“Screening, isolation & optimization for activity of amylase producing bacteria from soil sample”

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INDUSTRIAL BIOTECHNOLOGY

Submitted By

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Certificate

I hereby certify that the dissertation titled "**Screening, isolation & optimization for activity of amylase producing bacteria from soil sample"** submitted by **Garima Mishra**, Roll no. **2K20/IBT/04**, in the partial fulfillment of the requirements for the award of the degree of Master of Technology, Department of Biotechnology, Delhi technological University, Delhi is an authentic record of the work carried out by the student under my guidance. To the best of my knowledge, this work is original and has not been submitted in part or full for any Degree or Diploma to the University or elsewhere.

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CANDIDATE'S DECLARATION

I, **GARIMA MISHRA, 2K20/IBT/04** here by certify that the work which I presented in the major project entitled "**Screening, isolation & optimization for activity of amylase producing bacteria from soil sample"** in the fulfillment of the requirement for the award of the Degree of Master of Technology in Industrial Biotechnology and submitted to the Department of Biotechnology, Delhi Technological University, Delhi is an authentic record of my own, carried out during a period from 15Dec'21 – 26May'22, under the supervision of HOD & Prof. Pravir Kumar and Dr. Rashmi Kataria. The matter presented in the thesis has not been submitted by me for the award of any other degree at this or any other University.

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Abstract

Keywords : Amylase, Bacillus, Enzymes, Soil-bacteria

The amylases from the bacterial sources are the one among them, belongs to the genus bacillus have been studied deliberately for their commercial utilization. The output of the amylase functioning on the starch are known as Dextrins (alpha-1,4 linked glucose dimmers such as Maltose) Alpha-linked glucose trimers (Maltotriose) and branched oligosaccharides of 6-8 glucose subunits that carries both alpha-1,6 and alpha-1,4 linkages. At present, amylase producing bacteria are being isolated and are then characterized for their morphological and biochemical properties. The major objective to isolate & screen the amylase-secreting bacteria from abundant soil samples is to determine the hinderance to pathogenic bacteria. This study has been aimed to extract the various strains of Amylase, their screening and identification. The isolated bacteria are to be expected to produce high amount of amylase, to be identified using 16s RNA sequencing. The isolated bacteria will be gram-positive from different bacterial strains. The specific amylase activity will be tracked to check-out the enzyme kinetics and the results will be shown at the highest activity after the sequential incubation of various strains and thus incubation will be taken for the further analysis. This amylase-producing bacterial strains posses number of industrial applications in fruit, agricultural, medicinal industry.

List of abbreviation

OD : Optical density

Glc : Glucose

B. : Bacillus

AA : Amino acids

SSF : Solid state fermentation

SmF : Sub-merged state fermentation

DNA : Deoxy-ribonucleic acid

Nm : Nanometer

LPS : Lipo polysaccharide

mM : Milli molar

Min. : Minute

gm : Gram

uL : Micro liter

Hrs : Hours

mL : Milli liter

CHAPTER 1 : INTRODUCTION

Starch, after cellulose, a heterogeneous polysaccharide is considered most crucial industrially applicable raw material to be chemically or enzymatically processed into various product including the agrochemicals such as Mulches, pesticides, seed coatings, detergent industry as surfactants, co-builders, bleaching agents and bleaching activators, food industry as viscocity modifier and glazing agents, pharmaceuticals as diluent, binder, filler, disintegrants and drug delivery, in purification as flocculants and in textile industry as sizing, finishing and printing [1]. The glucose molecules connected to one-another through C1 O2 via glycosidic bond are the monomeric units of long chain polymers represents the n- values that varies from 50 to few thousands. The bonds are stable under the alkaline conditions but starch can be broken down when treated with enzymes or acids to glucose subunits [2]. The last component of the oligosaccharide chain contains the latent aldehyde group called as the reducing end group. Similarly, Amylases are the member of class enzymes catalyzes the hydrolysis of starch into its respective monomeric molecules with diversified applications extending from industry to life fate [3].

Amylases (E.C.3.2.1.1-1,4-alpha D-glucanohydrolase) class of enzymes hydrolyses the starch molecule to produce variety of products including the Dextrins, Maltose, Amino acids [4]. These monomeric units are the glucose molecules bonded to form polymers. The categorization of Amylase to alpha & beta Amylase where alpha-amylase acts on the starch to break them into the small sugar molecules, known as "Saccharification." [5]

In particular, Starch molecules are the carbohydrate made-up of two biomolecules namely amylase and amylopectin. The glc chains are joined to α-1,4 to form amylopectin with 1,6 linked branch points. The major function of amylase is to hydrolyze the linear chain connection between each glucose biomolecule which constituted the starch chain [6]. A linear chain of starch molecule have been considered Amylase where as the branched chain of 10 amylose is called as Amylopectin, particularly the largest molecule with an average DP around 2 million whose MW ranges from 05 and 106 g/mol with a standard polymerization of nearly 1000–10,000 glucose units [7]. The molecular weight of amylopectin is 1000 times higher than of amylose. Primarily, α-D-1,4 linkage in the amylose and straight segments of amylopectin posses α-D-1,6 linkage is found between each 20 to 30 anhydroglucose units [8]. Because the amylases are tolerant to extreme physical conditions, the micro biota of genus Bacillus build bio-factories with excellent functioning for the making of hydrolytic enzymes. The global market for enzymes corresponds around 50-60% of the amylase produced from the bacillus bacterial strain (Schallmey et al., 2004; Kiran et al., 2015; Dash et al., 2015; Ghazala et al., 2016; Ma et al., 2016) [9]. The various other micro-organisms are well-known to produce certain other enzymes like alkaline protease and amylase but the preference for the bacillus strain is expectedly high because of extravagant capacity to produce huge amount of functional enzymes [10]. The significant importance of these enzymes in the recent biotechnological applications varies from food industry, fermentation cultures, textile and paper industry, starch and syrup industry, detergent industry (Pandey et al 2000).

CHAPTER 2 : LITERATURE REVIEW

Amylase were the first enzymes to discovered and isolated (Payen 1833) [11]. The various sources of Amylase extraction includes plants, Pancreas and salivary glands of all animals, Small intestinal mucosa of many species and certain microbial sources. They are produced highly in few microbial strains including *A. Niger, B. Subtilis, B. Amlyloliquefaciens, B. Licheniformis, B. Stearothermophilus.* However, with the advancements of recent biotechnological approaches, the Amylase applications have been diversified to various functions and roles in medical biology, analytical chemistry, Textile industry, Breweries, distillaries as well as their crucial function in “Saccharification process” [12].

2.1. Starch Hydrolysis :

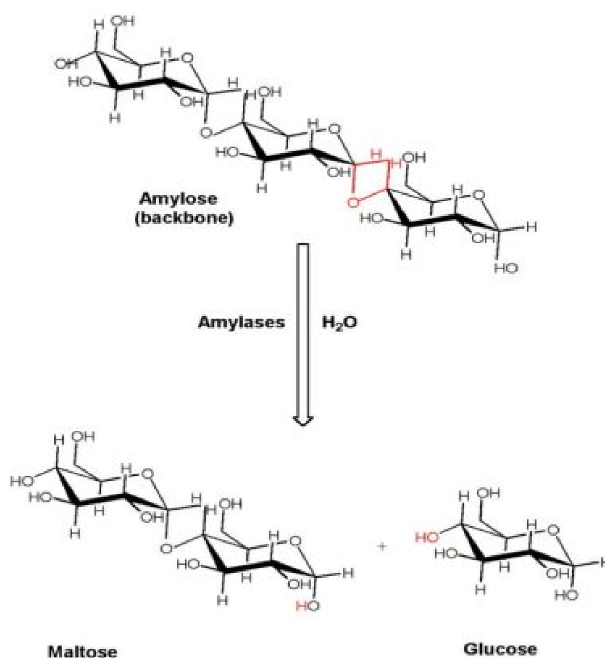


Figure 1 : Figure showing the enzymatic hydrolysis of starch using amylase enzyme.

2.2. Bacterial amylases :

Since the microorganisms have promising applications in paper, food, chemical, fermentation, paper, pharmaceuticals and textile industries, certain bacterial *Bacillus* species mostly *B. amyloliquefaciens* and *B. licheniformis, B. stearotermophilus, B.cereus* are widely used as a source for the enzyme production and other commercial purposes. The captivating advantage of the microbes is that the advanced genetic engineering techniques made it super easy to manipulate the organisms to desired traits like thermostability [13]. The microbes can be tailored to overcome the industrial needs by strain improvement, mutations and the other genetic changes

can be optimized accordingly. As the data has been set, the overall amylase production through bacterial strains is quite higher, cheaper and faster [14].

Advantages :

1 : As mentioned above, Bacterial production of amylase using soil-sample is more suitable for the formation of recombinant enzymes as a byproduct.

2 : Cheaper and faster production

3 : Ths isolation, refining and advancement of highly specific strains will floor-up an idea to screen-out and characterize the essential strains.

Like Dash et al. isolated a novel strain of *B. subtilis* BI19 producing amylase productively, optimized the growth parameters, increased the enzyme production rate around 3.06 folds. 3-D confirmational analysis of these amylases aims to improve their productivity [15].

One crucial exemplification is the crystalline shape of α-amylase isolated from *Anoxybacillus* has given a perception to this enzyme category that the studies on the 3-D shape can help into the alteration, advancement or genetically engineering of specific AA to upgrade the productivity, functioning and activity of any enzyme and protein [16].

