PROJECT TITLE

SCREENING AND PRODUCTION OF LIGNOCELLULOSIC ENZYMES FROM THERMOPHILIC BACTERIAL ISOLATES OF THE ANAEROBIC SYSTEM

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JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

WAKNAGHAT

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CERTIFICATE

This is to certify that the work entitled "**SCREENING AND PRODUCTION OF LIGNOCELLULOSIC ENZYMES FROM THERMOPHILIC BACTERIAL ISOLATES OF THE ANAEROBIC SYSTEM"** pursued by **Shubham Vashishtha** (123802) in partial fulfilment for the award of degree Masters of Technology in Biotechnology from Jaypee University of Information Technology, Wakhnaghat has been carried out under my supervision. This part of work has not been submitted partially or wholly to any other University or Institute for the award of any degree or appreciation.

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Date:

Place:

(Shubham Vashishtha)

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOLS	ABBREVIATIONS
LB	Luria Broth
UV	Ultraviolet
CMC	Carboxymethylcellulose
DNS	3,5-Dinitrosalicylic acid
dH_2O	Distilled water
OD	Optical density
PBS	Phosphate Buffer Saline
pH	Potential of hydrogen
°C	Degree Celsius
g	Gram
µl	Microliter
µg	Microgram
ml	Milliliter
mg	Milligram
rpm	Rotation per minute
%	Percentage
nm	Nanometers
BSA	Bovine Serum Albumin
MWCO	Molecular weight cut-off

CHAPTER 1

Review of literature

1.1. INTRODUCTION

Plant material mostly consists of lignocellulosic biomass also called as dry matter. It consists of cellulose, hemicellulose bound by an aromatic polymer called lignin which provides structural support to the plant system (1). The composition of these materials may vary accordingly based on the type of plant or species, e.g. Wood consist of high cellulose (50-60 %) and lignin content, whereas leaves and straw have more hemicellulose content (2). These components are discussed below in details:

- **Cellulose:** It is made up of a large number of β-D-glucose monomers depending upon its degree of polymerisation, linked via. β-(1,4)glycosidic linkage (3). The repeat unit of cellulose is cellobiose which can be further degraded to form glucose monomer. Cellulose is present have both crystalline and amorphous form present in it, out of which amorphous one is easy to degrade. Cellulose has many industrial applications and is used in mostly paper and pulp industry to make paper, paperboards, cardboard etc. Also, it is used in textile industry for making cotton and other fibres, this industry used cotton as a substrate which generally contains 90-95% of cellulose in it (4).
- **Hemicellulose:** It consists of monomer units of D-pentose and L-sugars. Mostly consist of xylose, mannose and arabinose 5 carbon sugars. Hemicellulose is degraded to form xylose and other monomers. It is mostly present in amorphous form and can be easily degraded with the help of weak acid/base instead of strong pretreatment methods usually followed for cellulose. Hemicellulose is present as 20-30 % of the total lignocellulosic content present in the plant. For laboratory use xylose is extracted from Birchwood which contains approximately 89% of hemicellulose (5, 6). Hemicellulose is used for production of various high value products like xylitol (used in pharmaceuticals), butanediol (used as solvent and in making of synthetic polymers), vanillin (used as antifoaming agent, in drug manufacturing and also as flavouring agent in food industry) and lactic acid (used in variety of industries like food, textile and pharmaceuticals) (7).
- **Lignin:** It is a complex aromatic polymer material which plays an important role in maintaining the structure of the plant. It binds together different vessels and fibres including cellulose and hemicellulose keeping them intact. Lignin have many industrial applications like,

a. It has antioxidant activity thus used in cosmetic industry.
b. In the making of plastics and polymers.
c. Applied in batteries with graphite thus increasing the battery life.
d. Used in slow release urea from efficient utilisation.

1.2. PRETREATMENT OF LIGNOCELLULOSIC BIOMASS

The derivatives of these biomolecules have various applications in industries as mentioned above. The problem that arises with their complete exploitation is the methods that are used for pretreatment. Pretreatment usually is a process for degrading the complex lignocellulosic biomass into smaller units e.g. Breaking of complete lignocellulosic material to cellulose, hemicellulose and lignin and further breaking them down to monomers for industrial use (8). The goals of pretreatment includes,

a) Improved production of monomers after treatment.
b) Reduced production of inhibitors in the product.
c) The method used should be cost effective.
d) Reduced wastage of substrate during conversion (9).
e) Reduced environmental hazards caused due to the substrate.

The pretreatment of this lignocellulosic material is done traditionally via. Different methods broadly divided into chemical, physiochemical and physical methods given in details below:

- **Chemical methods:** It includes the use of various chemicals for directly breaking the chemical structure of the lignocellulosic biomass. It included ozonolysis (use of ozone in degradation) and acid and alkali hydrolysis (use of strong acid or base based on the type of molecule targeted e.g. to remove hemicellulose week acids are used and for cellulose, strong acids are preferred) (10).
- **Physical methods:** It includes mechanical methods that are based on direct application of force and breaking materials like grinding, chipping and milling combinations (11). It also includes heating samples at a high temperature called pyrolysis.

- **Physicochemical methods:** Includes both physical stress and chemical conversion methodology eg. steam explosion. The samples are treated with high-pressure steam and then further pressure reduced in order to get explosive decomposition (9).

These methods are usually used for pretreatment in industries due to their efficiency but, they create a lot of pollution and require harsh methods. Presently use of enzymatic degradation methods for lignocellulosic material are in picture and preferred due to,

a. Simpler process compared to the use of harsh chemicals and sophisticated machines.
b. Low amount of energy required compared to other methods.
c. Better utilisation of substrate and less waste generated.
d. Good for the production of biofuels, as the pretreatment process can be integrated into the bio-ethanol production process.
e. The process is environmentally friendly.

Biological process for pretreatment uses set of enzymes for the degradation of lignocellulosic biomass; these enzymes are secreted by different organism like fungi; white rot, brown rot and soft rot fungi are used for degradation of cellulose, hemicellulose and lignin in waste. These fungi species act on the specific molecule and act on them like, white and soft rot fungi work on lignin and cellulose whereas, the brown rot act on cellulose only. For pretreatment of lignocellulosic material, white rot fungi are much more preferred (12). The study the of biological pretreatment effects on the Japanese red pine *Pinus densiflora* when exposed to three white rot fungi: *Stereum hirsutum, Ceriporia lacerata and Polyporus brumalis*. These fungi tested, *S. hirsutum* selectively degraded the lignin of the wood sample, rather than the holocellulose (cellulose and hemicellulose) component. After 8 weeks of pretreatment, the total weight loss was 10.7%, and the loss of lignin was highest among the tested samples (14.5%) with *S. hirsutum*. But, the holocellulose loss was lower, at 7.8%, than the losses in *C. lacerata and P. brumalis* (13).

The ratio of carbon to nitrogen for the pretreatment of lignocellulosic biomass is important because the efficiency of degradation depends upon this ratio. A definite ratio of nitrogen is required for the degradation of a single molecule of carbon, and this ratio changes with different organisms. The ratio of carbon and nitrogen ratios are high in fungi as compared to bacteria; hence, fungi are more capable of degrading lignocellulosic biomass. In contrast, the bacteria

have a high growth rate and are easy to grow and mutate accordingly thus, are preferred for use (14). The complete degradation of cellulose and hemicellulose is done by a cocktail of hydrolases enzymes having a different mode of action and activities. For degradation of cellulose, cellulase is of primarily use as it works on the β-1,4-glycosidic linkage. Hemicellulose is degraded with the help of xylanases, they cleave β-1,4- bond in β-1,4-xylan polysaccharide to form xylose. Depolymerisation of lignin involves the use of oxidative enzymes like laccases and lignin peroxidases (15). Some of the bacterial enzymes are discussed below:

- **Cellulase:** These are a group of enzymes that cleave β-1,4-glycosidic linkage in cellulose to glucose monomers or other smaller poly or oligosaccharides that are of high use, examples of different sources of cellulase include Fungal sources e.g. *Trichoderma, Humicola, Acremonium,* Bacterial sources e.g. *Clostridium thermocellum*. Different type of cellulases includes,
  1. **Endocellulase Endo-4-glucase:** Cleaves amorphous site of cellulose internally at random sites.
  2. **Exocellulase (Cellobiohydrolases)**: Cleaves two or four units from the end of endo-cellulase product chain.
  3. **B-glucosidases (Cellobiases)**: It degrades product of exocellulase into monosaccharides (16).

- **Hemicelluloses:** The enzyme that cleaves β-1,4- bond in β-1,4-xylan polysaccharide to form xylose like xylanases. They are also called as backbone degrading enzymes. Also, the enzymes removing the side groups are β-xylosidases. They help in complete degradation of cellulose and hemicellulose present in the biomass. As, leaves and fruits contain more amount of hemicellulose and thus, are important for industrial use.

- **Lignin-modifying enzymes**: These are the class of enzymes that oxidises the aromatic polymer present in plant cell wall i.e. lignin thus, belong to oxidoreductase class of enzymes. Different types of lignin modifying enzymes are;

1. **Lignin peroxidase:** These are glycoproteins that use hydrogen peroxide as an oxidant. These oxidises the non-phenolic lignin structures by taking one electron and generating free radicals which are further neutralised.
2. **Laccase:** It is a copper-containing enzyme that utilises molecular oxygen as oxidant and oxidises phenolic rings to phenoxyl radicals. It can also oxidise nonphenolic compounds under certain conditions, e.g. if the reaction mixture is supplemented with ABTS (17).

