et al*., 2009). The diameter of the zone of clearance is directly proportional to the amylolytic activity of the enzyme.

2.2.6 Physico-chemical Characterization of Amylases

Since the most of the characteristics of α-amylase hold the true for all the types of amylases, in general, the characteristics of α-amylase are considered for the all types of amylases.

2.2.6.1 Effect of Temperature

In 2008, Wang, S. and his colleagues characterized a hyperthermophilic amylase with optimal temperature of 95°C and a half-life of 5 hours at 90°C. They also reported that more than 40% and 30% activity of that amylase enzyme remained even after incubation for 2 hour and 3 hour at 100°C respectively.

2.2.6.2 Effect of pH

Amylases which produced from fungi and bacteria have different pH optima mostly in the range of 5 to 8. In the year 1996, α-amylases having pH optima of 3.0 (highly acidic) and 11 to 12 (highly alkaline) are reported by Bohdziewicz and Shinke respectively (Pandey *et al.,* 2006).

2.2.6.3 Effect of Metal ions

Prakash and Jaiswal (2010) reported that the cobalt and Manganese ions strongly activated the α-amylase produced from Soya bean seeds. Wang, H. *et al.* (2011) published that Magnesium ions enhanced the activity of glycoside hydrolases and Calcium ions inhibited the enzyme activity.

2.2.6.4 Effect of Inhibitors

Hauli *et al*. (2013) reported that the SDS, Triton-X-100 and CTAB strongly increases the activity of amylase produced from *Anoxybacillus* sp.

2.3 Genomic and Metagenomic DNA Analyses

Genomic and Metagenomic analyses are the revolutionary way to explore the desired gene in a gene pool because these analyses target the culturable and unculturable microbial population in the soil sample.

2.3.1 Genomic DNA Isolation

Genomic DNA is effectively isolated from the culturable organisms using the common laboratory chemicals (Sachinandhan *et al.,* 2010). The soil microbes are first isolated by spread plating the soil sample in the plates containing growth medium after serial dilution. Culturing the microbes by targeting the desired function (Abdulkadir and Waliyu, 2012) is an easy way to culture only the organisms that are producing amylase.

2.3.2 Metagenomic DNA Isolation

The same isolation method can be followed but the resultant DNA will contain humic substances. Humic substances (literally humic acids) are the major components of the natural organic matter present in soil. They consist of phenolic and carboxylic substituents. These humic acids cause interference in restriction digestion and PCR amplification of the DNA (Zhou *et al.,* 1996) so they should be removed from the DNA sample.

2.3.2.1 Removal of Humic Substances

In 2006, Dong *et al*., explained, the reason for the difficulty in humic acid removal from DNA, that both were long chain molecules carrying negative charge and also humic substances contain more hydroxyl and carboxyl groups whose physicochemical characteristics were nearly same to that of phosphate groups in the sugar-phosphate backbone of DNA.

Humic acids are effectively removed by various methods using Aluminium sulfate, Q-Sepharose, Polyethylene glycol (PEG) and divalent salts like calcium chloride (Dong *et al.,* 2006, Sharma *et al.,* 2007, Kumar, 2011 and Liu *et al.,* 2007). Among all these methods, the use of divalent salts has shown better results in the removal of humic acid without any damage to the DNA.

The sequence-based analysis with the isolated DNA samples using the conserved DNA sequence or the primer sequences used in the PCR amplification process. 16S rRNA sequencing is commonly used method for the conserved sequence (16s gene) to find the taxonomy of the microorganism from which genomic DNA is isolated. Sequencing on the

basis of the primer sequences used in the PCR amplification process helps to find about information about a specific sequence. This approach is generally time consuming but costlier than another approach. The targeted sequences are generally determined by using the BLAST algorithm (Purohit & Singh, 2013).

CHAPTER 3

SCOPE OF THE STUDY

Now-a-days amylases are industrially applied in starch industry, food industry, biofuel production, detergent industry, paper industry and textile industry. Even though amylases have various industrial applications, certain characteristics like thermostability, pH stability, optimum temperature and pH activity, effect of inhibitors and metal ions are building limitations to their commercial applications. To overcome this problem, improved varieties of amylases having better characteristics should be produced.

This study scopes on the search for improved and novel varieties of amylases in soil through genomic and metagenomic approaches.

CHAPTER 4

AIMS AND OBJECTIVES

This study aims at the isolation and characterization of novel amylases from the Phagwara soils through genomic and metagenomic analyses. The objectives of this study are

- ✓ Collection of soil samples from starch industrial site
- ✓ Screening and selection of genome of culturable soil microbes
- ✓ Isolation of high molecular weight metagenomic DNA, free from humic contaminants
- ✓ Isolation of genomic DNA from selected genome
- ✓ Genetic characterization of the isolated genomic and metagenomic DNA
- ✓ Physico-chemical characterization of the produced amylases