Amylase producing bacterial strains :

(a) *Bacillus* species (*B. subtilis*, *B. stearothermophilus*, *B. amyloliquefaciens*, *B. licheniformis*, *B. coagulans*, *B. polymyxa*, *B. mesentericus*, *B. vulgaris*, *B. megaterium*, *B. cereus*, *B. halodurans*, and *Bacillus* sp. Ferdowsicous).

(b) Halophilic strains includes *Haloarcula hispanica*, *Halobacillus* sp., *Chromohalobacter* sp., *Bacillus dipsosauri*, and *Halomonas meridiana*.

(c) *Corynebacterium gigantea*, *Rhodothermus marinus*, *Lactobacillus fermentum* , *Chromohalobacter* sp., *Caldimonas taiwanensis*, *Lactobacillus manihotivorans*, *Geobacillus thermoleovorans*, and *P. stutzeri.*

2.3. Table 1 - Amylase producing fungal strains :

Fungal strain	Function	Reference
A.terreus	Plays a role in the optimization of coding sequence for a particular protein using distinct alignment of alternative codons	17
S.cerevisiae	Basically, targets into the codon optimised activity	18

A.niger	Acts as co-factor/modulator, with the slight up-down of pH during fermentation medium, the metabolic activity of the microbes influences the consumption of N2 compounds and impacting the bio production of amylase.	19
C. guilliermondii	The free Ca2+ and Mg2+ ions regulates the exocytosis of eukaryotic proteins via the regulated secretion pathways. The transition of these metals intermingles with the surface charge of enzyme and affects the ionization of AA leading to the alteration of the structural confirmations of enzyme and renders it as unstable. Hence, biosynthesis of amylase occurs.	20
A.fumigatus	synthesizes the catabolically-inducible enzymes in the presence of their amylase substrate	21
A.awamori	Boosts up the growth efficiency and reduces lipid peroxidation	22
Malbranchea cinnamomea	It causes catabolic repression functions to alter the gene expression for the synthesis of α -amylase leading to high production.	22
A.tubingensis	Functions to encode the raw starch genes	22

Currently, A significant no. of Amylases are available commercially for functioning important role as starch hydrolysis in starch processing industry (Bernfeld 1955). Since, the amylase is found having quick reduction in size of the starch polymer, results to the oligosaccharides present in the water possessing the short retain adhesive capability [13]. Various experiments are been conducted to increase the biosynthesis and stability of Amylase enzyme. Especially, there are certain genotypic methods that are employed to acquire all the genetic information associated with bacterial strains of amylase producing enzymes of interest. Methods includes the PCR- premised on elements that are repeated out of which the ERIC and BOX and REP-PCR are available methods which allows detailed Bacillus genus genetic diversity analysis (Kumar et al., 2014; García et al., 2015) [23].

Glucoamylase are subjected to catalyse the reverse reaction that combines the dextrose molecules to give isomaltose and maltose. Likewise, α-amylase and glucoamylase are meant to sequence and saccharify sago starch, thus producing isomaltose. Although, a blend of pullulanase and glucoamylase while saccharification of sago starch produces glucose only and not the isomaltose [24]. The reverse reaction of dextrose causes the condensation of β-anomer of

D-glucopyranose either with the α- or a β-D-glucose molecule in the presence of glucoamylase, as mentioned below :

$$D\text{-glucose}+\beta\text{-}D\text{-glucose}\rightarrow\text{Dissacharide}+\text{H2O}.$$

Amylase have a strong historic background, first isolated & extracted in 1833 was discovered from an starch degrading enzyme by kirchohoff [16]. To a greater extent, Ohlsson explained the categorization of starch breaking enzymes in maltose are α-amylase & β-amylase on the basis of anomers formed from the enzymatic reaction (Gupta et al 2003).

2.4. Function of Amylase :

Amylase enzyme functions in digestion, break downs the carbohydrate into smaller monomers. The mechanism comprises of catalyzed substrate break down via double replacement mechanism that forms a covalent glycosyl-enzyme intermediate and is degraded through oxocarbenium ion as similar to transition state [25]. The carboxylic acids present at the active site plays the role of catalytic nucleophile throughout the production of intermediate. The second carboxylic acid functions either as the acid or base catalyst sticks around the equilibrium of transition state throughout the hydrolysis [26].

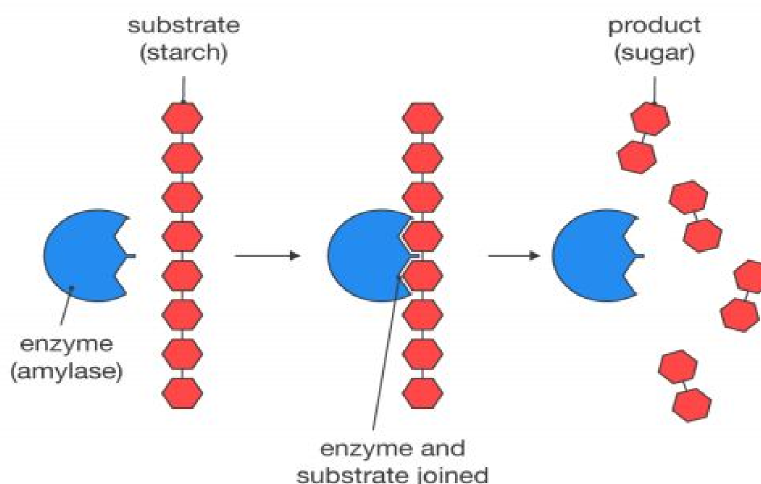


Figure 2 : Diagrammatic representation of function of Amylase enzyme

2.5. Structure of Amylase :

The composition of amylase protein made-up of three domains termed as A, B and C starts from the first amino terminal. The active sites are present at A-domain where as Calcium ions binds to the B-domain for the catalysis and stabilization at active sites. The C-domain consists of a globular domain whose action is still unspecified. Amylases are allosterically activated by chloride ions to modulate the pH and the maximum activity [27]. The active center of amylase have 5 branched sites to connect with the other glucose units in the substrate. Several studies

havebeen conducted for the broad recognition of the enzymatic mechanism of the glycosidases. The kinetic evidences supports the additional carbohydrate binding sites. The surface binding sites among them help the amylase to bind the starch granules. Since the structure of the starch determines the rate of starch digestion that could be a rapid digestion, slow or resistant. Various studies on beans and the structure of the enzyme-inhibitor have shown the enzymatic mechanism of the large family of α-amylase inhibitory proteins [28]. The more vigorous inhibitors have been derived from the pseudooligosaccharides among which the pseudotetrasaccharide, acarbose is important for clinical use.

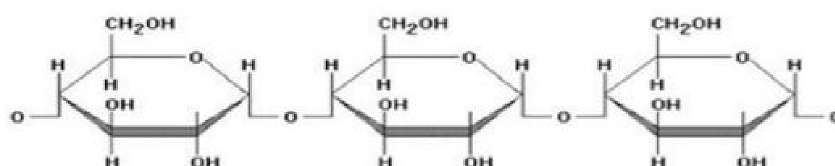


Figure 3 : Diagrammatic representation of structure of Amylase

Table 2 : Types of Amylase -

Basically, they are categorized into 4 groups –

S. No.	Enzyme	Function	Example
1	Endoamylases	Cleaves inner alpha-1, 4 bonds forms a-anomericby- products	α -amylase [29]
	Exoamylases	degrades alpha-1, 4 or alpha -1, 6 bonds of the outermost glucose units forms the alpha or beta anomeric by-products.	*β*-amylase or glucoamylase [29]

3	Debranching enzymes	Transfers the as whole from the branch to the main chain, then it will use its α1->6 glucosidase activity to hydrolyze the glucose from glycogen (glycosidic bonds) in pullulan and amylopectin for glycogen phosphorylase.	Pullalanase, Isoamylase [30]
4	Transferases	degrades α-1,4 glycosidic bond from donor and shifts a part of donor into glycosidic acceptor and produces another α-1,4 & α-1,6 glycosidic bond.	Amylomaltase, Cyclodextrin, Glycosyltransferase, Glucan branching enzyme [31]

Saccharification :

Saccharification, a depolymerization process of partial hydrolysis of amylose and amylopectin removing out the monomeric glucose subunits in a stepwise route from the non-reducing ends. The factors influencing rate of hydrolysis are the proportion of the compounds, the arrangement pattern of α-1,4 and α-1,6 bonds and linkages. Glucoamylase degrades the α-1,4 bonds so quick compare to α-1,6 links [32].

Example : The degradation rate of 1,4-α, 1,6-α and 1,3-α-bonds in tetrasaccharides lies in the ratio of 300 : 6 : 1

Amylase are extremely crucial in the regulatory mechanism of biogeochemical cycle of carbon possessing numerous applications in the biotechnological based paper & pulp, food, detergent, starch processing, leather, textile and pharmaceutical industries [33]. Because amylase are able to degrade the glycosidic linkage in oligosaccharides effortlessly, their extraction, characterization and commercialization is taking a speedy move in the industries. Human sources of amylase are salivary amylase where it initiates with the ingestion of the carbohydrates to degrade the compunds into small monomeric starch units. The hydrolyzed food tastes slightly sweet because the starch is converted into sugar [18]. The α Amylase secreted from the pancreas functions to break down the dietary starch into disaccharides and trisaccharides and the

transformed to glucose by certain other enzymes to release ATP via metabolic pathways [19]. Recently, A significant number of new enzymes belong to the amylase family associated with the hydrolysis of starch and other oligosaccharides havebeen studied well and find-out applicable commercially.