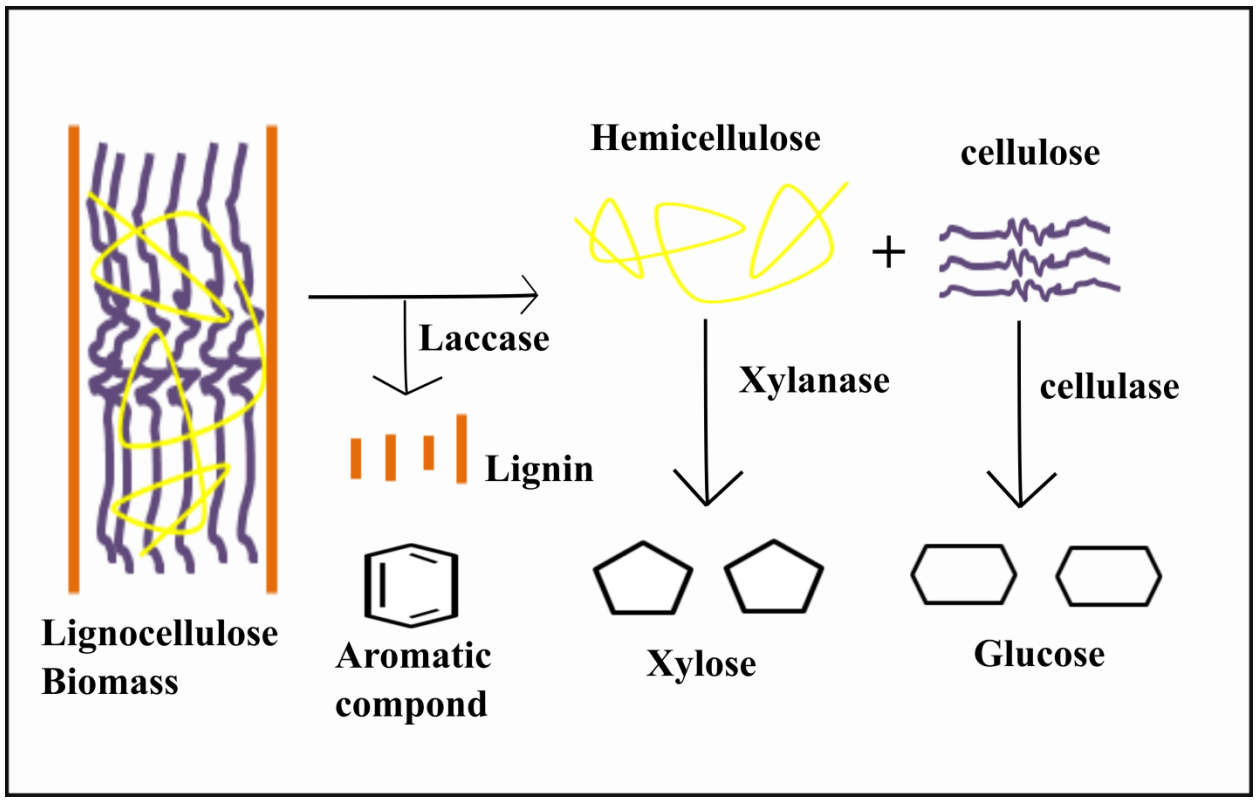

Figure 1: Enzymatic degradation of lignocellulosic biomass

- **Lignocellulosic enzymes with applications in industry**
  - **Cellulase:** Convert cellulose to mono/ oligo/ polysaccharide units (*Coffee, Textile, Paper and pulp industry)*
  - **Xylanase:** Converts hemicellulose (Xylan) to xylose (*Coffee, Food, Bleaching industry*)
  - **Pullulanase:** Converts Starch to glucose monosaccharide units (*Baking, Food, Detergent, Pharmaceutical industry*)

- **Amylase:** Converts starch to mono/ oligo/ polysaccharide units *(Food, Detergent, Pharmaceutical industry).*
- **Laccase:** These are oxidoreductases have potential use in *Textile, wine industry, dying in the textile industry and in teeth whitening.*

These enzymes are produced by many organisms and have high industrial importance. Most of the processes in industries are carried out at high temperature; some of the cultures attain self-high temperature during growth like in biogas plant. Also, maintenance of aerobic condition at large scale increases cost and resources resulting from pipes, aeration/air pumping etc. Also, some of the organisms can grow easily at very high or very low temperatures, these organisms are called extremophiles. Thus microbes that can withstand high temperature and can survive in scarce oxygen environment are preferred.

1.3. EXTREMOPHILES

Extremophiles are an organism that can survive in extreme environments and variously niches like hot water spring, cold areas, deserts, high salt, alkaline or acidic conditions. These organisms have developed various resistant factors and slightly modified enzymes in order to survive in harsh conditions. Some of the types of microorganisms discussed below;

- **Psychrophilic:** These are the organisms that can grow at a very low range of temperatures (-20° C to 10°C), and are also called cold-loving organisms. They can survive at very low temperatures either by converting into a glass (vitrification) or in the state of extreme dryness (desiccation). Some of them can also carry out various processes like *Lichen xanthoria*, which can perform photosynthesis at -26^{0}C (18). Some of the bacterial species that can survive at very low temperatures include *Holomona, Pseudomonas, Phychrobacter* species etc. They are also of very high industrial value due to specialised enzymes produced by them which can work at very low temperature. They are used in meat tenderization (protease), starch hydrolysis, wine clarification and stability, detergent and cleaning industry, tanning and hide industry and much more. These organisms are used in industries which require enzymes that can process at low temperatures. In food industry to preserve food to prevent it from spoilage and at the same time processing at 4 ^{0}C, In detergent industry enzymes earlier used required

lukewarm or hot water for processing but now they can easily act at low temperatures or room temperatures.

- **Mesophilic:** These are organisms that grow at moderate temperature i.e (20-40 ^{0}C). It consists of most of the organisms detected which survive at 37^{0}C (normal body temperature). Most of the human pathogens are mesophiles and cause infections to normal health. Common examples of mesophiles include *E. coli, Clostridium sp., Pseudomonas maltophilia;* most of the Lactic acid bacteria's e.t.c. These organisms are used in the majority of industries as they were initially detected and cultured for the production of many chemicals and in industrial processes ex. penicillin, wine, ethanol/spirit, cheese making e.t.c.

- **Thermophiles:** These are organisms that grow at high temperatures (45-122^{0}C). Most of them use different molecules for growth like sulphur thus, producing a beautiful ecosystem of bright colours. These are found in naturally occurring geothermal hot springs like in Yellowstone national park (*Thermus aquaticus*) and in deep sea vents. The GC content of these organisms is high which help them in surviving at high temperatures.The enzymes produced by these thermophiles are of high industrial importance.DNA polymerase which is the best example of thermostable enzyme produced by *Thermus aquaticus* bacteria isolated from hot water springs in the Yellow stone national park.This polymerase is active at high temperature (75-80^{0}C) as compared to normal polymerases thus, making PCR one of the most efficient process in present science.The process of starch liquefaction is practised in many industries for making high fructose syrups, dextrose syrups, glucose solutions e.t.c., are used in pharmaceuticals, textile, paper pulp, food industry. This process usually takes place at 55-60^{0}C and 7.8-8.5 pH thus, requiring thermophilic alkaline hydrolyses. The use of thermostable α-amylase and pullulanase is in practice to make the process better and efficient.

  Similarly, in biofuel production, the system is self-healing and in summers reach 70-80^{0}C, when compost is used. This system requires a cocktail of enzymes to degrade lignocellulose and hence require various thermophilic enzymes like cellulase, xylanase and laccase.

- **Halophiles:** These are organisms that grow at high concentrations of salt i.e. 20-30 %. They require sodium chloride for growth and maintaining similar osmotic concentration inside the cell so as to maintain isotonic concentration with the environment to survive and prevent cell lysis. These halophiles are of high importance in the industries, they are used in the fermentation of a food product having high salt content i.e. soy sauce, fermented beams e.t.c. *Halobacterium* used in industry ex. *Chromohalobacter beijerinckii*.
- **Acidophile/ Alkalophiles:** These organisms can survive at low or high pH range respectively. Alkalophiles create an acidic matrix around the cell wall with the help of gluconic acid, aspartic acid present on the surface thus neutralising the surface environment to survive. In contrast, the acidophiles use proton pump to remove protons from the cytosol in order to maintain its pH. Ex *Acetobacter.*
- **Anaerobes:** These are an organism that does not require oxygen for growth and development. Some of them can survive in both presence and absence of oxygen and are termed as facultative anaerobes ex. *Bacillus sonorensis*.

This study used 20 organisms that were anaerobically isolated from the biogas samples taken from the tattapani area. These organisms were isolated in strictly anaerobic conditions and identified using 16s rRNA sequencing. List of organisms identified and their details are given in table 1. The Study was focused on screening facultative anaerobes that can grow in aerobic conditions also.

Table 1. Organisms selected for initial screening

Organism code	Tentative identification	Growth condition	Past study	Reference
1	*Bacillus thermocopriae*	Facultative anaerobic	Only isolation studies available	(19, 20)
2	*Bacillus thermocopriae*	Facultative anaerobic	Only isolation studies available	(19, 20)
3	*Weissella oryzae*	Aerobic	Isolation studies and Silver nanoparticle production	(21, 22)
4	*Bacillus sonorensis*	Aerobic	Lipase action reported, industrial use	(23, 24)
5	*Weissella confusa*	-	Detection methods, cause endocarditis	(25)
6	*Leuconostoc fallax*	Facultative anaerobic	Isolation studies and phylogenetic analysis	(26, 27)
7	*Bacillus thermocopriae*	Facultative anaerobic	Only isolation studies available	(19, 20)
8	*Bacillus licheniformis*	Aerobic	Keratinase, protease, chitinase many applications	(28)
9	*Brevibacillus aydinogluensis*	-	Isolation study	(29)
10	*Brevibacillus aydinogluensis*	-	Isolation study	(29)
11	*Bacillus thermocopriae*	Facultative anaerobic	Only isolation studies available	(19, 20)
12	*Geobacillus thermodenitrificans*	Aerobic	Bioremediation and isolation studies	(30)
13	*Geobacillus*	Aerobic	Bioremediation and	(30)

	thermodenitrificans		isolation studies	
14	*Bacillus thermocopriae*	Facultative anaerobic	Only isolation studies available	(19, 20)
15	*Bacillus thermocopriae*	Facultative anaerobic	Only isolation studies available	(19, 20)
16	*Brevibacillus aydinogluensis*	-	Isolation study	(29)
17	*Brevibacillus aydinogluensis*	-	Isolation study	(29)
18	*Weissella cibaria*		Isolation, Industrial use glucan production	(31)
19	*Caldalkalibacillus ozonesis*	-	-	-
20	*Geobacillus thermodenitrificans*	Aerobic	Bioremediation and isolation studies	(30)

1.4. RATIONALE

- Lignocellulosic enzymes can be used for:
    - Pretreatment
    - Complex substrate degradation
    - Biofuel production and many industrial applications

There is need of thermostable enzymes for maintaining catalytic and process stability at high temperature along with efficiently catalysing the reaction. Large scale production requires enzymes that survive scarcity of oxygen thus facultative anaerobes are important that can survive high temperature. Stable enzymes are easy to reuse thus improving the cost of production. Also if a single organism capable of producing all of the lignocellulosic enzymes then, the single step process can lead to complete degradation of complex biomass into simple monomers.