CHAPTER 5

RESEARCH METHODOLOGY

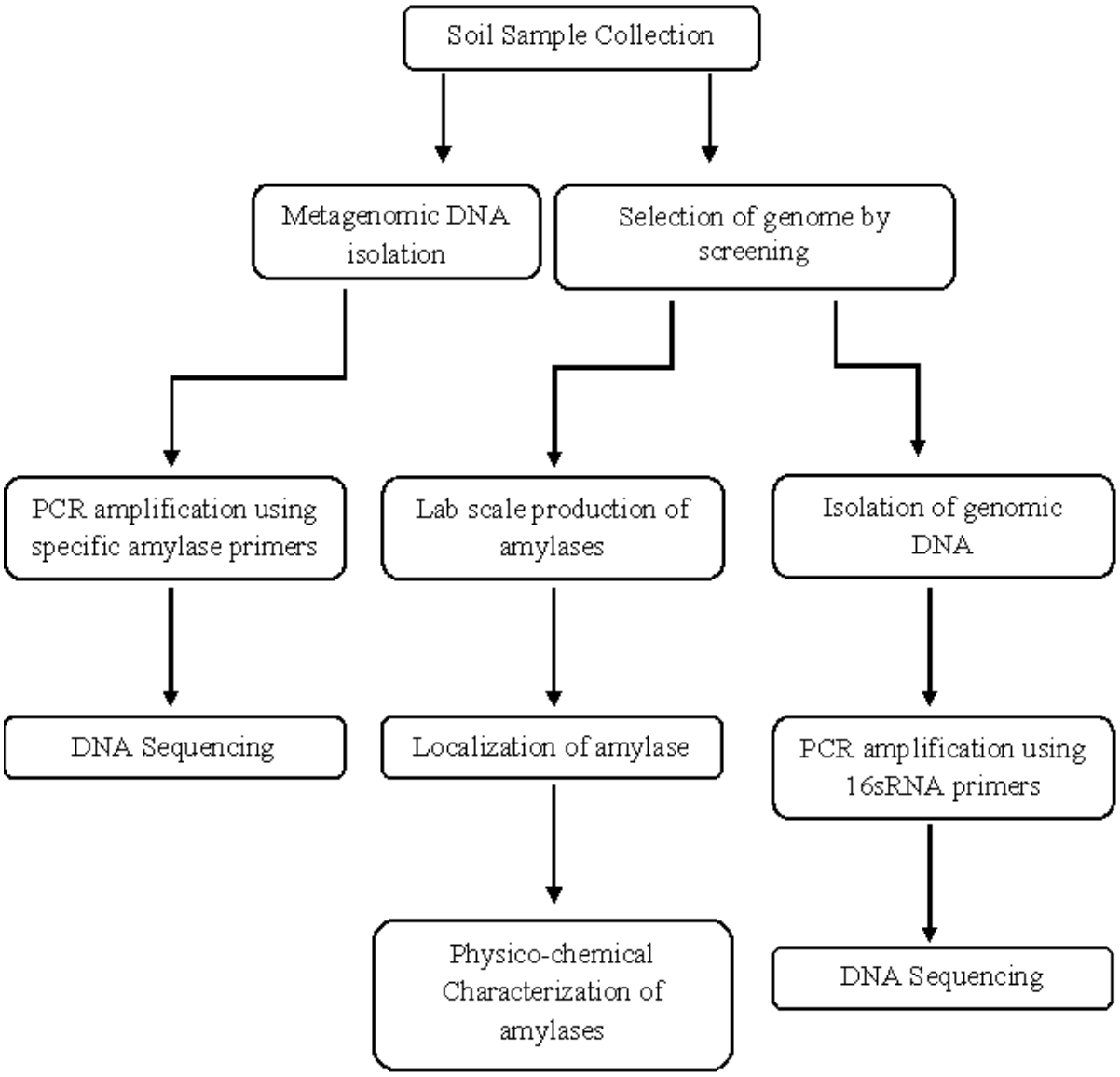

Figure 2: Scheme of the Research Methodology

5.1 Sample Collection

Industrial soil of Phagwara, Punjab [Longitude 31.13°N, Latitude 75.47°E] was collected. The soil sample collection site is exposed to different range of temperatures in different seasons ranging between 20 to 45°C (summer) and 0 to 15°C (winter). Surface of the soil was first removed to 1-2 inches depth. This was to remove the industrial wastes and spills. Approximately 1.5kg of the soil sample was collected in a sterile polyethylene bag using a sterile stainless steel spatula and finally stored at 4°C for analysis.

5.2 Screening and Selection of Genome

Soil enrichment was the first step in screening process. Amylolytic micro-organisms were firstly enriched by adding soil (1gm) in enrichment broth (50mL). Enrichment broth medium consisted of soluble starch (5g/L), peptone (5g/L), yeast extract (5g/L), $MgSO_4.7H_2O$ (0.5g/L), $FeSO_4.7H_2O$ (0.01g/L), NaCl (0.01g/L). Incubation was carried out in a shaker at 37°C and 120 oscillations per second for 24 hours. Amylases producing colonies were screened on agar plate, containing soluble starch (10g/L), peptone (5g/L), yeast extract (5g/L), $MgSO_4.7H_2O$ (0.5g/L), $FeSO_4.7H_2O$ (0.01g/L), NaCl (0.01g/L), agar agar (15g/L). Incubation was done at 37°C for 48 hours. The plates were stained with Gram iodine solution [I_2 (0.1%) and KI (1%)] and screened for amylolytic colonies. The colonies with the largest zone of clearance were isolated for further investigations.

5.3 Isolation of metagenomic DNA from soil

The optimized metagenomic DNA isolation protocol was modified form of Zhou *et al*., (1996). The modification done was the implementation of humic removal method by Lui *et al*., (2007). Soil (5gm) was mixed with extraction buffer [Tris-HCl (100mM, pH 8.0), sodium EDTA (100mM, pH8.0), NaCl (1.5M), CTAB (1%)], lysozyme (100µL), proteinase K (50µL) and RNase (10µL) were added in a sterile 50mL centrifuge tube and then incubated at 37°C for 30 minutes. SDS (1.5mL, 20%) was added to the mixture and now the incubation is at 65°C for 2 hour with continuous end-to-end inversions for every 15 minutes. After centrifugation at 6,000g for 10 minutes, the supernatants were transferred to fresh eppendorffs. Further extractions of DNA were done by adding extraction buffer (4.5mL) and SDS (0.5mL, 20%).