2.9. Table 3 - Timeline of industrial production of amylase :

Year	Industrial production
1811	Discovery of amylase by Kirchoff [34]
1833	Isolation of diastase (an amylase) by Anselme Payen and Jean-Francois Persoz [35]
1894	Industrial production of amylase from fungus [27]
1917	Boidin and Effront worked on *Bacillus subtilis* and *B. mesentericus* in SMF [36]
1925	Nomenclature of alpha-amylase by Kuhn [36]
1930	Discovery of another amylase which degrades beta-mannose by Ohlsson [36]
1959	Industrial and commercialized processing of dextrose powder and crystals using starch and alpha-amylase [18]
1990	Determination of 3-D structure of amylase [21]

2.9. Classification of Amylase :

1 : Endoamylase : Endoamylases, the sub-category of endoglycosidase are the class of amylase that hydrolyses the non-terminal α-1,4 glycosidic bond. Bacterial strains are ***B.lichiniformis, B. subtilis, B. amyloliquifaciens.***

Example : α Amylases

α Amylases : α Amylases **(EC 3.2.1.1) (CAS 9014-71-5)** (alternative names: 1,4-α-D-glucan glucanohydrolase; glycogenase) functions as starch hydrolysizing enzymes consists of highly conserved amino acid sequences. Amylases of the strain ***A. hydrophila* weighs 48-49 kDa, among which the largest found is around 70kDa named *A. hydrophila* JMP636** [9].

2 : Exoenzymes : Exoenzymes or the exoglucanases functions to cleave the cellulose chain at the reducing and the non-reducing ends to release the cellobiose or glucose. The Clostridium species have been found producing the botulinum exoenzymes which are not known as the toxins exactly because at certain temperatures it causes catastrophic effects to the cell function. Furthermore, The exoenzymes do not contain the binding domains associated with the cell [37]. As a result, they don't show the effects until added to a ruptured cell or inserted in-vitro to the

cellular cytoplasm. Once they enter inside the cell, Cells can show changes in the morphological functionality due to the alteration in the regulatory proteins. They have been found functioning mono(ADP-ribosyl)transferase activity similar to binary toxin.

β-amylase (EC 3.2.1.2) : *Β*-amylases **(EC 3.2.1.2),** arguably are the most important exoenzymes, break-downs the second α-1,4 glycosidic bond in the starch, acting from the non-reducing end and produces the disaccharide maltose [17]. *Β*-amylases can alone act and degrade the amylose to the maltose completely. The mechanism, however, includes the attack to the α-(1 → 6) linkages or α-(1 → 4) linkages close to the α-(1 → 6) links. Hence, it hydrolyzes the outermost unit of the amylopectin and drops a large portion (β-limit dextrins) where the outermost units are hydrolyzed to the monomers of either 2 or 3 glucose units next to α-(1 → 6) linkages [38].

The biosynthesis of *Β*-amylases doesnot occur throughout the germination but present in the *Β*-amylases as the starch endosperm. The 4 main isozymes occurs unbound or protein-bonded structures. The high amount of *Β*-amylases successively increases during malting as all the enzymes are released-out from the proteolytic enzymes [39]. Their activity is crucial during the initial stages of brewing to produce enough maltose, the essential fermentable sugar. The high amount of *β* -amylases are extracted from the seedlings in an inactive form prior to germination. Basically, *Β*-amylases needs the chain of atleast four glycosyl residues leading to the terminal degradation of the straight glucan units including the maltose (4-O-*α*-D-glucopyranosyl-*β*-D-glucose) and minor formation of maltotriose that is too less to be degraded by *β*-amylases. The breakdown of the subunits of polysaccharides requires maltose and isomaltose as the enzyme cannot pass-on *α*,1-6 linkages [19].

The major amount of maltose is extracted not only from malt but other important cereals too but *β*-amylase are extremely sensitive to temperature during brewing and rapid inactivation of *β*-amylase will result in the production of less maltose compared to other sugars and high amount of oligosaccharides.

3 : Direct-acting debranching enzymes : Direct-acting debranching enzymes are found in plants and bacteria are subjected to break-down *α*-1, 6-glucosidic bonds of unchanged substrate and categorized into **R-enzymes, pullulanases and isoamylases** depending upon the substrate specificity [23].

Isoamylase (EC 3.2.1.68) or (glycogen 6-glucanohydrolase) : Isoamylase, also called as glycogen 6-α-D-glucanohydrolase functions as the catalysis of the chemical reaction hydrolysis of alpha-D-glucosidic branch linkage α-(1 → 6) among the glycogen, amylopectin and their beta-limit dextrins, often hydrolysis amylopectin. Initial named as Amylosynthetase, (1 → 6) branches are called for its hydrolytic action [40]. There havebeen eight debranching enzymes screened-out and isolated from the human pancreatin purposely results in the split of starch into the fragments of glucose to maltodecaose, product outcomes depends upon the characteristics of the substrate hydrolyzed.

Pullulanase (EC 3.2.1.41) : Pullulanase, an α-(1 → 6) α-d-glucanohydrolase is a linear molecule breaks the α-(1 → 6) bonds among amylopectin and glycogen. The Bacillus acidopullulyticus, Klebsiella planticola, Bacillus deramificans, Bacillus cereus FDTA-13, thermophilic Bacillus sp.

AN-7 and Geobacillus stearothermophilus derived Pullulan 6-glucanohydrolase limits dextrinase and amylopectin 6-glucanohydrolase **(Table 4)** [41].

Enzyme	**EC number**	**Bond present**	**Substrate preference**	**Final products**	**Reference s**
Pullulanase type I	3.2.1.41	α-(1,6)	Oligo- and polysaccharides, Pullulan	Trimer (maltotriose)	42
Pullulanase type II (amylopullulanase)	3.2.1.41	α-(1,6) α-(1,4)	Pullulan, Poly- and oligosaccharide (starch)	(a) Trimer (maltotriose), composition of glucose (b) maltotriose and maltose	27
Pullulan hydrolase type I (neopullulanase)	3.2.1.135	α-(1,4) glucosidic bond	Pullulan	Panose	43
Pullulan hydrolase type II (isopullulanase)	3.2.1.57	α-(1,4) glucosidic bond	Pullulan	Isopanose	43
Pullulan hydrolase type III	3.2.1.—	α-(1,4) glucosidic bond and α-(1,6) glucosidic bond	Pullulan, amylose, starch and amylopectin	(a) Composition of panose, maltotriose, and maltose (b) Maltose and maltotriose	29

Advantages of pullalanase in starch saccharification process :

(a) The industrially essential starch contains 80% amylopectin where the branch points occurs on every 20-25 D-glucose molecules. Hence, amylopectin contains 4% to 5% of α-1,6 glycosidic linkages [44].

(b) During saccharification, Initially, starch is gelatinised and solubilised at high temperatures (Above 50 degree) and the large subunits are broken down into the linear or branched monomers (maltodextrins).

4 : Transferases :

Transferases plays role in the transfer of the functional group from the donor to accepter. Particularly, in the bacterial transferases, they transfer the sugar moieties by forming a glycosidic bond (Braton, Snajdrova, Jeanneau, Koca and Imberty, 2006) In biotransformation, the sub-classes of the glucosyl transferases are often used. A huge family of enzymes are implemented in the synthesis of the polysaccharides, oligosaccharides and glycol conjugates. The transfer of sugar molecules to the polyphenol increases the water solubility and stability of flavonoids (Xiao, Cao, Wang, Zhao & Wei 2009).

2.10. Production methods : For the commercial and industrial purposes, Amylases are produced using two methods, namely **(Table 5)** -

1 : Solid-state fermentation

2 : Submerged-state fermentaion

3 : Traditional method for enzyme production

Solid-state fermentation : The SSF seems to have great potential to produce microbiological products such as enzymes, biofuels, food, pharmaceuticals, industrially important chemicals and animal feedstock. For the processing of food waste, the biochemical approaches have better designed bioreactors providing SSF an alternative to produce useful products [45].

Submerged-state fermentation : The SSF transforms the substrates molecules of microbes into desired biological compounds, fermented products such as alcohol and CO2. It gives high technology observatory samples and easy handling which allows enzyme production on large quantity, mainly occurs in the presence of excess water.

Characterstics	SSF	SMF
Definition	Media as raw materials : Feedstuff Functional ingredients : Growth factors High fermentation productivity	SMF is carried out by microbes grown in the liquid media [34].
Substrate	Soluble sugar such as wheat bran, cane sugar, rice bran, beet sugar, high fructose corn syrup and sugarcane bagasse	Polymerized insoluble substrate such as Xylan, Pectin, Mannan [34]
Scale-up	Industrially important equipments are available	New designed reactors are needed [28]

Contamination	High chances of single strain bacteria to be contaminated	Fungi has comparatively low chances of getting contaminated [18]
Metabolic heating	Temperature control is easy	Heat transfer is comparatively low [18]
pH control	Easy	Substrate buffers are required [22]
Aseptic parameters and control	Heat sterilization or autoclave	Vapor treatment, non-sterile conditions [27]
Agitation	Continuous agitation (mixing) for aeration and mixing	Essential
Microbial cells	DMF (Probiotics), high density microbial cells	A homogenized solution of microbes throughout the media [27]
Water content	40-80%	More than 95%

Table 5 : Tabular representation of difference between SSF and SMF.

2.11. Bacterial source and their mode of fermentation (Table 6) :

S. No.	Bacterial source	Production Method	Reference
1	*B.coagulans*	SSF	
2	*B.licheniformis M27*	SSF	
3	*B.mesentericus*	SSF	
4	*Halobacillus sp MA-2*	SmF	
5	*B.polymyxa*	SSF	
6	*B.vulgarus*	SSF	
7	*B.cereus MTCC 1305*	SSF	
8	*Halomonas meridiana*	SmF	

9	*B.licheniformis GCB-U8*	SmF	

- SSF : Solid-state fermentation
- SmF : Submerged-state fermentation

Tabular representation of bacterial sources and their mode of fermentation for industrial purposes.