1.5. OBJECTIVES OF THE PROJECT

- Screening of facultative anaerobes from the set of anaerobes.
- Selection of lignocellulosic enzymes producing facultative anaerobes by qualitative analysis.
- Selection of lignocellulosic enzymes producing facultative anaerobes by quantitative analysis.
- Comparative analysis of substrate degrading efficiency of selected facultative anaerobes producing lignocellulosic enzymes.
- Protein purification from efficient lignocellulosic enzymes producer.
- Optimisation of various lignocellulosic enzymes.

CHAPTER 2

MATERIALS AND METHODS

2.1. REVIVAL OF GLYCEROL STOCKS

- Initially, 20 thermophilic Nobel anaerobic organisms were selected and their tentative names are given further in the study.
- LB was prepared and poured into test tubes for autoclaving in duplets for 20 thermophilic bacterial isolates (tagged 1-20), autoclaved at 121^0 C at 15 psi for 15 mins.
- Bacterial isolates maintained as glycerol stocks at -80^0 were collected from the cell repository.
- Cultures were kept on ice for an hour to bring them to 4^0 C and then kept at room temperature for culturing. (*Note: cultures were not directly brought to room temperature in order to prevent heat shock and damage to cell lines*)
- Tubes of LB media were inoculated with 10 µl of glycerol stock for the respective organism.
- Cultured test tubes were incubated at 45^0 C for 48 hours in aerobic conditions.
- Tubes were observed after incubation to check growth.

- **TO CHECK CONTAMINATION IN THE REVIVED CULTURES**
  - LB agar media was prepared with 1.5 % agar (Merck) concentration in duplets and autoclaved.
  - Autoclaved media was poured in plates of LB media and kept under UV sterilisation for 30 mins to solidify. Plates were then marked 1-20 tag.
  - Plates after solidification were kept at 45^0 C for 48 hours in an incubator to check contamination if any.
  - From the seed cultures prepared above a small amount of inoculum was taken using sterile inoculation loop.
  - Simple and quadrant streaking was done on the plates from each sample respectively.
  - Plates after inoculation were incubated at 45^0 C for 36 hours for bacterial isolates.
  - Results were observed and noted after incubation.

- **SUBCULTURE OF THE REVIVED COLONIES AND PREPARATION OF GLYCEROL STOCKS FOR LONG TERM STORAGE**
    - After contamination check, LB media was prepared for subculture and growth of the organism.
    - At the same time, 30% glycerol was made and autoclaved.
    - 100 ml of LB broth was made for each organism and 10 ml broth in test tubes was prepared for subculturing and seed cultures and autoclaved.
    - From the above-prepared plates, a small amount of inoculum was taken using sterile inoculation loop and test tubes were inoculated and incubated for 48 hours respectively.
    - After growth in seed culture media 500 µl of culture was taken and pipetted in 1.5ml Eppendorf and an equal volume of glycerol (30%) was added and sealed using parafilm and stored at -80^0 C.
    - Also for subculture 100 µl of inoculum was taken and flasks were inoculated for 24 hours and 36 hours respectively.

2.2. QUALITATIVE ANALYSIS OF VARIOUS LIGNOCELLULOSIC ENZYME ACTIVITY

- **Qualitative analysis of amylase activity by starch iodine test (32)**

Media used: Starch agar media

Theory: The starch agar test is done to check for the production of amylase enzyme by the organism. The iodine reacts with starch to form a dark blue-colored complex. Clear area around the growth of the culture after the addition of iodine indicates the breakdown of starch by Amylase.

Protocol:

- Using the specific composition (annexures 2) the starch agar media was made and autoclaved, media was poured in plates accordingly for duplets.

- Plates were inoculated with 7 culture isolates (2, 4, 7, 11, 12, 16, and 17) which showed growth and simple streaking was done.
- Plates were incubated at 45^0 C for 48 hours respectively.
- After growth plates were flooded with gram iodine and observed.
- The experiment was performed in duplets for confirmation of results.

- **Qualitative analysis of pullulanase activity by pullulan degrading test (33)**

Media used: Pullulan agar media

Theory: Pullulan is a polysaccharide polymer consisting of maltotriose units, also known as α-1, 4, α-1, 6-glucan. Pullulanase produced by bacteria degrades pullulan at the α-1,4 linkage and both 1,4 and 1,6 in amylopullulanase to give glucose monomer thus making media colourless from the area where it degrades the substrate making the test positive.

Protocols:

- The seed culture was prepared for all strains in LB media in 10 ml test tubes.
- 500 ml of Pullulan agar media was made and autoclaved, media was poured in plates in duplets.
- Plates were inoculated with isolates of bacterial culture (2, 4, 7, 11, 12, 16, and 17) and simple streaking was done.
- Plates were incubated at 45^0 C for 48 hours respectively.
- After growth plates were flooded with gram iodine and observed.

- **Qualitative analysis of Cellulase activity by Carboxymethyl cellulose (CMC) degrading test (34)**

Media used: CMC agar media

Theory: Carboxymethylcellulose (CMC) or cellulose gum is a cellulose derivative with Carboxymethyl groups (-CH2-COOH) bound to some of the hydroxyl groups of the glucopyranose monomers that make up the cellulose backbone. Cellulase acts on cellulose and converts it into mono/polysaccharide units. Degradation of cellulose causes the substrate to degrade and forming clear zones in the media which were further visualised clearly using iodine.

Protocols:

- The seed culture was prepared for all strains in LB media in 10 ml test tubes.
- 500 ml of CMC agar media was made and autoclaved, media was poured in plates in duplets.
- Plates were inoculated with isolates of bacterial culture (2, 4, 7, 11, 12, 16, and 17) and simple streaking was done.
- Plates were incubated at 45^0 C for 48 hours respectively.
- After growth plates were flooded with gram iodine and observed.

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- **Qualitative analysis of Xylanase activity by Xylan-degrading test (35)**

Media used: Xylan agar media

Theory: Xylan is a group of hemicelluloses that are found in plant cell walls and some algae. Xylans are polysaccharides made from units of xylose (a pentose sugar). Xylanase acts on hemicellulose (Xylan) and converts in into xylose. Thus, Xylan present in media is degraded by xylanase which forms clear zones and is clearly detected by iodine solution.

Protocols:

- The seed culture was prepared for all strains in LB media in 10 ml test tubes.
- 500 ml of Xylan agar media was made and autoclaved, media was poured in plates in duplets.
- Plates were inoculated with isolates of bacterial culture (2, 4, 7, 11, 12, 16, and 17) and simple streaking was done.
- Plates were incubated at 45^0 C for 48 hours respectively.
- After growth plates were flooded with gram iodine and observed.

- **Qualitative analysis of Laccase activity by Guaiacol degrading test (36)**

Media used: Guaiacol agar media

Theory: Laccases are copper-containing oxidase enzymes found in many plants, fungi, and microorganisms. Laccases act on phenols and similar molecules, performing one-electron oxidations. They are also called lignin modifying enzymes. Guaiacol being a phenolic compound is degraded by laccase and produces characteristic brown colour colonies.

Protocols:

- The seed culture was prepared for all strains in LB media in 10 ml test tubes.
- 500 ml of Guaiacol agar media was made and autoclaved, media was poured in plates in duplets.
- Plates were inoculated with isolates of bacterial culture (2, 4, 7, 11, 12, 16, and 17) and simple streaking was done.
- Plates were incubated at 45^0 C for 7 days and observed.

- **Qualitative analysis of Pectinase activity by Pectin degrading test (37)**

Media used: Pectin agar media

Theory: Pectin is a complex polysaccharide consisting mainly of esterified D-galacturonic acid resides in an alpha-(1-4) chain. Pectin is degraded by pectinase and produces clear zones which are positive tests for pectinase activity.

Protocols:

- The seed culture was prepared for all strains in LB media in 10 ml test tubes.
- 500 ml of Pectin agar media was made and autoclaved, media was poured in plates in duplets.
- Plates were inoculated with isolates of bacterial culture (2, 4, 7, 11, 12, 16, and 17) and simple streaking was done.
- Plates were incubated at 45^0 C for 48 hours respectively.

2.3. QUANTITATIVE ACTIVITY ANALYSIS OF VARIOUS ENZYMES

- **Using DNS reagent for quantitative assay of amylase, xylanase and Cellulase activity.**

Chemicals used: DNS reagent, Starch solution *(1% starch solution prepared in 100 ml 0.02 M Sodium phosphate buffer pH 7),* Buffer (*0.02 M sodium phosphate buffer pH 7 with 0.006 M sodium chloride),* 1mg/ml Glucose solution *(0.1 g Glucose in 100 ml of dH_2O),* 1 mg/ml Xylose*(0.1 g xylose in 100ml dH_2O).*

Theory: This tests for the presence of free carbonyl group (C=O), the reducing sugars. It involves the oxidation of the aldehyde functional and ketone functional group. Also, 3, 5-dinitrosalicylic acid (DNS) is reduced to 3-amino-5-nitrosalicylic acid under alkaline conditions, as shown below:

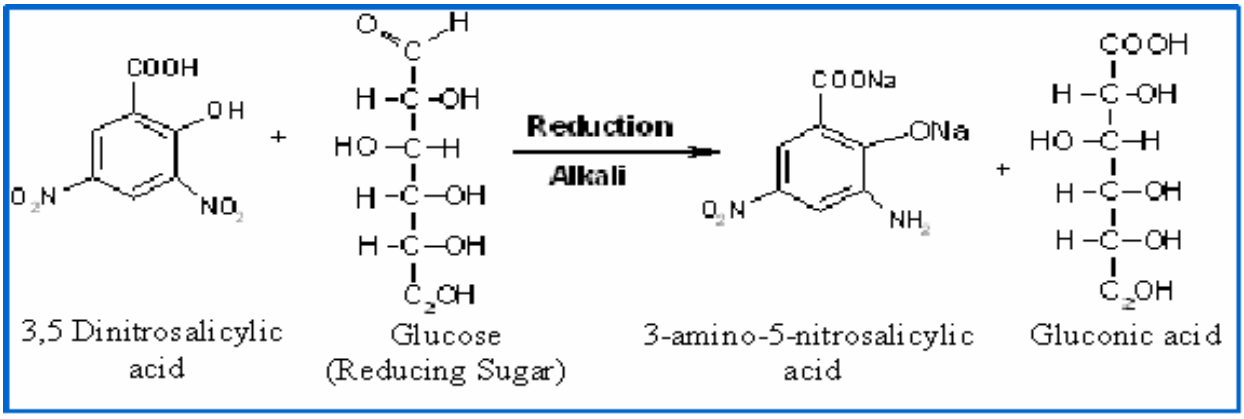


Figure 2: Principle reaction of DNS reagent

The substrates used for testing enzyme activity may vary depending upon the type of enzyme but, the principle followed remains same in all.