The DNA was extracted using chloroform:isoamyl alcohol (24:1) extraction. The aqueous phase was recovered after centrifugation at 14,000g for 15 minutes at 4°C and the DNA was precipitated by treating with two volumes of ice-cold absolute ethanol and sodium acetate (3M, 1/10 volume). In the end, the tubes are centrifuged at 12,000g for 5 minutes at 4°C and the pellet was washed with 70% ethanol, dried and resuspended in 1X TE buffer.

5.3.1 Humic acid removal

The humic contamination was removed by treating with $CaCl_2$ (2%) and incubated at room temperature for 1 hour. These tubes are centrifuged at 14,000g for 20 minutes at 4°C and the supernatant was re-precipitated by treating with ice-cold absolute ethanol (2 volumes) and sodium acetate (3M, 1/10 volume). In the end, humic contamination free, high molecular weight DNA was harvested by 15,000g for 15 minutes at room temperature. The pellet was washed with 70% ethanol, dried and resuspended in 1X TE buffer.

5.3.2 Metagenomic DNA quantification

The integrity of the DNA was checked by electrophoresis on 0.8% Agarose gel. The DNA purity was quantified by spectrophotometry with a UV/VIS spectrophotometer. The DNA concentration was evaluated at a wavelength of 260nm. The absorbance ratios A260/A280 (DNA/Proteins) and A260/A230 (DNA/ Humic acids) were used to determine the purity of the soil metagenomic DNA.

5.4 Isolation of genomic DNA

The selected colony was grown overnight and culture (8mL) broth was centrifuged at 6,000g for 8 minutes. The pellet (biomass) was resuspended in extraction buffer [Tris-HCl (100mM, pH 8.0), sodium EDTA (100mM, pH8.0), NaCl (1.5M), CTAB (1%)] and enzyme combination of lysozyme (20µL), proteinase K (10µL) and RNase (5µL) was added and incubated at 37°C for 30 minutes. Now, SDS (30µL, 10%) was added to the samples and incubated at 65°C for 30 minutes with continuous inversions for every 5 minutes. After centrifugation at 6,000g for 10 minute, the supernatants were transferred to fresh tubes. The DNA was extracted using chloroform:isoamyl alcohol (24:1) extraction. The aqueous phase was recovered after centrifugation at 14,000g for 15 minutes at 4°C and the DNA was precipitated by treating with

two volumes of ice-cold absolute ethanol and sodium acetate (3M, 1/10 volume). In the end, the tubes are centrifuged at 12,000g for 5 minutes at 4°C and the pellet was washed with 70% ethanol, dried and resuspended in 1X TE buffer.

5.4.1 Genomic DNA quantification

The integrity of the DNA was checked by electrophoresis on 0.8% Agarose gel. The DNA purity was quantified by spectrophotometry with a UV/VIS spectrophotometer. The DNA concentration was evaluated at a wavelength of 260nm. The absorbance ratio A260/A280 (DNA/Proteins) was used to determine the purity of the isolated genomic DNA.

5.5 Physico-chemical Characterization

Physico-chemical analysis was performed to determine the effects of temperature, pH, metal ions and inhibitors on the activity of the amylases. The enzyme was produced in the Luria Broth medium (50mL) by using the Ssta1 colony (the colony selected for genomic analysis).

5.5.1 Enzyme Localization

Firstly, Localization of the enzyme was done to find whether the enzyme is an endoenzyme or exoenzyme. The Ssta1 culture was centrifuged at 6,000g for 10 minutes. Now, the supernatant (S) contains the exoenzymes. The cell pellet was suspended in phosphate buffer. All the endoenzymes were released by disrupting cells using glass beads. After centrifugation at 8,000g for 5 minutes, the cell free extract (cfu) contains all the endoenzymes.

These two enzyme samples were analyzed for amylase activity by DNS method. The nature and localization of the enzyme can be determined using the observation of activity of the enzyme in a sample.

5.5.2 Effect of Temperature

Effect of temperature on enzyme activity was studied using the glucose standard curve using 1% soluble starch as substrate. To determine the temperature optima, the enzyme and substrate were allowed to react at different temperatures ranging from 10^0C to 90^0C for 30 minutes in

phosphate buffer [pH 7.0]. The reaction was stopped by adding DNS reagent and incubated at 95^0C for 5 minutes.

To measure the most stable temperature for amylase activity, the enzyme was pre-incubated at different temperatures (30 ^{0}C to 90 ^{0}C) for 1 hour followed by the same protocol for temperature optima study. The optical density readings were recorded for all the samples at 540nm using a control for each samples which do not have any enzyme and all the others remain the same.

Table 1: Protocol for temperature studies

Temperature (^{0}C)	10	20	30	40	50	60	70	80	90
Buffer (µL)	300	300	300	300	300	300	300	300	300
Substrate (µL)	500	500	500	500	500	500	500	500	500
Enzyme (µL)	200	200	200	200	200	200	200	200	200
DNS Reagent (mL)	2	2	2	2	2	2	2	2	2

5.5.3 Effect of pH

Effect of pH on enzyme activity was studied using the glucose standard curve using 1% soluble starch as substrate. To determine the temperature pH, the enzyme and substrate were allowed to react in different pH buffers ranging from 3 to 11 for 30 minutes at optimum temperature. The reaction was stopped by adding DNS reagent and incubated at 95^0C for 5 minutes. The optical density readings were recorded for all the samples at 540nm using a control for each samples which do not have any enzyme and all the others remain the same.