2.12. Process parameters :

1 : Temperature - The two temperatures optimized for the growth of the bacteria are the temperature throughout the growing bacteria, the accurate temperature for the highest enzyme growth is achieved. The temperature required for the *Bacillus* strains including *B. licheniformis* at and *B. subtilis* is 42°C whereas the formation of amylase lies around 50–55°C for the thermophilic strains. The optimum temperature required for growth of α-amylase is around 45, 46 and 50 °C respectively. In *B.s*, the growth temperature is 42°C which is 5°C greater to amylase production nearly 37°C. Thermophilic archaebacterial α-Amylases are vigorous and growth occurs at high temperatures. *Pyrococcus* enzymes are optimally active around 100°C. They show optimum activity at the angre of 95-100°C for the maximum enzyme activity and production. *Penicillium fellutanum* and *Pyrococcus furiosus* exhibits enzymatic activity of 98±4.6 U/ml around 30° C [48].

2 : pH - pH is considered an important point for the stable enzyme production. Mainly, enzymes are sensitive to pH. Here's why a control over the pH is important during its production cycle. Usually, Amylases show their activity at pH 6.5-7.5. The *Bacillus* strains such as *Bacillus amyloliquefaciens* gives enzyme at the pH around 7.0. *H. meridiana for the* screening and identification of α-Amylase posses highest results at pH 7.0, quite a stable under alkaline conditions. The pH needed for the enzyme production from *Bacillus* sp. Occurs at 6.5 [49].

The bacterial strain for Amylases are found advantageous in the economically feasible bulk production capacity and with the use of advanced genetic engineering techniques, microbes are easy to modify with the desired traits (Lonsane et al 1990). Various medical applications include

1 : Aging support

2 : Digestive health support to prevent oxidative stress

3 : Immune support system

4 : Healthy inflammation management.

2.13. Industrial applications of Amylase :

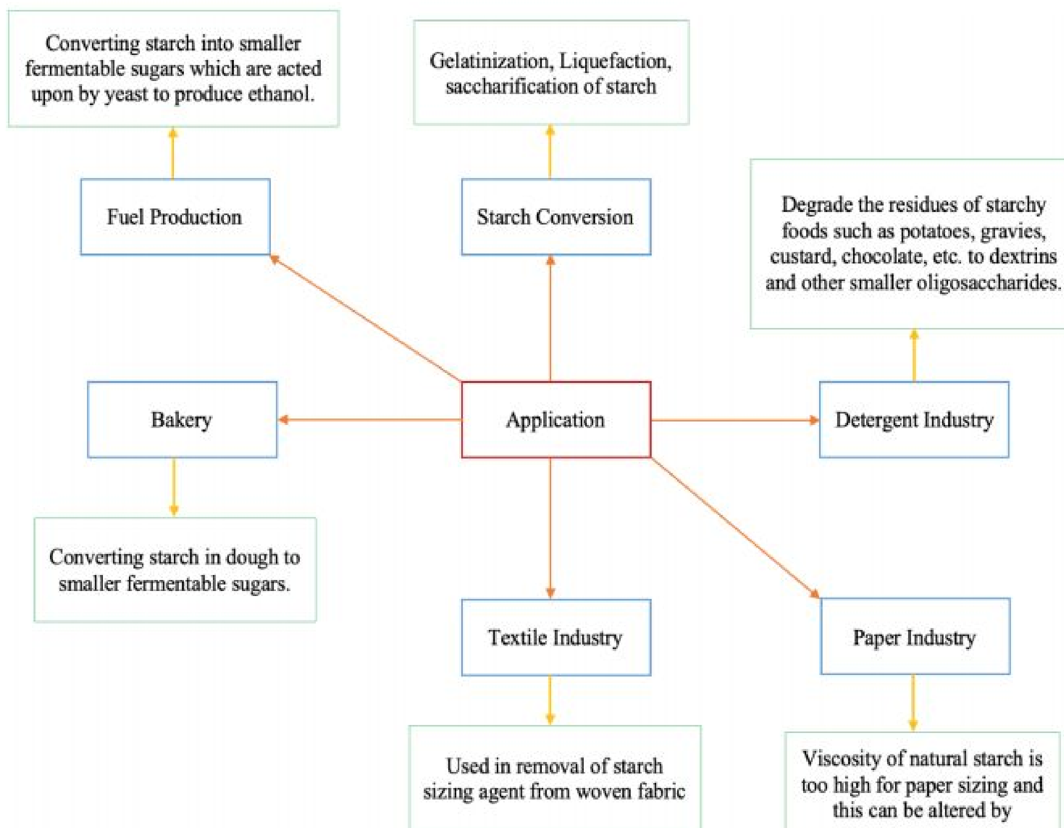


Figure 4 : Flowchart representation of the applications of Amylase

Various other applications of Amylase includes :

1 : Beta-amylase has its application as the end product maltose is required as sweetener in food, pharmaceuticals & fruit industry because of its mild sweetness and lack of color formation.

2 : *β*-Amylases functions in delaying the starch retrogradation. Thus, maintains the quality of bread and prevents it from staling. Additionally, it has various industrial applications with potential in bringing out up to date progress in the biotechnology.

3 : In the textile industry, amylase are used in the weaving. Starch functions for warping. Once the weaving is finished, *B. subtilis* α-amylase is used to remove starch. Dextrins are used for improving the better viscosity, filler and food additives produced from the liquefaction of starch from alpha-amylase [50]

4 : The *B.subtilis*, or *B.licheniformis strains producing* α-amylases are mainly utilized for the liquifaction of starch for the production of high conversion glucose syrup.

5 : In the medical biology, for the treatment of Pancreatitis used to determine the level of amylase in the blood Pancreatitis is diagnosed by regulating the amount of amylases present in blood, gives destructed cells for amylase production and renal failure leads to uneasy excretion.

6 : Alpha-amylase produces Malt, The enzyme production takes place throughout the seed and grains germination. The β-amylase and α-amylase secretes the precursor protein named β/α amylase (BAAM)

7 : For industrial purpose, Isomaltose is used as byproducts to downgrade the general glucose production which are not used for high-fructose syrup as a sweetener.

8 : The productivity of saccharification reaction is enhanced by including some particular amylopectin-debrancing (subunits) enzyme. If these enzymes such as Pullulanase specially degrade the subunits into amylopectin units carrying the hydrolysis.

If a debranching enzyme like pullulanase and glucoamylase are utilised at the same time during saccharification, the pullulanase will hydrolyze the branching in the amylopectin residues, followed by the glucoamylase hydrolysis of linear 1,4-glucosidic bonds. As a result, the highest dextrose concentrations achievable are higher.

9 : The ability to screen novel and better enzymes, their fermentation and purification on a wide scale, and enzyme formulations define the potential industrial applications of enzymes.

For the production of amylase, the crude extract is important for industrial applications such as pharmaceuticals, purification of enzyme is extremely important is done by ion-exchange chromatography, hydrophobic interaction gel filtration, chromatography, polyethylene glycol/Sepharose gel separation, immunoprecipitation, and aqueous two-phase and gradient systems, where the size and charge of the amylase determine the method chosen.

CHAPTER 3 : MATERIALS AND METHODS

3.1. Media used :

All the media and glass wares were sterilized at the temperature 121°C and the pressure at 100kPa (15psi) was provided for 15 minutes in an vertical autoclave. All media were prepared in distilled water according to the manufacturer's instructions.

Table 7 : Luria Bertani Agar, Miller - Starch – Agar media (g/L)

Manufacturer : Sigma – Aldrich, st. Louis, Missouri USA

Ingredients	g/L
Tryptone	10.00
Yeast extract	5.00
Sodium chloride	10.00
Agar	15.00

Final pH at 25°C, 7.5 ± 0.2

Table 8 : Luria Bertani Broth, Miller (g/L)

Ingredients	g/L
Tryptone	10.00
Yeast extract	5.00
Sodium chloride	10.00

Final pH at 25°C, 7.5 ± 0.2

Formula adjusted, standard to suit performance parameters.

3.2. Soil Collection and Isolation of Bacteria. The soil sample have been collected from the different sites near fruit stalls from Delhi Technological University, Delhi. These soil samples have been used to isolate amylase producing bacteria using the serial dilution method. Basically, 1 gm of the soil sample was dissolved in the 9ml of sterilized distilled water in different test tubes, heat shock has been given at 90°C for 15 min followed by cooling to room temperature. Further, The serial diluted culture (10-1 to 10-7) was taken to spread on the nutrient agar plates. Then, incubated at 50°C for 24–48 hours. The bacterial colonies have been found next day named 10^{-2}. , 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} respectively. The bacterial colonies showing clear difference in morphology were inoculated further by sub – culturing and streaking on the nutrient agar (3A, 3B, 3C, 3D, 4A, 4B, 4C, 5A, 5B, 5C, 5D respectively) and then kept on incubation at 50°C for 24–48 hours. The pure isolates were picked-up and transferred to the fresh poured starch agar plates and incubated at 50°C for 48 hrs. The bacterial colonies were at at 4°C for the further experiments and studies.