A standard curve for glucose:

- Components were added as shown in the table (2) and incubated respectively.

Table 2: Glucose standard curve process						
Tube no.	**Glucose Concentration (µg/ml)**	**Glucose Volume (ml)**	**dH_2O (ml)**	**DNS reagent (ml)**	**Heat in Boiling water bath for 5 mins And cool them**	**dH_2O (ml)**
1	0	0	1	3		6
2	200	0.2	0.8	3		6
3	400	0.4	0.6	3		6
4	600	0.6	0.4	3		6
5	800	0.8	0.2	3		6
6	1000	1	0	3		6

- After completion 200 µl of the sample was loaded in the ELISA plate wells and OD was taken at 540 nm in ELISA plate reader.

A standard curve for Xylose:

- Components were added as shown in the table (3) and incubated respectively.

Table 3: Xylose standard curve process						
Tube no.	**Xylose Concentration (µg/ml)**	**Xylose Volume (ml)**	**dH_2O (ml)**	**DNS reagent (ml)**	**Heat in Boiling water bath for 5 mins And cool them**	**dH_2O (ml)**
1	0	0	1	3		6
2	200	0.2	0.8	3		6
3	400	0.4	0.6	3		6
4	600	0.6	0.4	3		6
5	800	0.8	0.2	3		6
6	1000	1	0	3		6

- After completion 200 µl of the sample was loaded in the ELISA plate wells and OD was taken at 540 nm in ELISA plate reader.

a. Amylase activity assay:

Media used: Amylase production media i.e. Starch media (Annexure)

Chemicals used: DNS reagent, phosphate buffer (pH 7) (Annexure), *Substrate:* 1% starch solution (1 gm starch in 100 ml phosphate buffer).

Test for enzyme activity

- Selected cultures were grown in starch media for 72 hrs.
- Cultures were collected in 50 ml each sterile Tarson tubes and centrifuged at 5000 rpm for 10 mins at 4^0 C, Supernatant was collected in new Tarson 50 ml tubes and kept on ice.
- Test tubes were taken and marked accordingly.
- Substrate control Cs (*substrate + buffer*) was used and incubated for 10 mins at 45^0 C.
- Further proceeded as per the table (4).

Table 4: Amylase activity assay

Tube no.	Enzyme (ml)	Incubate for 3 mins at 45^0 C	Substrate (ml)	Buffer (ml)	Incubate for 10 mins at 45^0 C	DNS (ml)	Heat in boiling water for 5 mins	dH_2O (ml)
Cs	0.0		0.5	0.5		3		6
4_1	0.5		0.5	0.0		3		6
4_2	0.5		0.5	0.0		3		6
7_1	0.5		0.5	0.0		3		6
7_2	0.5		0.5	0.0		3		6
12_1	0.5		0.5	0.0		3		6
12_2	0.5		0.5	0.0		3		6

(Note: Marking goes as Cs- substrate control

4, 7, 12 are codes for organisms (table1))

b. Xylanase activity assay:

Media used: Production media for xylanase i.e. Xylan media (Annexure).

Chemicals used: DNS reagent, Citrate buffer (pH 5.3) (Annexure), *Substrate:* 1% Xylan solution (1 gm xylose in 100 ml citrate buffer).

Test for enzyme activity

- Selected cultures were grown in Xylanase production media for 72 hrs.
- Cultures were collected in 50 ml each sterile Tarson tubes and centrifuged at 5000 rpm for 10 mins at 4^0 C, Supernatant was collected in new Tarson 50 ml tubes and kept on ice.
- Test tubes were taken and marked accordingly.
- Substrate control Cs (*substrate + buffer*) was used and incubated for 10 mins at 45^0 C.
- Further proceeded as per the table (5).

Table 5: Xylanase activity assay

Tube no.	Enzyme (ml)	Incubate for 3 mins at 45^0 C	Substrate (ml)	Buffer (ml)	Incubate for 10 mins at 45^0 C	DNS (ml)	Heat in boiling water for 5 mins	dH_2O (ml)
Cs	0		1	1		3		5
4_c	1		0	1		3		5
41	1		1	0		3		5
7c	1		0	1		3		5
71	1		1	0		3		5
12c	1		0	1		3		5
121	1		1	0		3		5

(Note: Marking goes as Cs- substrate control

4, 7, 12 are codes for organisms (table1))

c. Cellulase activity assay:

Media used: Cellulase production media i.e. CMC media (Annexure).

Chemicals used: DNS reagent, Citrate buffer (pH 4.8) (Annexure), *Substrate:* 2% CMC solution (2 gm CMC in 100 ml citrate buffer).

Test for enzyme activity

- Selected cultures were grown in Cellulase production media for 72 hrs.

- Cultures were collected in 50 ml each sterile Tarson tubes and centrifuged at 5000 rpm for 10 mins at 4^0 C, Supernatant was collected in new Tarson 50 ml tubes and kept on ice.
- Test tubes were taken and marked accordingly.
- Substrate control Cs (*substrate + buffer*) was used and incubated for 10 mins at 45^0 C.
- Further proceeded as per the table (6).

Table 6: Cellulase activity assay

Tube no.	Enzyme (ml)	Incubate for 3 mins at 45^0 C	Substrate (ml)	Buffer (ml)	Incubate for 10 mins at 45^0 C	DNS (ml)	Heat in boiling water for 5 mins	dH_2O (ml)
Cs	0.0		0.5	0.5		3		6
4_1	0.5		0.5	0.0		3		6
4_2	0.5		0.5	0.0		3		6
7_1	0.5		0.5	0.0		3		6
7_2	0.5		0.5	0.0		3		6
12_1	0.5		0.5	0.0		3		6
12_2	0.5		0.5	0.0		3		6

(Note: Marking goes as Cs- substrate control

4, 7, 12 are codes for organisms (table1))

2.4. QUANTITATIVE ANALYSIS OF ENZYME ACTIVITY BY DNS METHOD USING SPECIFIC SUBSTRATE.

Substrate used: Untreated pine needle powder

Chemicals used: DNS reagent, Starch solution *(1% starch solution prepared in 100 ml 0.02 M Sodium phosphate buffer pH 7),* Buffer (*0.02 M sodium phosphate buffer pH 7 with 0.006 M sodium chloride),* 1mg/ml Glucose solution *(0.1 g Glucose in 100 ml of dH_2O),* 1 mg/ml Xylose*(0.1 g xylose in 100ml dH_2O).*

A. Preparation of seed culture for substrate media.

Chemical Used: PBS buffer (Annexures).

- 50 ml each LB broth media was prepared for the selected bacterial isolates (4, 7, and 12) and autoclaved.
- Media was inoculated with cultures and kept for incubation at 45° C for 48 hours.
- After incubation, the cultures were centrifuged at 5000 rpm for 10 minutes and the pellet was retained.
- Pellet was further dissolved in PBS buffer and OD was measured at 600nm.
- Dissolved cells were further used for culturing.

B. Production of the enzyme for enzyme activity analysis.

Media used: Pine needle specific media (Annexures)

Protocols:

- Pine needle specific media was made and autoclaved.
- Media was further inoculated with the Dissolved cell sample cultures and incubated at 45° C.
- The activity of various enzymes (Amylase, Cellulase, and Xylanase) was analysed at an interval of 24 hours and results were analysed.

C. Amylase substrate degradation and activity check.

Chemicals used: DNS reagent, phosphate buffer (pH 7) (Annexure), *Substrate:* 1% starch solution (1 gm starch in 100 ml phosphate buffer).

Protocols:

- At time zero and at time 24 hours 2 ml culture was collected in autoclaved 2 ml centrifuge tubes in LAF.
- Tubes were then centrifuged at 5000 rpm for 10 minutes.
- The supernatant was collected and transferred into fresh tubes and kept at 4° C.
- Test tubes were taken and marked accordingly.

- Both controls were taken Enzyme control (*enzyme + buffer*) and substrate control (*substrate + buffer*) and incubated for 10 mins at 45^0 C.
- Further proceeded as per the table (7).

Table 7: Amylase activity assay

Tube no.	Enzyme (ml)	Incubate for 3 mins at 45^0 C	Substrate (ml)	Buffer (ml)	Incubate for 10 mins at 45^0 C	DNS (ml)	Heat in boiling water for 5 mins	dH_2O (ml)
Cs	0.0		0.5	0.5		3		6
4_c	0.5		0.0	0.5		3		6
41	0.5		0.5	0.0		3		6
7c	0.5		0.0	0.5		3		6
71	0.5		0.5	0.0		3		6
12c	0.5		0.0	0.5		3		6
12_1	0.5		0.5	0.0		3		6

(Note: Marking goes as Cs- substrate control

4, 7, 12 are codes for organisms)

Subscript c donates controls for respective organisms**)**

- Samples were cooled and OD took at 540 nm.
- The process was further repeated for 48, 72, 96 hours interval following the same table.

D. Xylanase substrate degradation and activity check.

Chemicals used: DNS reagent, Citrate buffer (pH 5.3) (Annexure), *Substrate:* 1% Xylan solution (1 gm xylose in 100 ml citrate buffer).