Table 2: Protocol for pH studies

pH	3	4	5	6	7	8	9	10	11
Buffer (µL)	300	300	300	300	300	300	300	300	300
Substrate (µL)	500	500	500	500	500	500	500	500	500
Enzyme (µL)	200	200	200	200	200	200	200	200	200
DNS Reagent (mL)	2	2	2	2	2	2	2	2	2

5.5.4 Effect of Metal Salts

From many reviews, amylase activity has been influenced by various metal ions and their intensity of influence depends on their concentration in the reaction environment. Effect of metal ions on amylase activity was studied by addition of pre-specified concentrations of respective metal ions to the amylase assay protocol.

The enzyme-substrate mixture was allowed to react in different metal ion concentrations in optimum pH for 30 minutes at optimum temperature. The reaction was stopped by adding DNS reagent and incubated at 95^0C for 5 minutes. The concentrations of metal ions and the assay constituents are described in the Table 7. The optical density readings were recorded for all the samples at 540nm using a control which do not has any metal ions and all the others remain the same.

Table 3: Protocol for Metal Salts studies

Metal Salts	$HgCl_2$		$CuSo_4$		$CaCl_2$		$FeCl_3$		$MnCl_2$		$MgCl_2$		$CoCl_2$		$ZnCl_2$	
Concentration (mM)	2	5	2	5	2	5	2	5	2	5	2	5	2	5	2	5
Buffer (µL)	300	300	300	300	300	300	300	300	300	300	300	300	300	300	300	300
Substrate (µL)	500	500	500	500	500	500	500	500	500	500	500	500	500	500	500	500
Enzyme (µL)	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200
DNS Reagent (mL)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

5.5.5 Effect of Inhibitors

The enzyme activity was affected by several inhibitors on the basis of the interaction between them. Effect of inhibitors on amylase activity was studied by addition of pre-specified concentrations of inhibitors to the amylase assay protocol. The enzyme-substrate mixture was allowed to react in different environments having different inhibitor concentrations in optimum pH for 30 minutes at optimum temperature. The reaction was stopped by adding DNS reagent and incubated at 95^0C for 5 minutes. The concentrations of metal ions and the assay constituents are described in the Table 7. The optical density readings were recorded for all the samples at 540nm using a control which do not has any inhibitor and all the others remain the same.

Table 4: Protocol for Inhibitor studies

Inhibitors	SDS		CTAB		T-X-100		T-20		T-80		PMSF		UREA		G-HCl		H_2O_2	
Concentration (mM)	2%	5%	2%	5%	2%	5%	2%	5%	2%	5%	1mM	5mM	1M	2M	1M	2M	5%	10%
Buffer (µL)	300		300		300		300		300		300		300		300		300	
Substrate (µL)	500		500		500		500		500		500		500		500		500	
Enzyme (µL)	200		200		200		200		200		200		200		200		200	
DNS Reagent (mL)	2		2		2		2		2		2		2		2		2	

5.6 Genetic Characterization of DNA

The genetic characterization of the DNA samples is of two steps: i) Amplification and ii) Sequencing. In the first step, genomic DNA is amplified using the 16S rDNA primers to find the organism from which the DNA is isolated. On the other hand metagenomic DNA is amplified using the specific amylase primers, specific for amylases, to find the type of amylase present in the environment from where the soil sample was collected.

5.6.1 16S rRNA Amplification

To find the genome selected for amylase characterization, PCR amplification was conducted for the isolated genomic DNA sample using the 16S rDNA primer pair: forward primer (16Sf) [5'-GAATTTGATCCTGGCTCAG-3'] and reverse primer (16Sr) [5'-GGCTACCTTGTTAC GACTT-3'] (Matsuda *et al*., 2006). The PCR reaction mixture set for a volume of 20µL. After the reaction, the amplification was checked by electrophoresis on 1% Agarose gel with a 100bp

ladder DNA. Now the reaction is set for 200µL volume and the amplified DNA mixture was further forwarded for sequencing.

Table 5: 16S rRNA PCR amplification reaction mix (20µL)

Molecular grade water	12µL
Forward primer	1µL
Reverse primer	1µL
dNTP mix	1µL
Template DNA	2µL
Taq Polymerase	1µL
Total	20µL

Table 6: PCR Protocol for 16S rDNA amplification

Initial Denaturation	Amplification (30 cycles)			Final Extension	Hold
94°C	94°C	56°C	72°C	72°C	4°C
2 minutes	30 sec	45 sec	90 sec	5 minutes	---

5.6.2 Amylase Gene Amplification

To find the metagenomic DNA isolated from soil, PCR amplification was conducted using specific amylase primers the DNA primer pair: forward primer (APAmy11F) [5'-GGN TTY ACN GCN ATH TGG ATH -3'] and reverse primer (Amy11R) [5'-RTA DAT DAT YGG

DAT YCC RTC-3']. The PCR reaction mixture set for a volume of 20µL. After the reaction, the amplification was checked by electrophoresis on 1% Agarose gel with a 1kbp ladder DNA.

Now the reaction is set for 200µL volume and the amplified DNA mixture was further forwarded for sequencing.