Figure 5 : Diagrammatic representation of bacterial isolates from the serial dilution spread on starch-agar plate

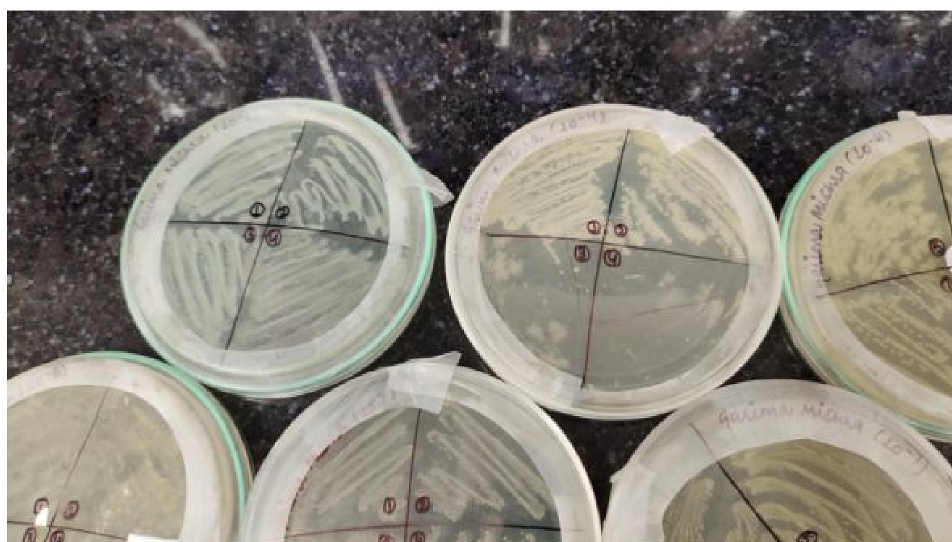


Figure 6 : Picture showing the further streaking of the isolated bacterial colonies.

3.3. Screening and Selecting Amylase-Producing Bacteria

The isolated and purified bacteria grown on the starch – agar plates were taken further for the amylase production. The isolated colonies were sub-cultured in the fresh poured starch – agar plates and incubated at 50°C for 2 days. After incubation, the plates were flooded with 1% iodine solution **(Gram's iodine: 250 mg iodine crystals added to 2.5 gm potassium iodide solution and 125 mL of water, stored at room temperature).** Further, the plates were kept undisturbed

for 5-10 min and the iodine was discarded via decanting from all the plates. The formation of the zone of clearance was observed and the diameters of the clear zones of the isolates were measured using the ruler as the Halo : Colony ratio. 10^{-4} and 10^{-8} were showing the maximum zone of clearance.

Then, the highest zone of clearance isolates were inoculated in the starch agar media at 50°C for 2 days, flooded the plates again with the two drops of iodine solution and noted-down the time taken to decolorize the iodine solution. 10^{-5}, 10^{-8} and 10^{-4} were showing the quick decolorization.

3.4. Identification and Characterization of Bacterial Isolates

3.4.1. Macroscopic and Microscopic Characterization of Isolates.

The morphological and biochemical properties of the isolates have also been recorded. The colony size, color, shape and texture was determined. Gram staining, spore staining and motility tests were performed following the protocols respectively.

3.4.2. Biochemical Characterization –

For the characterization of the bacterial isolates, certain biochemical tests have been conducted. The catalase and casein hydrolysis tests were recorded and Citrate utilization, urea hydrolysis and growth on anaerobic condition were also noted. Oxidase and triple sugar iron tests have also been performed following the standard protocols.

3.5. Growth curve : The growth profile and the enzyme production activity by the enzyme has also been studied. The liquid culture using the Liquid broth, distilled water, starch mentioned in the table below. The liquid media was inoculated for 16 hours and further the readings were taken for 30 hours. This experiment was performed in the duplicates and 1 blank solution.

Table 9 : Composition of the liquid media

Ingredients	g/L
Soluble starch	10.00
Peptone	10.00
Yeast extract	20.00
KH_2PO_4	0.05
$MnCl_2.4H_2O$	0.015
$MgSO_4.7H_2O$	0.25
$CaCl_2.2H_2O$	0.05
$FeSO_4.7H_2O$	0.01

Optimum pH and temperature were kept constant while performing the experimental work. Growth activity have been analyzed for the 30 hours straight and calculated taking the OD using the spectrophotometer and DNSA assay. They total yield of the amylase was increasing at the

initial stages and started declining after that due to the deficiency of glucose concentration in the media. The growth for the liquefying action showed that the maximum production of enzyme was taking place and found declining after that. It was also reported that the amylase production in the low volumes don't decrease to the extent as in high volumes as 100mL. These reasons have already been explained further **(Graph 1)**.

3.6. Total reducing sugar assay by DNSA method : 3,5-Dinitrosalicylic acid (DNSA, IUPAC – 2-hydroxy-3,5-dinitrobenzoic acid), an aromatic compound reacts with reducing sugars and some other reducing molecules to produce 3-amino-5-nitrosalicylic acid that absorbs light and gives optical range at 540nm (for glucose). This measures the presence of free carbonyl group (C=O) hence, called reducing sugars. This mainly involves the oxidation of aldehyde group present in the glucose and ketone in the fructose. Simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino,5-nitrosalicylic acid under alkaline conditions **(Graph 2).**

Requirements :

1. DNSA
2. Sodium potassium tartarate
3. 2 N sodium hydroxide (2N NaOH)
4. Dinitro salicylic acid (DNSA)
5. Distilled water
6. Sample

Procedure :

- Prepare 20 mL of 2N NaOH.
- Weigh 1 g DNSA and dissolve in 20 mL NaOH with the help of a magnetic stirrer
- Weigh 30 g of sodium potassium tartarate and dissolve in 50 mL dH2O.
- Slowly pour sodium potassium tartarate solution in the DNSA and NaOH solution and made the volume up to 100 mL (Note: Wait for the two to mix properly).
- Decant the contents in a brown bottle. Filter if necessary.

Table 10. Protocol :

- Take eight tubes and label them as Blank and 1 to 7.
- Make dilutions of glucose standards
- Add 3 ml of DNSA reagent to all the eight test tubes. Mix well.
- Keep in boiling water bath for 15 minutes.
- After cooling to room temperature in a cold water bath, record the absorbance with a spectrophotometer at 540nm.
- First, take the absorbance (OD) of Blank and make it zero.
- Take the OD of all the tubes (No. 1-7). Wash the cuvettes each time after taking OD.

S.No.	Glucose stock concentration (1mg/mL)	Glucose working concentration (µg/mL)	Glucose working volume (µL)	Distilled water (µL)
1	1	0	0	1000
2	1	0.25	25	975
3	1	0.5	50	950
4	1	1	100	900
5	1	1.25	125	875
6	1	1.5	150	850
7	1	2	200	800

500µL of each dilution was taken in separate test tubes and mixed with 500µL of 3,5-dinitrosalicylic acid (DNS). The test tubes were then boiled at 100ºC for 10 minutes. Different shades of brown color were developed in different test tubes which were determined spectrophotometrically at 540nm. The glucose concentrations and absorbance values were plotted on the X and Y axis respectively.

3.7. Determination of Amylase enzyme activity :

This follows the DNS method where the bacterial isolates with the maximum colonies (10^{-5}, 10^{-8} and 10^{-4}) were choosen for the specific Amylase enzyme activity. The isolates were sub-cultured in the liquid broth media (g/L) and incubated further at 37°C, 150 rpm for 1,2 and 3 days. The clear supernatant (crude extracellular amylase enzyme extract) was obtained after centrifugation at 10000 rpm for 15 min at 4°C. The crude extract was concentrated using MWCO 10kDa ultracentrifuge protein concentrator.

The DNS method was used to determine the amylase activity of the each bacterial isolate with high zone of clearance. 1 mL of crude enzyme was mixed with 1 mL of 1% starch solution in o.o5 M sodium-phosphate buffer with pH 7.0. The samples were kept on incubation at 37°C for 30 min. After incubation, 0.5 mL of DNS solution was added to each test tube to stop the reaction. Further, boiled at 100°C for 5 min in the water bath. The color intensity of the sample is then observed by measuring the optical density (OD) using the spectrophotometer at 575nm. Then, The reading was compared with the pre-prepared blank solution (with crude enzymes). The OD values of the samples at T_{30min} were deducted from the samples of T_{0min} because there's still some glucose remains in the T_{0min} samples after the enzyme induction process for 1,2,3 days. All these process have been carried out in duplicates. The Glucose concentration produced from

each solution was obtained from the standard curve of glucose. Finally, the glucose activity was calculated.

Table 11. Acetate buffer preparation :

Component	Amount	Concentration
Sodium Acetate (mw : 83.03 g/mol)	7.721 g	0.09413 M
Acetic acid (mw : 60.05 g/mol)	352.5 mg	0.005871 M

1. Prepare 800 mL of distilled water in a suitable container.
2. Add 7.721 g of Sodium Acetate to the solution.
3. Add 352.5 mg of Acetic Acid to the solution.
4. Adjust solution to final desired pH using HCl or NaOH
5. Add distilled water until the volume is 1 L.

DNS preparation : Following are the steps to follow :

Dissolve 1g of3,5 dinitrosalicyclic acid in 20mL 2M NaOH. Then, add 30g sodium potassium tartrate and dilute to a final volume upto 100mL using distilled water.

Note : 1u of the amylase activity is defined as the amount of amylase needed to catalyze the formation of reducing sugar equivalent to 1 µmol of glucose per min under standard assay conditions. The crude enzyme of the bacterial isolate with the maximum activity have kept separately for the further studies.