Protocols:

- At time zero and at time 24 hours 2 ml culture was collected in autoclaved 2 ml centrifuge tubes in LAF.
- Tubes were then centrifuged at 5000 rpm for 10 minutes.
- The supernatant was collected and transferred into fresh tubes and kept at 4^0 C.
- Test tubes were taken and marked accordingly.
- Both controls were taken Enzyme control (*enzyme + buffer*) and substrate control (*substrate + buffer*) and incubated for 10 mins at 45^0 C.

- Further proceeded as per the table (8).

Table 8: Xylanase activity assay								
Tube no.	**Enzyme (ml)**	**Incubate for 3 mins at 45^0 C**	**Substrate (ml)**	**Buffer (ml)**	**Incubate for 10 mins at 45^0 C**	**DNS (ml)**	**Heat in boiling water for 5 mins**	**dH_2O (ml)**
Cs	0		1	1		3		5
4_c	1		0	1		3		5
41	1		1	0		3		5
7c	1		0	1		3		5
71	1		1	0		3		5
12c	1		0	1		3		5
121	1		1	0		3		5

(Note: Marking goes as Cs- substrate control

4, 7, 12 are codes for organisms)

Subscript c donates controls for respective organisms**)**

- Samples were cooled and OD took at 540 nm.
- The process was further repeated for 48, 72, 96 hours interval following the same table.

E. Cellulase substrate degradation and activity check

Chemicals used: DNS reagent, Citrate buffer (pH 4.8) (Annexure), *Substrate:* 2% CMC solution (2 gm CMC in 100 ml citrate buffer).

Protocols:

- At time zero and at time 24 hours 2 ml culture was collected in autoclaved 2 ml centrifuge tubes in LAF.
- Tubes were then centrifuged at 5000 rpm for 10 minutes.
- The supernatant was collected and transferred into fresh tubes and kept at 4^0 C.
- Test tubes were taken and marked accordingly.
- Both controls were taken Enzyme control (*enzyme + buffer*) and substrate control (*substrate + buffer*) and incubated for 20 mins at 45^0 C.
- Further proceeded as per the table (9).

Table 9: Cellulase activity assay								
Tube no.	**Enzyme (ml)**	**Incubate for 3 mins at 45^0 C**	**Substrate (ml)**	**Buffer (ml)**	**Incubate for 20 mins at 45^0 C**	**DNS (ml)**	**Heat in boiling water for 5 mins**	**dH_2O (ml)**
Cs	0.0		0.5	0.5		3		6
4_c	0.5		0.0	0.5		3		6
41	0.5		0.5	0.0		3		6
7c	0.5		0.0	0.5		3		6
71	0.5		0.5	0.0		3		6
12c	0.5		0.0	0.5		3		6
12_1	0.5		0.5	0.0		3		6

(Note: Marking goes as Cs- substrate control

4, 7, 12 are codes for organisms)

Subscript c donates controls for respective organisms**)**

- Samples were cooled and OD took at 540 nm.
- The process was further repeated for 48, 72, 96 hours interval following the same table.

2.5. PROTEIN PURIFICATION BY AMMONIUM PRECIPITATION AND ACTIVITY ASSAY

Theory: **(38)** Most of the proteins are soluble in water and their solubility depends upon the pH and nature of the solution they are mixed in. Ammonium precipitation is a method to precipitate out proteins present in the solution by addition of salts. Ammonium sulphate is preferred for this process due to its high solubility at low temperature. In aqueous solutions, a protein remains soluble in solution but, when salt is added water molecules become more attracted towards the salt ions as compared to proteins. Thus, proteins present in the solution aggregates and precipitates this process are called “salting out”. The precipitate can then be collected by centrifugation and the protein pellet is redissolved in the buffer.

Protocols:

- Cultures of the selected organisms with good enzymatic activity were taken and grown in LB media at 45 °C for 72 hrs.
- Cultures were collected in 50ml tarson tubes and centrifuged at 5000 rpm for 10 mins and the supernatant was collected in new tarson in duplets.
- From the table of ammonium sulphate precipitation for 0-30 % cut amount of ammonium sulphate was calculated.
- 8.2 g of ammonium sulphate each was slowly added pinch by pinch to the medium containing protein and was continuously stirred at 4^0 C.

Figure 3: Setup for Ammonium sulphate precipitation

- Ammonium sulphate was mixed completely and the media was kept at 4^0 C for overnight precipitation.
- Next day sample was centrifuged at 10000 rpm for 15 mins, the supernatant was taken and collected in a new tarson tube and the pellet was resuspended in sodium phosphate buffer and the assay was done for xylanase with the DNS reagent, as per in table 10, and OD was taken at 540 nm.

- Now for the second cut of 30-60% amount of ammonium sulphate was calculated from the table.
- 9.05 g of ammonium sulphate each was slowly added pinch by pinch to the medium containing protein and was continuously stirred at 4^0 C
- Ammonium sulphate was mixed completely and the sample was kept at 4^0 C for overnight precipitation.
- Next day sample was centrifuged at 10000 rpm for 15 mins, supernatant was taken and collected in a new tarson tube and pellet was resuspended in sodium phosphate buffer and the assay was done for xylanase with the DNS reagent following the same protocol as above in table 10.
- Now for the third cut of 60-90% amount of ammonium sulphate was calculated from the table.
- 10.05 g of ammonium sulphate each was slowly added pinch by pinch to the medium containing protein and was continuously stirred at 4^0 C
- Ammonium sulphate was mixed completely and the sample was kept at 4^0 C for overnight precipitation.
- Next day sample was centrifuged at 10000 rpm for 15 mins, supernatant was taken and collected in a new tarson tube and pellet was resuspended in sodium phosphate buffer and the assay was done with the DNS reagent following the same protocol as above.
- Similarly, the above experiment was performed with the other set of samples and OD was taken for all the samples at 540nm.

Table 10: Activity table for xylanase after ammonium precipitation								
Tube no.	**Enzyme (ml)**	**Incubate for 3 mins at 45^0 C**	**Substrate (ml)**	**Buffer (ml)**	**Incubate for 10 mins at 45^0 C**	**DNS (ml)**	**Heat in boiling water for 5 mins**	**dH_2O (ml)**
Cs	0		1	1		3		5
30% 4c	1		0	1		3		5
30% 4	1		1	0		3		5
60% 4c	1		0	1		3		5

(Note: Marking goes as Cs- substrate control

4, 7, 12 are codes for organisms)

Subscript c donates controls for respective organisms**)**

2.6. DIALYSIS OF PURIFIED PROTEIN OBTAINED FROM AMMONIUM PRECIPITATION

Theory: Dialysis is used for the removal of ammonium sulphate from the samples based on the molecular weight cut-off (MWCO). The membrane consists of pores that allow small salt molecules to pass out of the membrane and restricts proteins to pass in an isotonic solution. Thus, removing salts from the sample and purifying it.

Protocols:

- Dialysis membrane was taken and washed in running distilled water thoroughly and then kept wet.
- One end of the bag was carefully tied using a thread.
- The bag was filled with the samples from ammonium precipitation and the other end of the bag was tied.
- Dialysis bag was then dipped in respective buffers and left undisturbed at 4 °C.

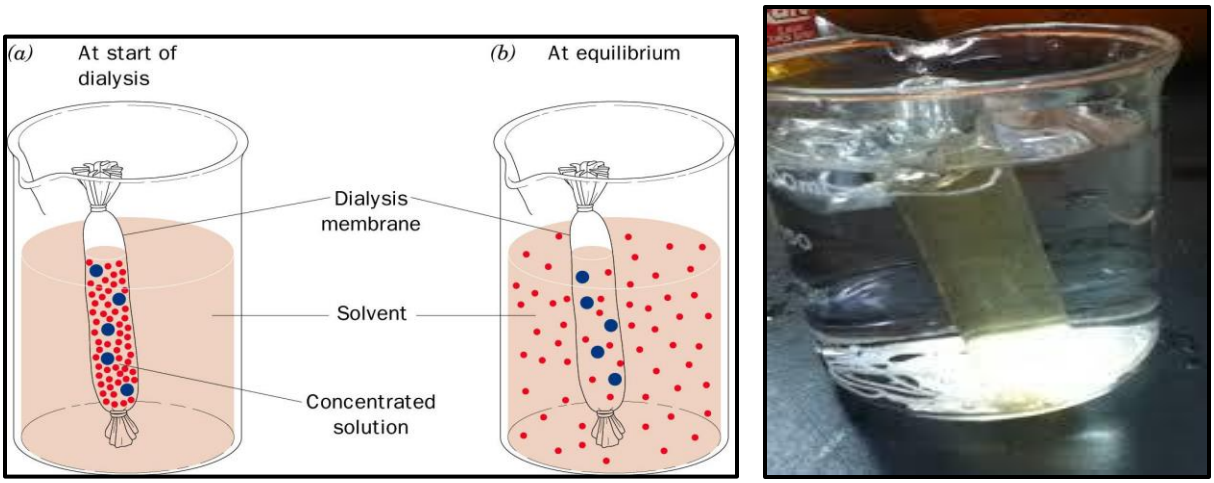


Figure 4: (1) Principle of dialysis. (2) Setup for dialysis

- After 8 hours the buffer was replaced with fresh buffer and the bag was again left undisturbed for the next 8 hours.
- Dialysis solution was taken out in a vial and was centrifuged at 7000 RPM for 10 minutes.
- Pellet and supernatant were collected in separate vials.
- Activity assay for xylanase was performed using DNS test using table 11.

Table 11: Activity table for xylanase after Dialysis

Tube no.	Enzyme (ml)	Incubate for 3 mins at 45^0 C	Substrate (ml)	Buffer (ml)	Incubate for 10 mins at 45^0 C	DNS (ml)	Heat in boiling water for 5 mins	dH_2O (ml)
Cs	0		1	1		3		5
30% 4c	1		0	1		3		5
30% 4	1		1	0		3		5
60% 4c	1		0	1		3		5

(Note: Marking goes as Cs- substrate control

4, 7, 12 are codes for organisms)

Subscript c donates controls for respective organisms**)**

2.7. PROTEIN ESTIMATION USING BRADFORD METHOD

Chemicals used: Bradford reagent, BSA standard solution (1 mg/ml)

Theory: The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilise the anionic form of the dye, causing a visible colour change.

a. A standard curve for Bradford using BSA:

- Components were added as shown in the table (12) and incubated respectively.