Table 7: Gene specific PCR reaction mix (20µL)

Molecular grade water	12µL
Forward primer	1µL
Reverse primer	1µL
dNTP mix	1µL
Template DNA	2µL
Taq Polymerase	1µL
Total	20µL

Table 8: Gene specific PCR amplification protocol

Initial Denaturation	Amplification (30 cycles)			Final Extension	Hold
94°C	94°C	60°C	72°C	72°C	4°C
2 minutes	30 sec	45 sec	90 sec	5 minutes	---

CHAPTER 6

RESULTS & DISCUSSIONS

6.1 Screening and Selection of Genome

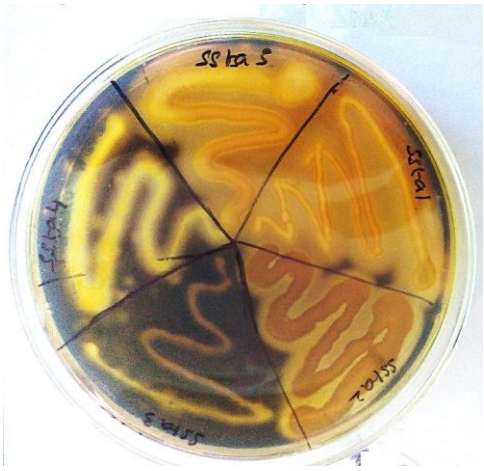

Figure 3: Starch agar plate stained with Iodine Solution

Ssta1, Ssta2 and Ssta5 were showing largest zone of clearance. The larger the zone of clearance, the larger is the amylase activity. Among these three colonies, only Ssta 2 was selected and isolated by choice for further investigations.

6.2 Isolation of metagenomic DNA from soil

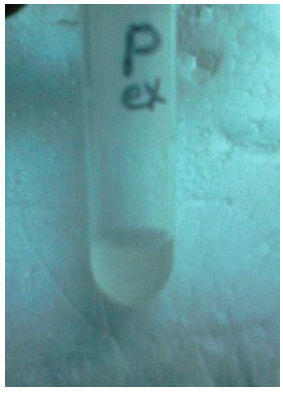

Figure 4: Metagenomic DNA before $CaCl_2$ treatment

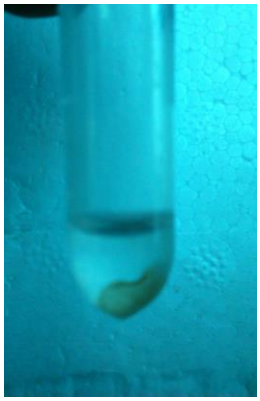

Figure 5: Metagenomic DNA after $CaCl_2$ treatment

These two eppendorffs contain the metagenomic DNA before and after calcium chloride treatment. Before calcium chloride treatment, the sample contains both the DNA and humic contaminations. Because of the high level humic contamination, the sample is turbid and light brownish in color (Figure 4). After the calcium chloride treatment, the humic contaminants bind to calcium ions and get precipitated during centrifugation. Because of the low concentration of humic substances, the supernatant is clear and colorless (Figure 5). This observation shows that the humic contaminations in the metagenomic DNA are more effectively removed by calcium chloride treatment.

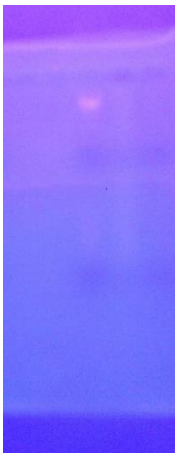

Figure 6: 0.8% Agarose gel containing metagenomic DNA

Soil metagenomic DNA was isolated and examined by electrophoresis on a 0.8% Agarose gel. Lane 3 in the gel contains the metagenomic DNA isolated from the soil after the removal of humic contaminations by calcium chloride treatment.

DNA purity was determined by spectrophotometric analysis. The A260/230 and A260/280 ratios shows the range of humic and protein contaminations. The results were tabulated in Table 9. The results showed that the yield of DNA was high in the first extraction but it has the low DNA purity. In the second extraction, DNA purity was high and better but the yield of DNA was less.

Table 9: Spectrophotometric Analysis of DNA purity

Extracts	**DNA purities**		**DNA yields (µg/mL)**
	A260/230 Ratio	**A260/280 Ratio**	
I	0.95	1.18	2.078
II	1.56	1.78	0.627

6.3 Isolation of genomic DNA

The colony (Ssta2) was cultured in Luria broth and the genomic DNA was effectively isolated from the culture. Lane 1 has the genomic DNA isolated from the colony Ssta2.

Figure 7: 0.8% Agarose gel containing genomic DNA isolated from Ssta2

DNA was quantified by spectrophotometric analysis. The A260/280 ratio indicates the protein contamination. The yield of genomic DNA was 2.058µg/mL.

6.4 Physico-chemical Characterization

The enzyme samples (S and cfu) were analyzed amylase activity by DNS assay. Amylase activity was observed in both the samples. This indicates that the amylase produced, by Ssta1, was an exoenzyme. Thus the supernatant (S) sample was used for further investigations.

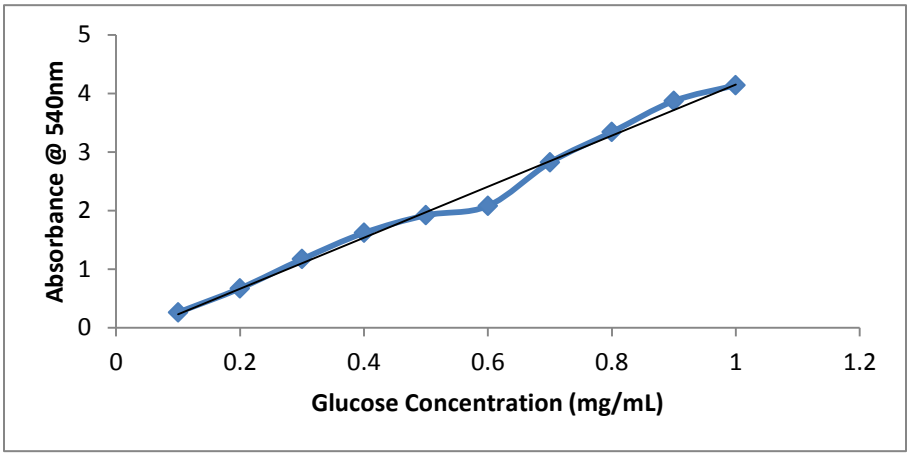

Figure 8: Glucose standard graph

Enzyme activity was analyzed against different temperatures, pH, metal ions and inhibitors by DNS assay. Glucose standard graph (Figure 8) was used to determine the enzyme activity.