The specific activity of the selected isolates, the Folin-lowry method was used for total protein estimation. The formula used to measure specific enzyme activity are :

Specific acitivty (U/mg) = Enzyme activity (U/ml) / Extracellular protein concentration (mg/ml)

3.8. Production of Amylase

Before the production of amylase, the inoculum was prepared by transferring a loopful of amylase-producing bacterial cultures in Erlenmeyer flask (250 mL) containing amylase screening broth (starch broth) (50 mL) and incubated at 50°C in an incubator shaker at 250 rpm for 24 hrs. Production of amylase was carried out using soluble starch as the substrate [31]. -e selected isolates were separately cultured at 50°C temperature, pH 7, and 250 rpm for 48 hrs in 100 mL starch broth (1% starch, 0.5% peptone, and 1.5% yeast extract) after inoculated with 4 mL of an overnight bacterial culture. 6 mL of the sample was withdrawn from flasks after 48 and 72 hrs of incubation. -e cell-free supernatant obtained after centrifuging the culture broth at 10,000 rpm for 10 min was used as the crude enzyme source. -e crude extract was used for characterization of the enzyme activity and stability under different conditions.

CHAPTER 4 : RESULTS AND DISCUSSION

4.1 Soil sample site : Here, The soil samples for the bacterial isolation and screening of Amylase-producing bacteria were collected from the soil near to fruit stalls Delhi Technological University, Delhi.

4.2 Serial dilution and the bacterial spread plate :

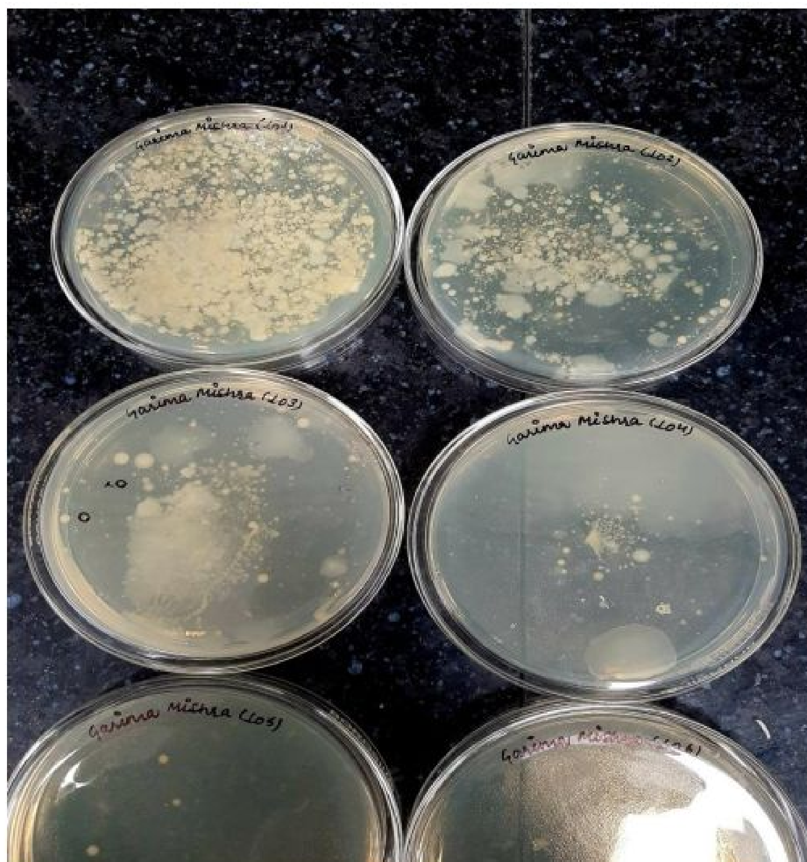


Figure 7 : Starch – Agar poured petriplates with bacterial isolates

4.3 Streaked plates of different dilutions :

Figure 8 : Starch-agar media plated individual colonies of the isolates 10^{-5}, 10^{-8} and 10^{-4}

4.4 Primary screening using gram's Iodine staining : Based on the morphology of each colony, total 42 colonies were isolated. Later, The iodine's gram staining confirmed the presence of Amylase. 10^{-5}, 10^{-8} and 10^{-4} were showing the clear results of zone of clearance around the colonies on the well plate assay, decolorization for the plate assay method. **Table 12** refers the zone of clearance variation among the stained isolates for the starch hydrolysis ranging from 0.5 to 0.8cm.

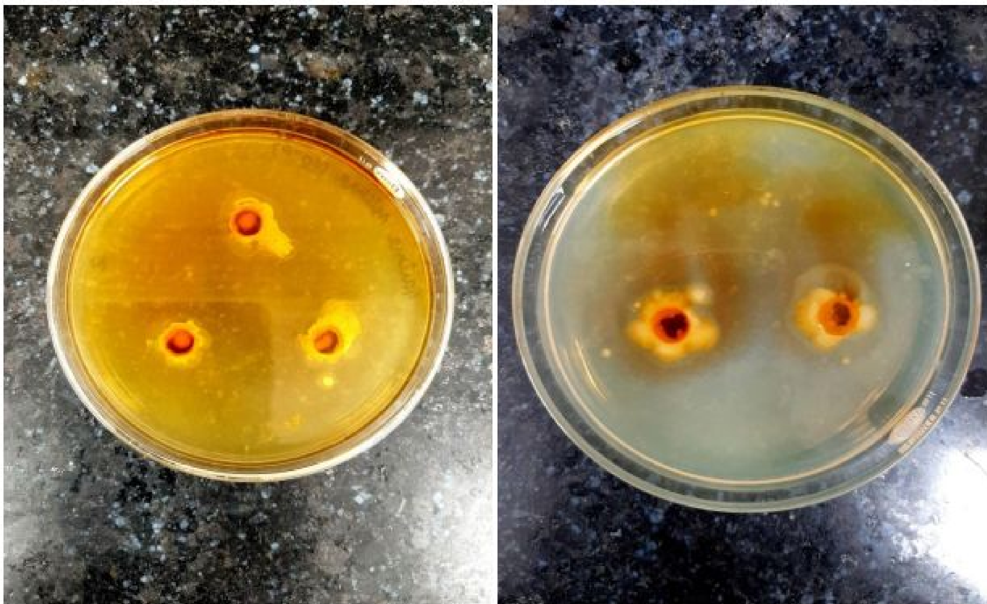


Figure 9 : Gram's iodine stained potential isolates of 10^{-5} and 10^{-8}

Figure 10 : Gram's iodine stained potential isolates of 10^{-5}, 10^{-4} and 10^{-8}

4.4.1. Table 12 - Colony range, diameter and their difference :

Streaked plate	Colony no.	Colony diameter (cm)	Clear zone diameter (cm)	Colony diameter – Clear zone diameter (cm)
10^{-4}	1	0.3	0.5	0.2
10^{-4}	2	0.2	0.3	0.1
10^{-4}	3	0.5	0.7	0.2
10^{-4}	4	0.3	0.5	0.2
10^{-5}	1	0.3	0.6	0.3
10^{-5}	2	0.2	0.4	0.2
10^{-5}	3	0.4	0.6	0.2
10^{-8}	1	0.4	0.8	0.4
10^{-8}	2	0.3	0.5	0.2
10^{-8}	3	0.4	0.7	0.3

4.4.2 Decolorization time using gram's iodine staining (Table) :

Figure 11 : Presentation of the gram's iodine stained 10^{-5}, 10^{-4} and 10^{-8} plate showing decolorization

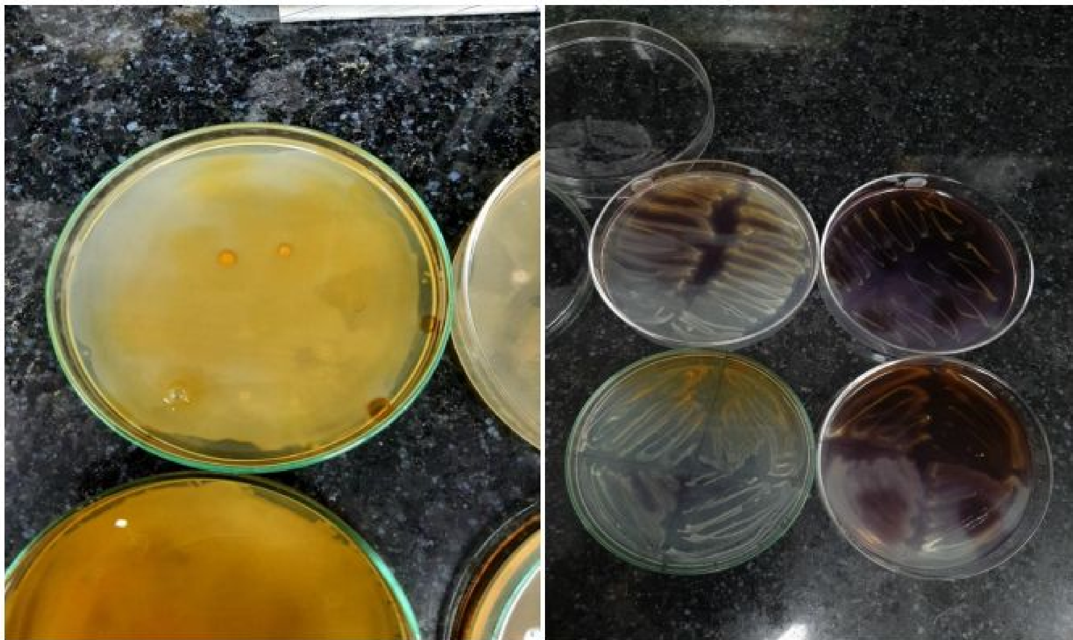


Figure 12 : Starch hydrolysis test on starch-agar plate of 10^{-5}, 10^{-4} and 10^{-8} isolates confirming the presence of Amylase by washing-out the colonies

Table 13 : Washing-out time of the potential isolates 10^{-5}, 10^{-4} and 10^{-8}