Table 12: Standard curve for Bradford method

Tube no.	BSA Concentration (µg/ml)	BSA Volume (µl)	dH_2O (µl)	Amount of sample added to microtiter plate in triplet(µl)	Bradford reagent (µl)	Let it rest for 5 mins and take OD at 595 nm.
1	0	0	30	10	200	
2	200	6	24	10	200	
3	400	12	18	10	200	
4	600	18	12	10	200	
5	800	24	6	10	200	
6	1000	30	0	10	200	

- After completion 200µl of the sample loaded in the ELISA plate wells and OD was taken at 595 nm in ELISA plate reader.

b. Protein estimation for unknown samples:

- Samples of proteins at different steps were taken (*crude enzyme, different cuts of ammonium precipitation, dialysis*)
- The supernatant was used in case of crude enzyme and for other two, the samples used after final centrifugation and buffer addition were used.
- With respective controls of samples, experiment was performed

- Further proceeded as per the table (13).

Table 13: Protein concentration of unknown samples					
Tube no.	**Unknown sample(µl)**	**LB media (µl)**	**Buffer**	**Bradford reagent(µl)**	**Let it rest for 5 mins and take OD at 595 nm.**
Crude control	**0**	**10**	**0**	**200**	
Crude sample	**10**	**0**	**0**	**200**	
Phosphate control	**0**	**0**	**10**	**200**	
Ammonium precipitation 30% 4	**10**	**0**	**0**	**200**	
Ammonium precipitation 60% 4	**10**	**0**	**0**	**200**	
Ammonium precipitation 90% 4	**10**	**0**	**0**	**200**	
Dialysis 30% 4	**10**	**0**	**0**	**200**	
Dialysis 60% 4	**10**	**0**	**0**	**200**	
Dialysis 90% 4	**10**	**0**	**0**	**200**	

CHAPTER 3

RESULTS

3.1. REVIVAL OF GLYCEROL STOCKS, CHECK FOR CONTAMINATION AND SUBCULTURING

The cultures used for the study were cultured in aerobic conditions at 45°C to check the thermophilic facultative anaerobes. The cultures were revived and only 7 of the strains showed growth in aerobic conditions and were further used.

3.2. QUALITATIVE ANALYSIS OF VARIOUS LIGNOCELLULOSIC ENZYME ACTIVITIES

a. Qualitative analysis of amylase activity by a starch iodine test.

Table 14: Results of qualitative analysis of amylase activity			
Numbering	**Organisms**	**Plate 1**	**Plate 2**
2	***Bacillus thermocopriae***	-	-
4	***Bacillus sonorensis***	+	+
7	***Bacillus thermocopriae***	++	++
11	***Bacillus thermocopriae***	+	+
12	***Geobacillus thermonitrificans***	+	+
16	***Brevibacillus aydinogluensis***	-	-
17	***Brevibacillus aydinogluensis***	-	-

As seen in the table (14) strains 4,7, 12 showed the most activity and larger zone of clearance.

Figure 6: Starch agar plates after flooding the plates with Gram Iodine sol^{n}

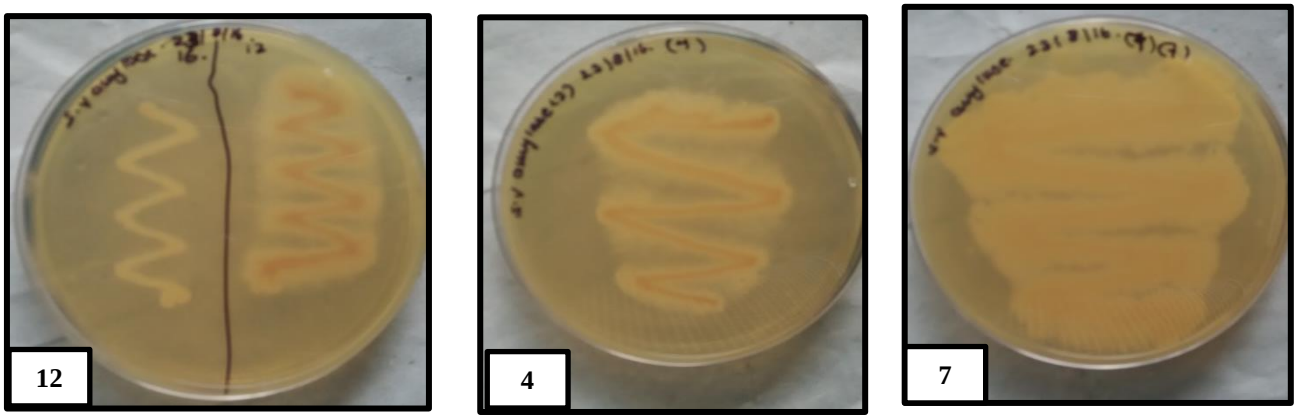


Figure 7: Starch agar plates before flooding the plates with Gram Iodine sol^{n}

b. Qualitative analysis of pullulanase activity by pullulan degrading test

Table 15: Results of qualitative analysis of pullulanase activity

Numbering	Organisms	Plate 1	Plate 2
2	*Bacillus thermocopriae*	+	+
4	*Bacillus sonorensis*	+	+
7	*Bacillus thermocopriae*	++	++
11	*Bacillus thermocopriae*	-	-
12	*Geobacillus thermonitrificans*	+	+
16	*Brevibacillus aydinogluensis*	-	-
17	*Brevibacillus aydinogluensis*	-	-

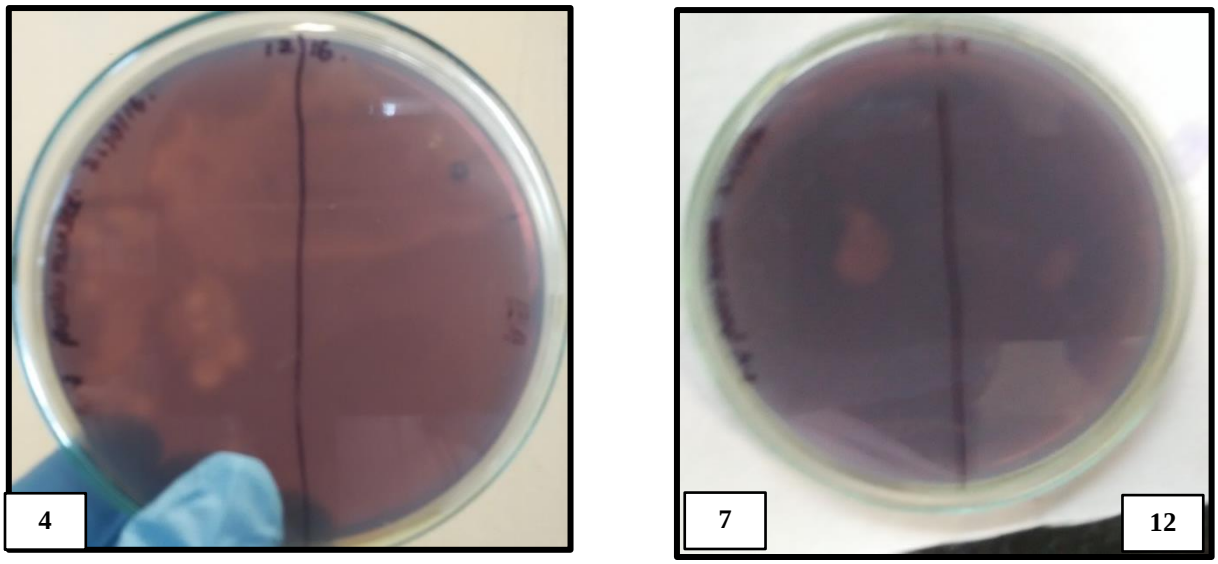
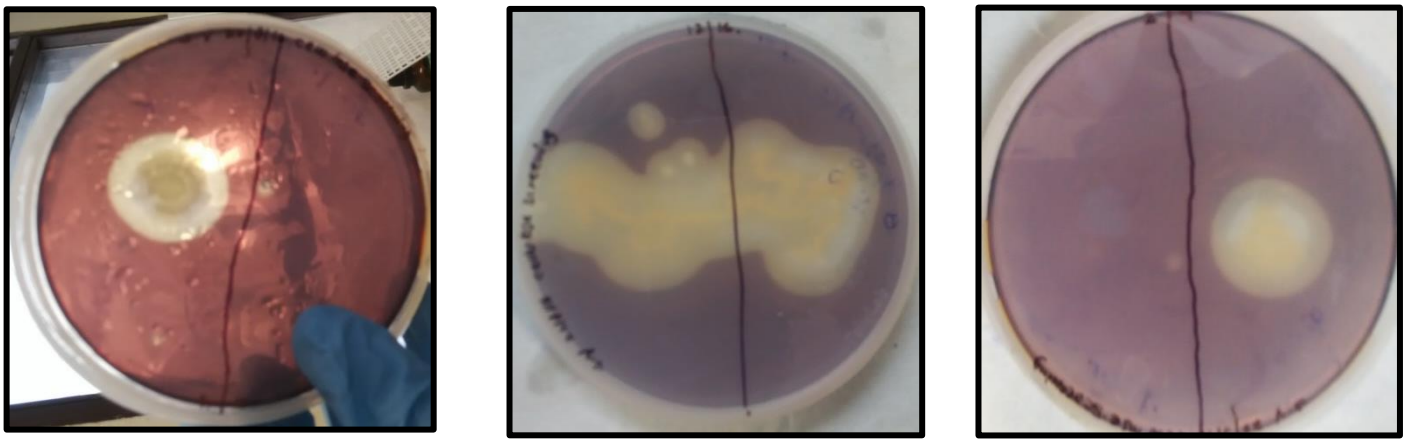


Figure 8: Pullulan agar plates showing zone of clearance with gram iodine

As seen in the table (15) strains 4,7, 12 showed positive for the activity. Strain 2 also showed some activity but not as high to visualise.

c. Qualitative analysis of Cellulase activity by Carboxymethyl cellulose (CMC) degrading test