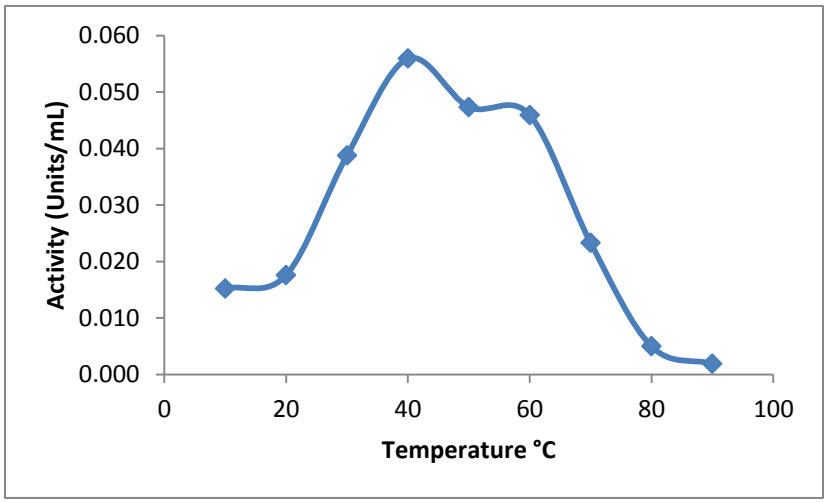

Figure 9: Effect of temperature on amylase activity

The optimum temperature for the amylase activity was analyzed using DNS assay. Figure 9 shows the amylase activity at different temperatures using the buffer solution of pH 7.0. The optimum temperature for maximum activity of amylase produced by Ssta2 colony was 40°C.

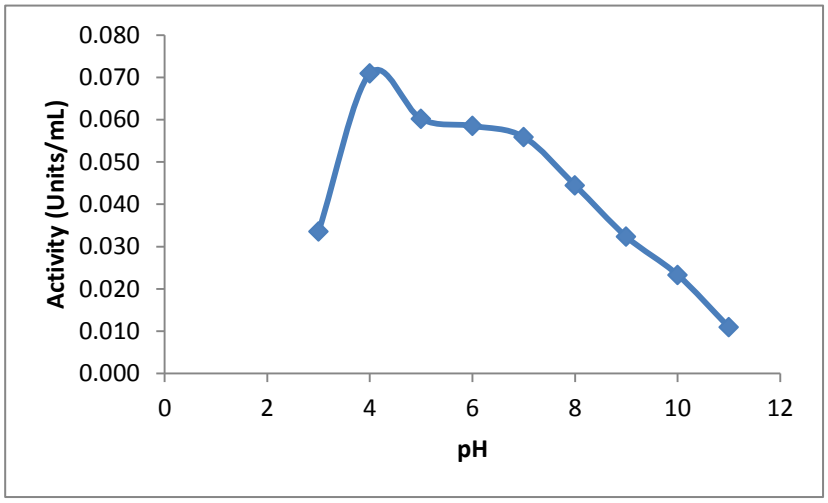

Figure 10: Effect of pH on amylase activity

The optimum pH for the amylase activity was analyzed using DNS assay. Figure 10 shows the amylase activity at 40°C using the different buffer solutions. The optimum pH for maximum activity of amylase produced by Ssta2 colony was pH 4.

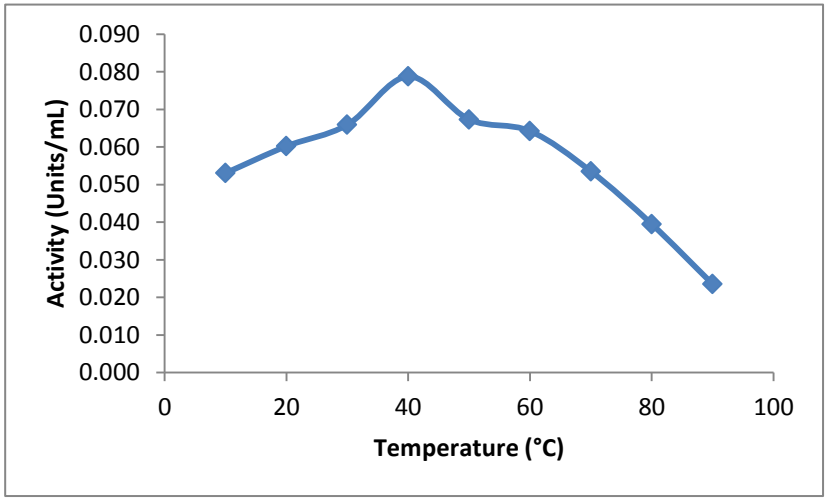

Figure 11: Effect of temperature on amylase stability

Stability of the enzyme activity of amylase produced by Ssta2 colony at different temperature was analyzed using DNS assay. Figure 11 shows the stability of the enzyme activity at different temperatures at its optimum pH 4. The activity of amylase was more stable at 40°C.