Streaked plate	Isolate	Decolorization time (min)
10^{-4}	1	3
10^{-4}	2	1.5
10^{-4}	3	4
10^{-4}	4	3
10^{-5}	1	4
10^{-5}	2	2
10^{-5}	3	4
10^{-8}	1	5
10^{-8}	2	3
10^{-8}	3	4

4.5. Identification and Characterization of Bacterial Isolates

4.5.1. Macroscopic and Microscopic Characterization of Isolates

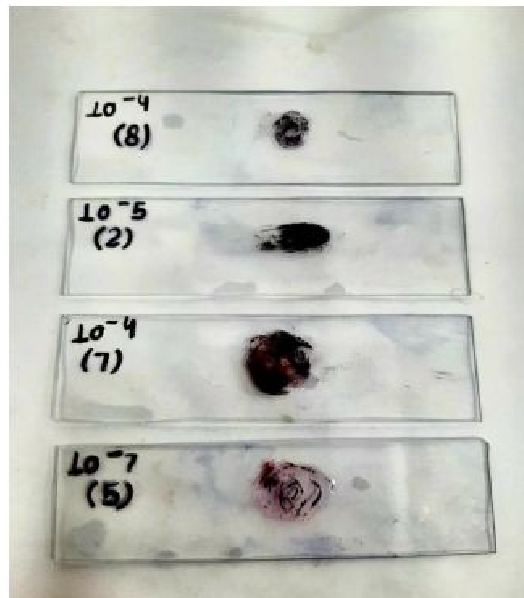


Figure 13 : Gram's iodine staining to study the morphology of the cells

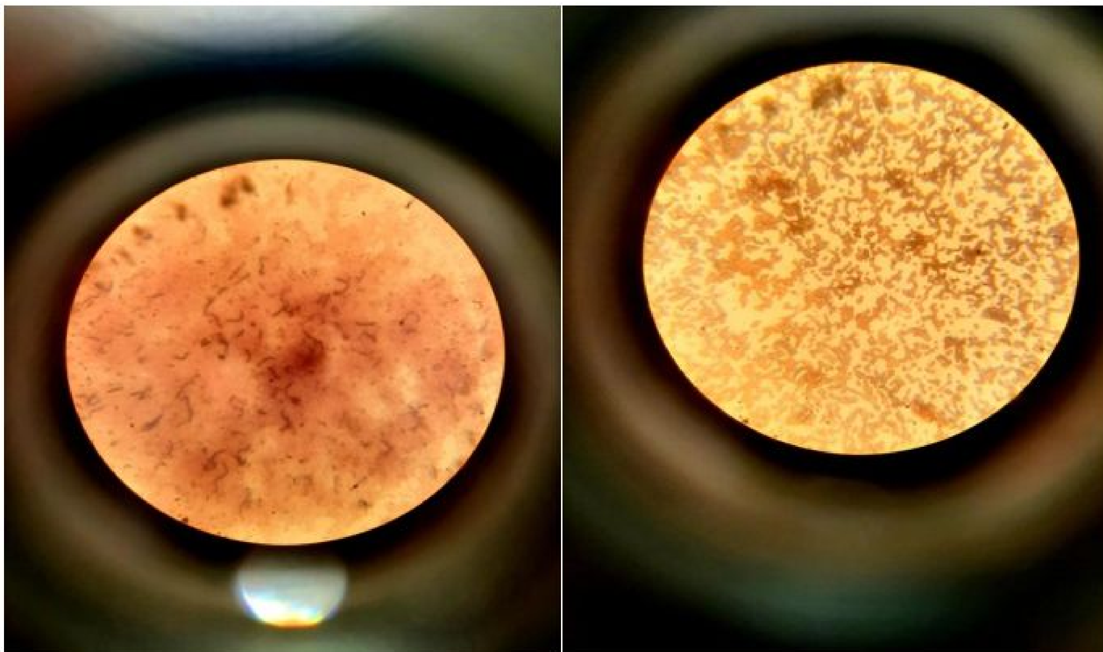


Figure 14 : Gram's iodine microscopic screening of isolate 10^{-7}(5) and 10^{-5} (4) respectively.

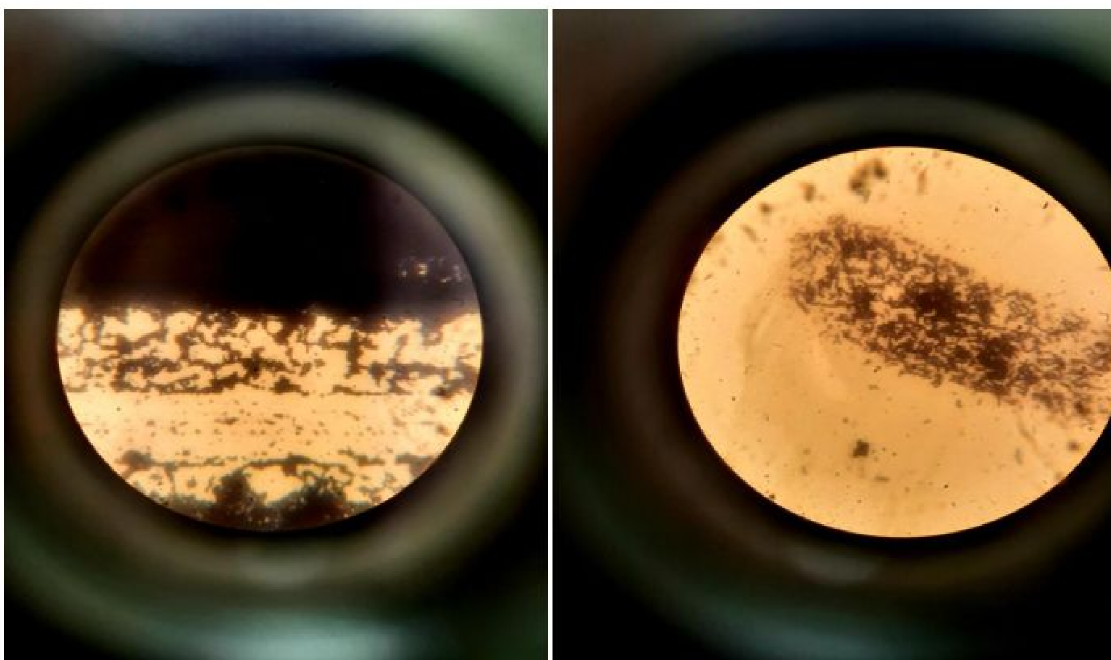
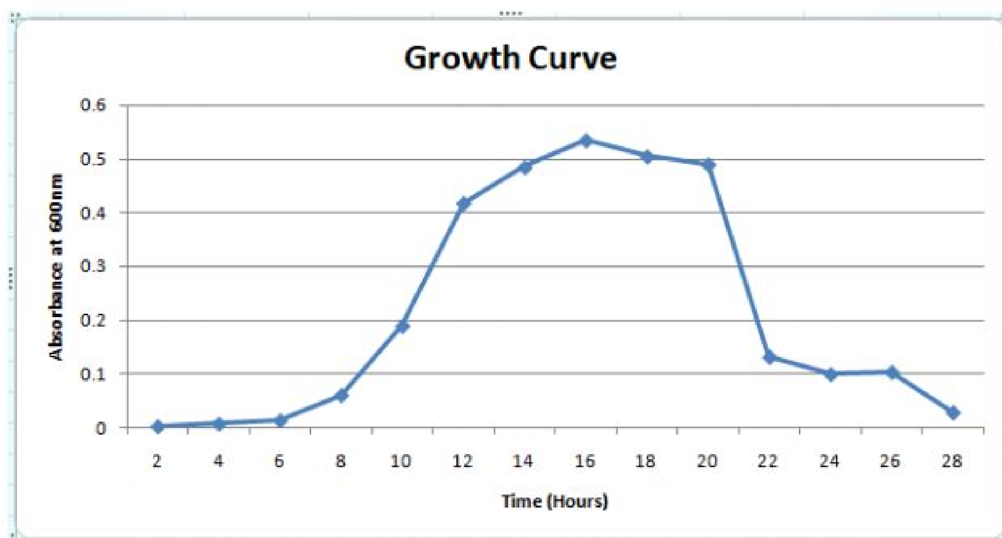


Figure 15 : Gram's iodine microscopic screening of isolate 10^{-5} (2) and 10^{-4} (8) respectively.