Table 16: Results of qualitative analysis of cellulase activity			
Numbering	**Organisms**	**Plate 1**	**Plate 2**
2	***Bacillus thermocopriae***	+	+
4	***Bacillus sonorensis***	+	+
7	***Bacillus thermocopriae***	++	++
11	***Bacillus thermocopriae***	-	-
12	***Geobacillus thermonitrificans***	+	+
16	***Brevibacillus aydinogluensis***	-	-
17	***Brevibacillus aydinogluensis***	-	-



4 **12** **7**

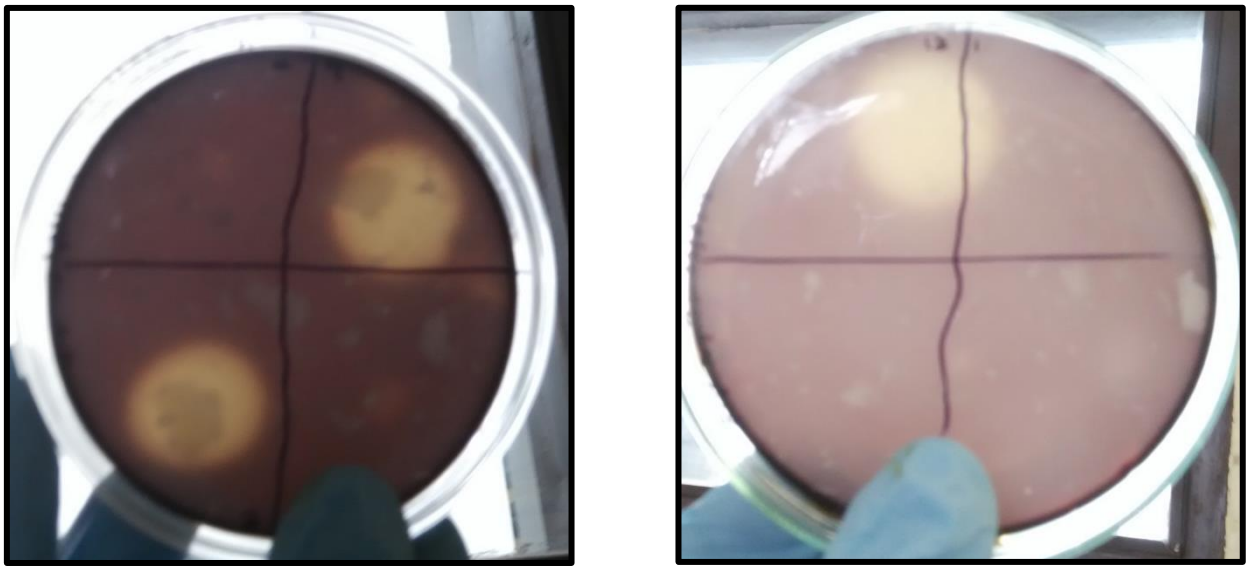
Figure 9: CMC agar plates showing zone of clearance with gram iodine

As seen in the table (16) strains 4, 7, 12 showed positive for the activity. Strain 2 also showed some activity but not as high to visualise.

d. Qualitative analysis of Xylanase activity by Xylan-degrading test

Table 17: Results of qualitative analysis of xylanase activity			
Numbering	**Organisms**	**Plate 1**	**Plate 2**
2	***Bacillus thermocopriae***	-	-
4	***Bacillus sonorensis***	++	++
7	***Bacillus thermocopriae***	++	++
11	***Bacillus thermocopriae***	-	-
12	***Geobacillus thermonitrificans***	+	+
16	***Brevibacillus aydinogluensis***	-	-
17	***Brevibacillus aydinogluensis***	-	-

As seen in the table (17) strains 4,7, 12 showed positive for the activity.

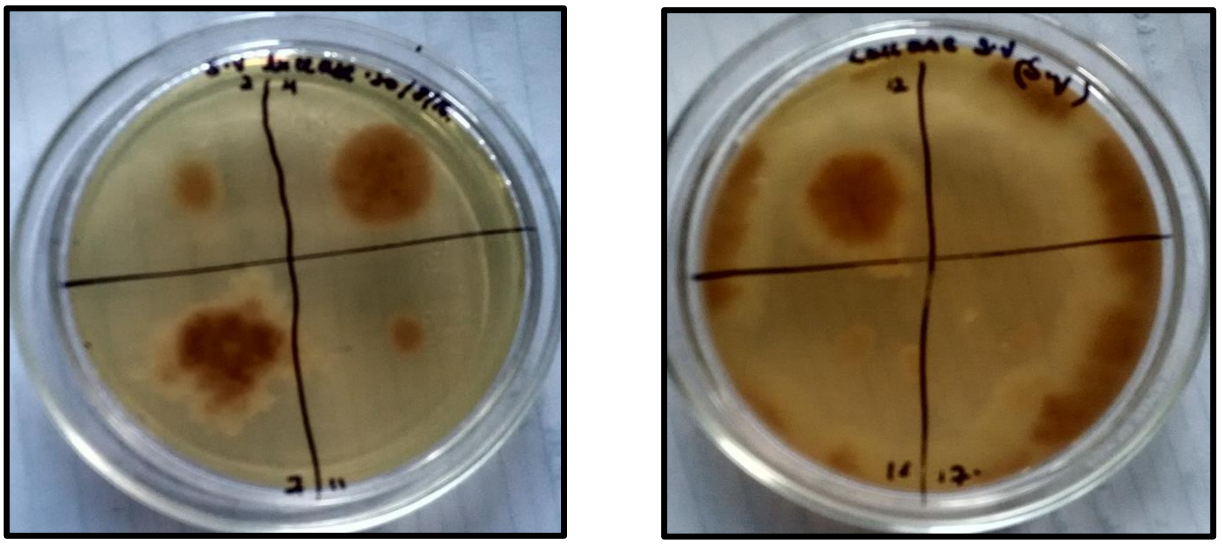


4 **7** **12**

Figure 10: CMC agar plates showing zone of clearance with gram iodine

e. Qualitative analysis of Laccase activity by Guaiacol degrading test

Table 18: Results of qualitative analysis of guaiacol activity			
Numbering	**Organisms**	**Plate 1**	**Plate 2**
2	***Bacillus thermocopriae***	-	-
4	***Bacillus sonorensis***	++	++
7	***Bacillus thermocopriae***	++	++
11	***Bacillus thermocopriae***	-	-
12	***Geobacillus thermonitrificans***	+	+
16	***Brevibacillus aydinogluensis***	-	-
17	***Brevibacillus aydinogluensis***	-	-



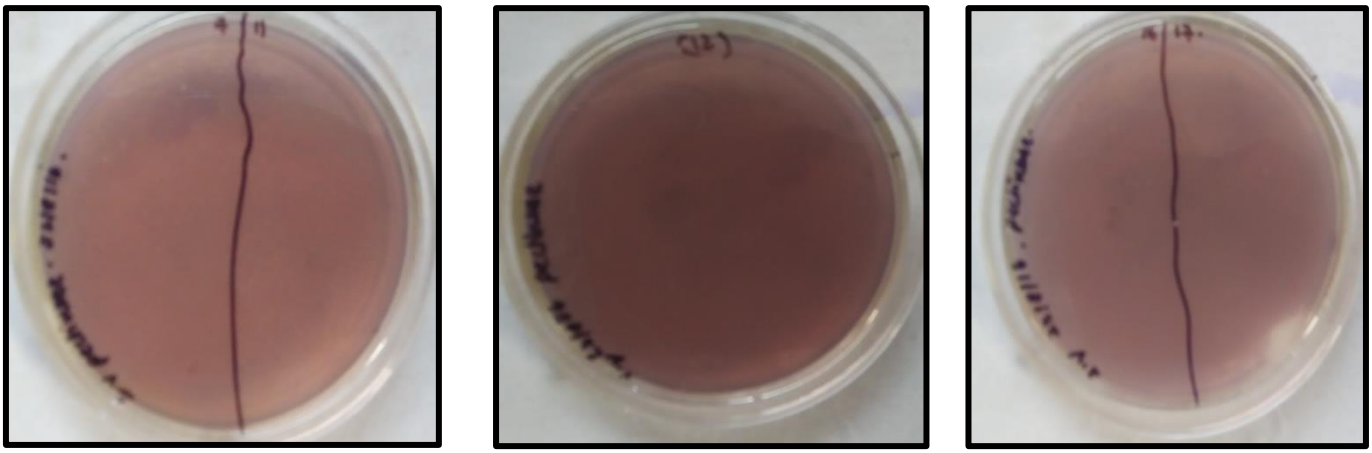
7 **4** **12**

Figure 11: Guaiacol agar plates showing formation of characteristic brown color

As seen in the table (18) strains 4, 7, 12 showed positive for the activity.

f. Qualitative analysis of Pectinase activity by Pectin degrading test

Table 19: Results of qualitative analysis of pectinase activity			
Numbering	**Organisms**	**Plate 1**	**Plate 2**
2	***Bacillus thermocopriae***	**-**	**-**
4	***Bacillus sonorensis***	**-**	**-**
7	***Bacillus thermocopriae***	**-**	**-**
11	***Bacillus thermocopriae***	**-**	**-**
12	***Geobacillus thermonitrificans***	**-**	**-**
16	***Brevibacillus aydinogluensis***	**-**	**-**
17	***Brevibacillus aydinogluensis***	**-**	**-**



4 **7** **12** **16** **17**

Figure 12: Pectin agar plates with gram iodine

In the case of pectinase, all enzymes showed negative results.

From the above data, it was seen three isolates 4, 7 and 12 showed the positive activity of all the enzymes (except pectinase). These isolates were selected for further analysis.

3.3.QUANTITATIVE ACTIVITY ANALYSIS OF VARIOUS ENZYMES

Substrate used: Glucose (1mg/ml), Xylose (1mg/ml)

A standard curve for glucose:

Absorbance was plotted after blank reduction as shown in the table (20).