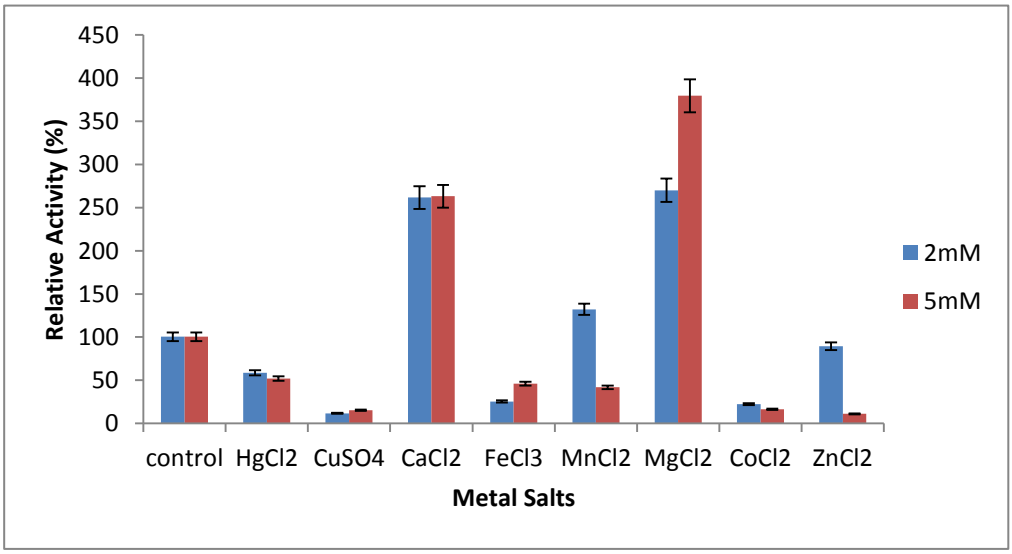

Figure 12: Effect of Metal salts on amylase activity

Influence of metal ions on the enzyme activity of amylase produced by Ssta2 colony was analyzed using DNS assay with the control in which there was no metal salts added. Figure 12 shows the influence of metal ions on the enzyme activity at its optimum temperature and optimum pH.

The results showed that $HgCl_2$, $CuSO_4$, $FeCl_3$, $CoCl_2$ decreased the enzyme activity at the same rate irrespective of their concentration. $MnCl_2$ increased the enzymes activity at 2mM concentration and decreased the activity at 5mM concentration. $ZnCl_2$ does not affect the activity much at 2mM concentration and decreased the activity drastically at 5mM concentration. $CaCl_2$ increased the enzyme activity by 2.5 times (approximately) irrespective of its concentration. $MgCl_2$ increased the enzyme activity effectively with increasing concentration. The relative activities of the enzyme (in percentage) by influence of metal salts were tabulated in Table 10.

Table 10: Effect of Metal salts on amylase activity

Metal Salts	Relative Activity (%)	
	2mM	5mM
$HgCl_2$	59	52
$CuSO_4$	12	15
$CaCl_2$	262	263
$FeCl_3$	25	46
$MnCl_2$	132	42
$MgCl_2$	270	380
$CoCl_2$	22	16
$ZnCl_2$	89	11

Effect of inhibitors on the enzyme activity of amylase produced by Ssta2 colony was analyzed using DNS assay with the control in which there was no inhibitor added. Figure 13 shows the effect of inhibitors on the enzyme activity at its optimum temperature and optimum pH.

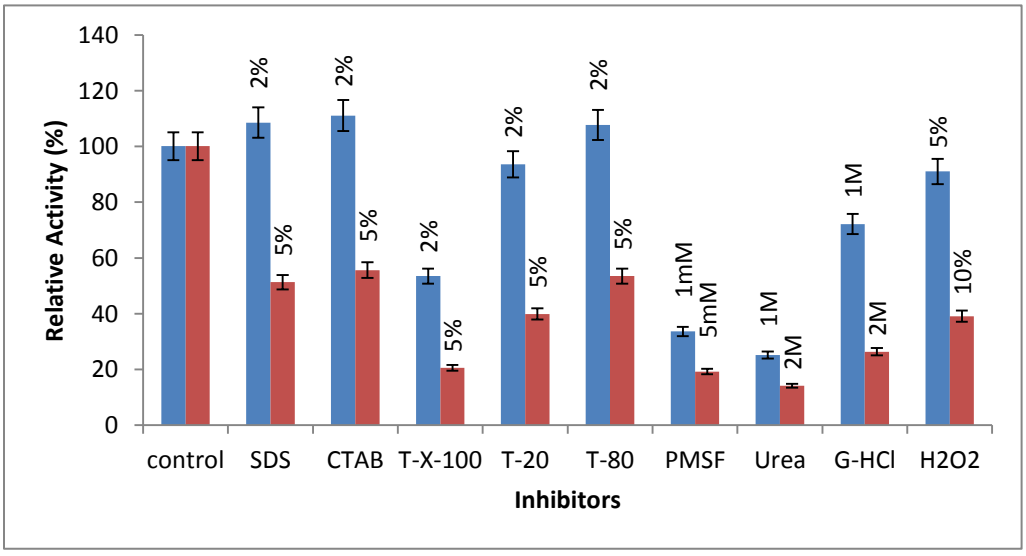

Figure 13: Effect of Inhibitors on amylase activity

The results showed that SDS, CTAB, T-80 increased slightly the enzyme activity at 2% concentration and decreased the activity at 5% concentration. T-20 and H_2O_2 decreased the enzyme activity slightly at 2% and 5% concentrations respectively and decreased the activity at 5% and 10% concentrations respectively. T-X-100, PMSF, Urea and G-HCl decreased the activity drastically with increasing concentrations. The relative activities of the enzyme (in percentage) by influence of inhibitors were tabulated in Table 11.