4.6 Growth curve of 10^{-8} (1) isolate :

S.No.	Time (Hours)	Absorbance (600nm)
1	0	0.00
2	2	0.008
3	4	0.014
4	6	0.061
5	8	0.190
6	10	0.418
7	12	0.437
8	14	0.495
9	16	0.572
10	18	0.486
11	20	0.365
12	22	0.280
13	24	0.161
14	26	0.104
15	28	0.074
16	30	0.010

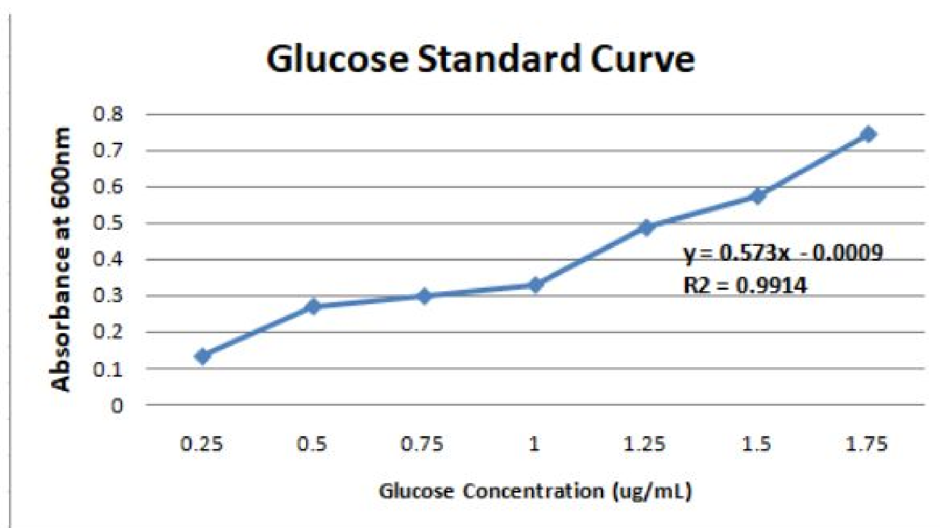


Graph 1 : Graphical representation of growth curve of the potential 10^{-8} (1) isolate for 30 hours

4.7 Glucose standard curve

The following absorbance was observed for the different glucose concentrations -

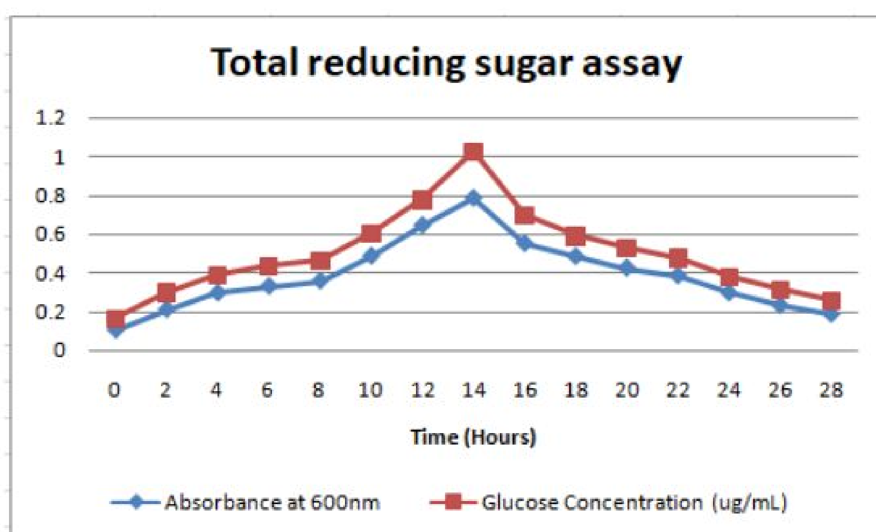
S.No.	Glucose concentration (µg/mL)	Absorbance (600 nm)
1	0.25	0.134
2	0.50	0.271
3	0.75	0.299
4	1.00	0.330
5	1.25	0.487
6	1.50	0.573
7	1.75	0.573
8	2.00	0.743
9	2.25	0.882



Graph 2 : Graphical representation of glucose concentration at different OD (600nm)

4.8 Total reducing sugar assay

S. No.	Time (Hours)	Absorbance (600nm)	Glucose concentration (µg/mL)
1	0	0.103	0.061945
2	2	0210	0.08834
3	4	0.295	0.09026
4	6	0.330	0.102258
5	8	0.357	0.107057
6	10	0.486	0.117615
7	12	0.645	0.132493
8	14	0.786	0.237596
9	16	0.551	0.143531
10	18	0.484	0.107057
11	20	0.425	0.100818
12	22	0.384	0.0917
13	24	0.295	0.083061
14	26	0.230	0.080181
15	28	0.185	0.070589

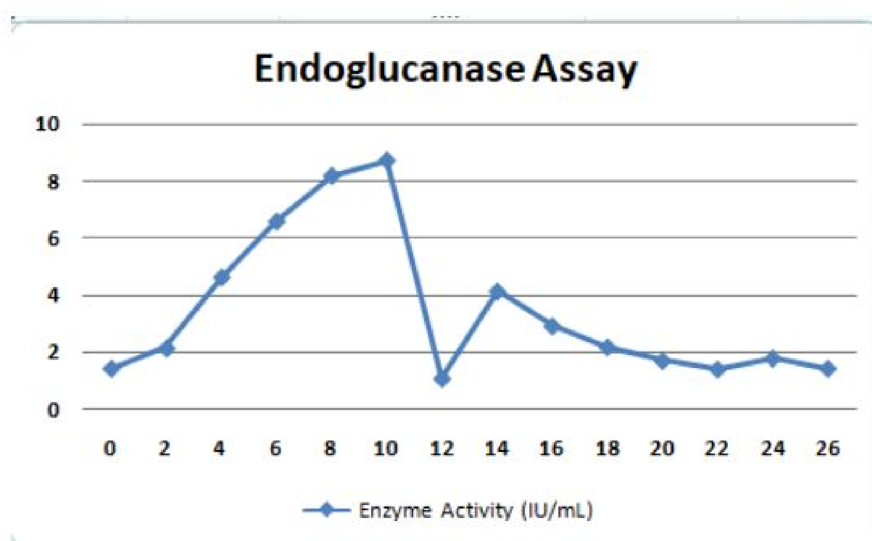


Graph 3 : Graph showing the standard spectrophotometric total reducing sugar estimation for reducing sugar concentration

4.9 Endoglucanase activity assay

S.No.	Time (Hours)	Absorbance (60nm)	Glucose concentration (µg/mL)	Enzyme activity (IU/mL)
1	0	0.343	0.003874335	1.434938
2	2	0.398	0.005794012	2.14593037
3	4	0.465	0.012512879	4.63439963
4	6	0.520	0.017791988	6.58962519
5	8	0.584	0.02211126	8.18935556
6	10	0.783	0.023551017	8.72259889
7	12	0.950	0.029310046	1.08555726
8	14	1.140	0.111856127	4.14281952
9	16	0.885	0.078741711	2.91635967
10	18	0.740	0.059065029	2.18759367
11	20	0.684	0.046107214	1.70767459
12	22	0.535	0.03794859	1.40550333
13	24	0.433	0.004834173	1.79043444

14	26	0.375	0.003874335	1.43493889



Graph 4 : Graphical determination of degradation of sugar into monomeric units.

$$\text{Enzyme activity} = \frac{\text{Product concentration} \times \text{Total reaction volume} \times \text{Dilution factor}}{\text{Molecular weight} \times \text{Enzyme volume} \times \text{Incubation time}}$$

where, Product concentration= amount of glucose liberated

Total reaction volume= 1mL (500µL substrate+500µL enzyme)

Dilution factor= 10

Molecular weight of glucose= 180g/mol

Enzyme volume= 500µL

Incubation time= 30 min

CHAPTER 5 : CONCLUSION

The amylase, being the most extensive starch hydrolyzing enzymes are highly diversified in the plant, animal and micro-organisms. In the recent years, the bacterial strains are being to isolate the amylase enzymes for different purposes from the starch degradation to industrial importance. The isolation of amylase from different soil samples, their morphological characterizationscreening and optimization by gram staining and other biochemical assays such as Oxidase test, Catalase test, IMViC test (MRVP test, Indole test, Simmon's citrate test); etc. are performed In-vitro to produce up-to-date variants. The genetic alterations, strain improvement are quite a easy to bring-out an enzyme with desired traits. Hence, α -Amylase are isolated from from various fungal sources, yeasts, bacteria and actinomycetes whereas enzymes derived from the bacterial strains have a control over many applications in industry. Primarily, the day-to-day significance of sustainable development has encouraged humans to bring enzymatic reactions to produce biodegradable products. Bacterial strains for the amylase production using the SSF are commercially useful for the production of fructose syrup, food industry, environmentally safe detergents, starch degradation, paper and pulp industry and baked products.

CHAPTER 6 : FUTURE PROSPECTS

Among all the categories of the enzymes, Amylases have grabbed the highest attention due to their easy availability, economical and applications in many industrial and medicinal purposes. The modernized technologies like white biotechnology, green tech and pinch technology will cope-up the industrial production in huge volumes. All these techniques will be implemented better with the establishment of fermentation technologies in genetically engineered bacteria or fungi and the complementation of other biotechnology approaches. The high-throughput screening and processing with the potential microbial strains, genetic manipulation of amylase-producing strains according to the desired traits. All these collectively will help to increase the amylase production for industrial, medicinal, research and various purposes. This will bring a revolution to the different commercial sectors like food, paper, textile and medicine. Additionally, The point-of-care measurements of the amylase should bring in the trend and needed to be handed carefully.

CHAPTER 7 : REFERENCES

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I hereby certify that the dissertation titled "**Screening, isolation & optimization for activity of amylase producing bacteria from soil sample**" submitted by **Garima Mishra**, Roll no. **2K20/IBT/04**, in the partial fulfillment of the requirements for the award of the degree of Master of Technology, Department of Biotechnology, Delhi technological University, Delhi is an authentic record of the work carried out by the student under my guidance. To the best of my knowledge, this work is original and has not been submitted in part or full for any Degree or Diploma to the University or elsewhere.

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I, **GARIMA MISHRA, 2K20/IBT/04** here by certify that the work which I presented in the major project entitled "**Screening, isolation & optimization for activity of amylase producing bacteria from soil sample**" in the fulfillment of the requirement for the award of the Degree of Master of Technology in Industrial Biotechnology and submitted to the Department of Biotechnology, Delhi Technological University, Delhi is an authentic record of my own, carried out during a period from 15Dec'21 – 26May'22, under the supervision of HOD & Prof. Pravir Kumar and Dr. Rashmi Kataria. The matter presented in the thesis has not been submitted by me for the award of any other degree at this or any other University.

Garima Mishra

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