Table 11: Effect of Inhibitors on amylase activity

Inhibitors	Relative Activity (%)	
	2%	5%
SDS	109	51
CTAB	111	56
T-X-100	53	23
T-20	94	40
T-80	108	53
	1mM	5mM
PMSF	34	19
	1M	2M
Urea	25	14
G-HCl	72	26
	5%	10%
H_2O_2	91	39

6.5 Genetic Characterization of DNA

The genomic DNA was amplified by PCR using 16S rDNA primers. Figure 14 shows the gel containing the PCR amplified gene product along with 100bp ladder DNA. Lane 1 shows the 100bp ladder DNA and Lane 2 shows the 16S rRNA gene amplified PCR product.

Figure 14: 1% Agarose gel containing 100bp ladder DNA and PCR product

The 16S rRNA gene sequence was sequenced for the PCR amplified genomic DNA and the result gave the 1.427kbp length 16S r DNA nucleotide sequence.

ATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAGCACAGAGA
GCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCC
TGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAA
GACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGAT
TAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGA
GAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTG
TGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGTAAG
GTTAATAACCTCTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGT
GCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCG
TAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACC
TGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATT
CCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGC
GGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAG
GATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGTGCC
CTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACG
GCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAG
CATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACA
GAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCAT
GGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGAGACTGCCAGTG

ATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAG
GGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGACCTCGCGAGAGCA
AGCGGACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCA
TGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCC
CGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTA
GGTAGCTTAACCTTCGGGAGG

The gene specific amplification was done for metagenomic DNA and the amylase gene sequence present in the metagenomic DNA was sequenced. Figure 15 shows the gel containing the PCR amplified gene product along with 1kbp ladder DNA. Lane 2 shows the 1kbp ladder DNA and Lane 3 shows the amylase gene specific amplified PCR product.

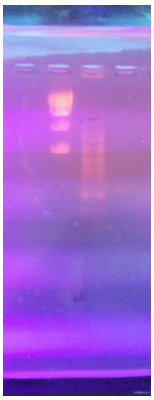

Figure 15: 1% Agarose gel containing 1kbp ladder DNA and PCR product

The result gave the nucleotide sequence of the amylase gene in the metagenomic DNA. The nucleotide sequence was translated into the amylase sequence of 495 amino acids. This sequence was aligned for sequence homology in blastp and the result showed that the enzyme has 96% homology alpha-amylase family.

MKNPTLLQCFHWYYPTVGELWPEVEALAPSLNEIGINMVWLPPAYKGASVGYTVGY
DTYDLFDLGEFDQKGSVATKYGDKAQLLAAINALKEHNIAVLLDVVVNHRMGADEK
EALKVQRVDEQDRTQIDEEIIECEAWTRYTFPVRAGQYSQFVWDYKCFSGIDHIENPTE
DGVFKIVNDYTGEGWNEQVDEELGNFDYLMGANIDFRNHAVTEDIKYWARWVMEQ
TGCDGFRLDAVKHIPAWFYKAWIEHVQEVVPQPLFIVAEYWSHEVEKLQQYIDLVEA
KSMLFDAPLNMKFHEASRQGRDYDMSQIFSGTLVEADPFHGVTLVTNHETQPLQALD
APVEPWFKPLAYALLLLRENGVPSVFYADLFAASYEDTGGDGETYAIEMPVIEQLHELI

DARQRFAHGVQTLWFDHPNCIAFSRTGTDEDPGCVVIMSNGDEAEKTLTLGENYGQK
RWRDFLGNREEIVETDGEGCATFTCNGGSVSVWVLEDVL

CHAPTER 7

CONCLUSION

The genomes for amylase production were selected and isolated from the soil through enrichment method. Among the isolated genomes, one of the best genome, Ssta2, was selected and analysed for amylase production and characterization. Simultaneously, metagenomic DNA was isolated from the soil and the PCR inhibitors (humic contaminations) were effectively removed by calcium chloride treatment. The final yields were approximately 2.08µg/mL in the first extraction and 0.63µg/mL in the second extraction.

Genomic DNA was isolated from the selected genome with high purity and yield. The amylase produced from the Ssta2 colony was characterized for physico-chemical properties. The results showed that the enzyme produced was an exoenzyme and have optimum temperature of 40°C and optimum pH of pH 4. Enzyme activity was more stable at 40°C. Metal salts such as $HgCl_2$, $CuSO_4$, $FeCl_3$, $CoCl_2$ drastically reduced the enzyme activity. $ZnCl_2$ and $MnCl_2$ did not influence the enzyme activity much at 2mM concentration and decreased the enzyme activity at 5mM concentration. $MgCl_2$ and $CaCl_2$ were effective activators for enzyme activity of amylase produced from Ssta2. Detergents like SDS, CTAB, T-80 increased slightly the enzyme activity at 2% concentration and decreased the activity at 5% concentration. T-20 and H_2O_2 decreased the enzyme activity slightly at 2% and 5% concentrations respectively and decreased the activity at 5% and 10% concentrations respectively. T-X-100, PMSF, Urea and G-HCl decreased the activity drastically with increasing concentrations.

Overall results showed that, amylase produced by Ssta2 colony was stable and more active at 40°C in acidic pH 4. $MgCl_2$ and $CaCl_2$ were the effective activators and $CuSO_4$, $CoCl_2$, PMSF and Urea were the most effective inhibitors. The amylase gene sequence in the soil was explored and the 16S rDNA sequence of the Ssta2 sequence was explored.

The studies on active site of the enzyme produced, activator and inhibitor kinetics, Phylogenetic relationship of the explored amylase gene sequence and the 16S rDNA sequence are the future studies to study, in detail, the catalytic nature of the enzyme.

CHAPTER 8

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