Table 20: Results for glucose standard curve		
Tube No.	**Glucose concentration(mg/ml)**	**Absorbance of sample at 540nm**
2	0.2	0.86
3	0.4	1.82
4	0.6	2.64
5	0.8	3.36
6	1	3.97

Final O.D = O.D of Sample – OD of Blank

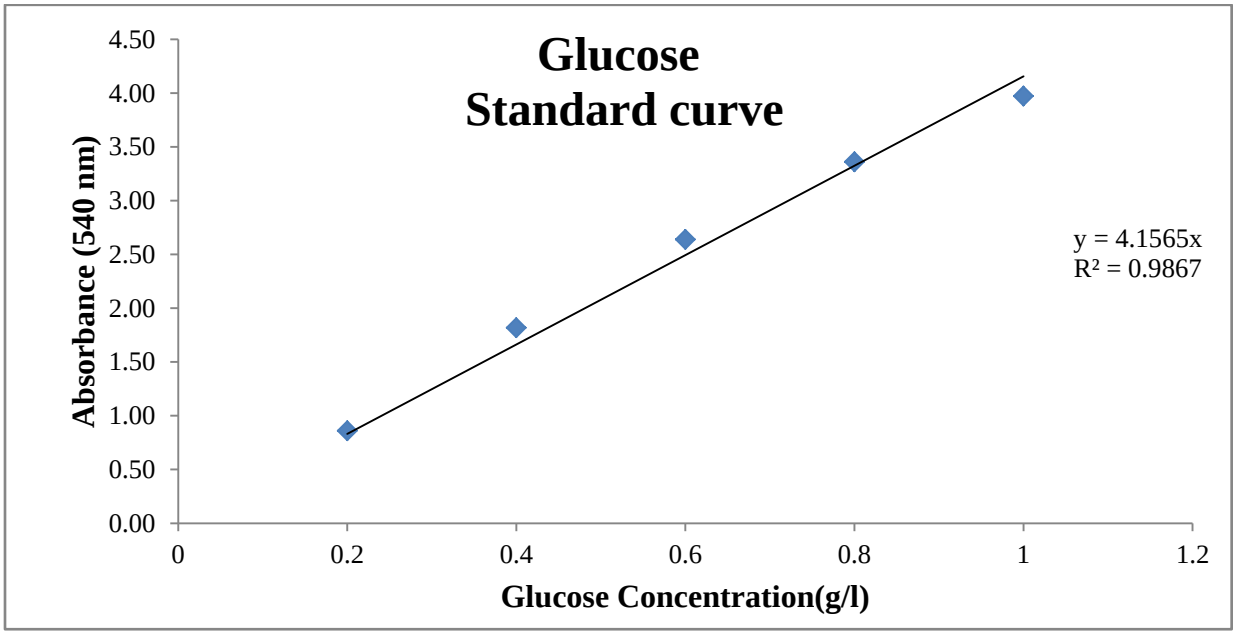


Figure 13: Standard curve of glucose using DNS method

A standard curve for Xylose:

Absorbance was plotted after blank reduction as shown in the table (21).

Table 21: Results for Xylose standard curve		
Tube No.	**Xylose concentration(mg/ml)**	**Absorbance of sample at 540nm**
2	**0.2**	1.103
3	**0.4**	2.01
4	**0.6**	3.216
5	**0.8**	3.959
6	**1**	4.559

Final O.D = O.D of Sample – OD of Blank

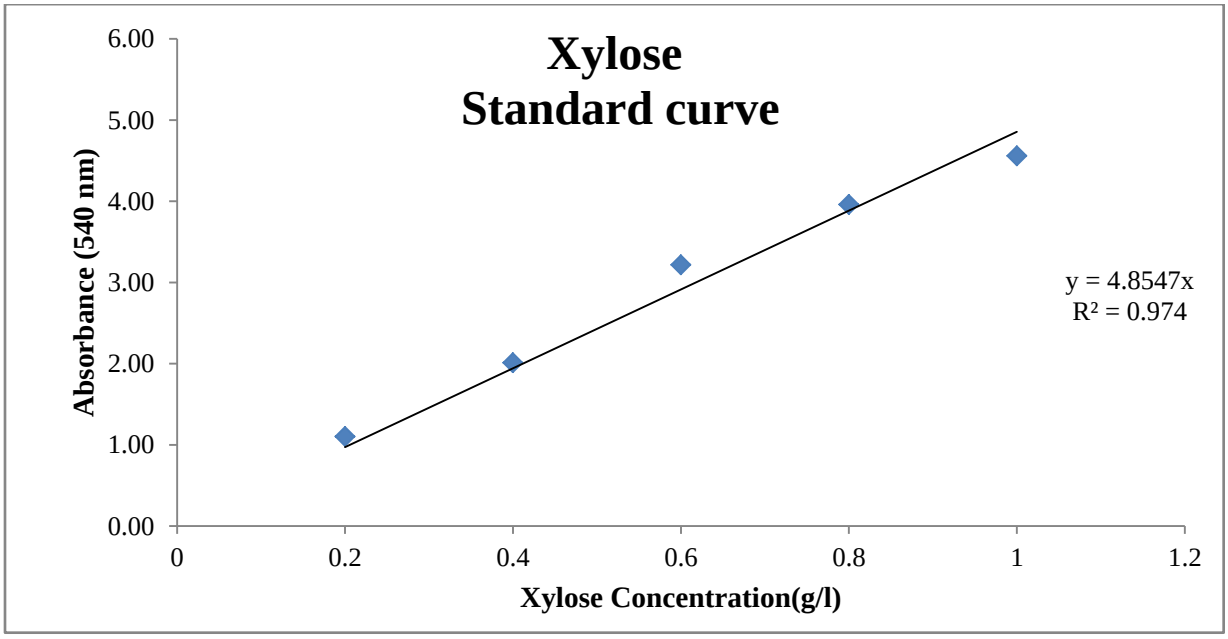


Figure 14: Standard curve of xylose using DNS method

a. **Amylase activity assay:** O.D of samples were taken at 540nm and Enzyme activity was calculated as,

- First Final O.D of the sample was calculated as,
- *Final O.D: O.D of sample – O.D of control*
- Unknown concentration of glucose was calculated from the standard plot, and using that the Enzyme activity was calculated by the formulae,

Enzyme activity (IU/ml) = (Concentration of Glucose (mg/ml)*1000)/ (Incubation time (mins)*Volume of Enzyme (ml)*molecular weight of glucose)

Table 22: Results of amylase activity by DNS method

Sample code	Absorbance of sample	Unknown Glucose concentration (mg/ml)	Enzyme activity(IU/ml)
4_1	0.428	1.86	2.069
4_2	0.347	1.55	1.719
7_1	0.442	1.92	2.129
7_2	0.512	2.19	2.432
12_1	0.373	1.65	1.832
12_2	0.324	1.46	1.620

(Note: Marking goes as Cs- substrate control

4, 7, 12 are codes for organisms (table1))

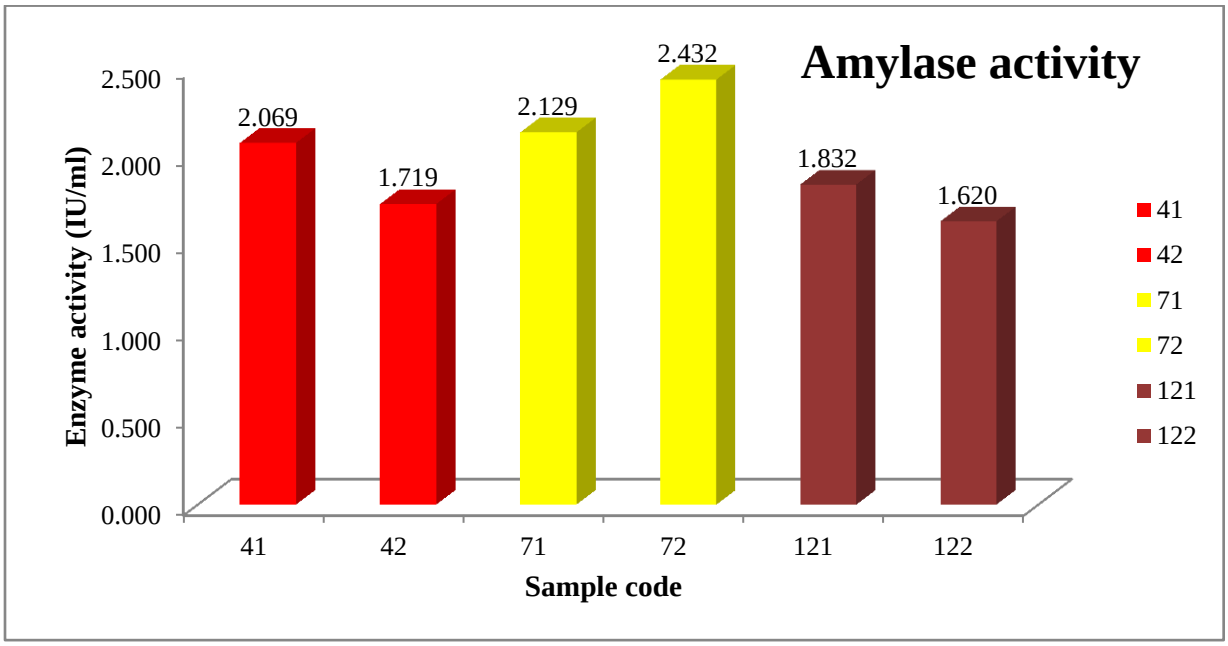


Figure 15: Amylase activity of different organisms using DNS method

b. Xylanase activity assay: O.D of samples were taken at 540 nm and Enzyme activity was calculated as,

- First Final O.D of the sample was calculated as,
- *Final O.D: O.D of sample – O.D of control*
- Unknown concentration of glucose was calculated from the standard plot, and using that the Enzyme activity was calculated by the formulae

Enzyme activity (IU/ml) = (Concentration of xylose (mg/ml)*1000)/ (Incubation time (mins)*Volume of Enzyme (ml)*molecular weight of xylose)

Table 23: Results of xylanase activity by DNS method

Sample code	Absorbance of sample	Unknown concentration (mg/ml)	Enzyme activity(IU/ml)
4_1	0.024	0.42	0.278
4_2	0.03	0.44	0.296
7_1	0.112	0.81	0.538
7_2	0.111	0.80	0.535
12_1	0.033	0.46	0.305
12_2	0.045	0.51	0.340

(Note: Marking goes as Cs- substrate control

4, 7, 12 are codes for organisms (table1))

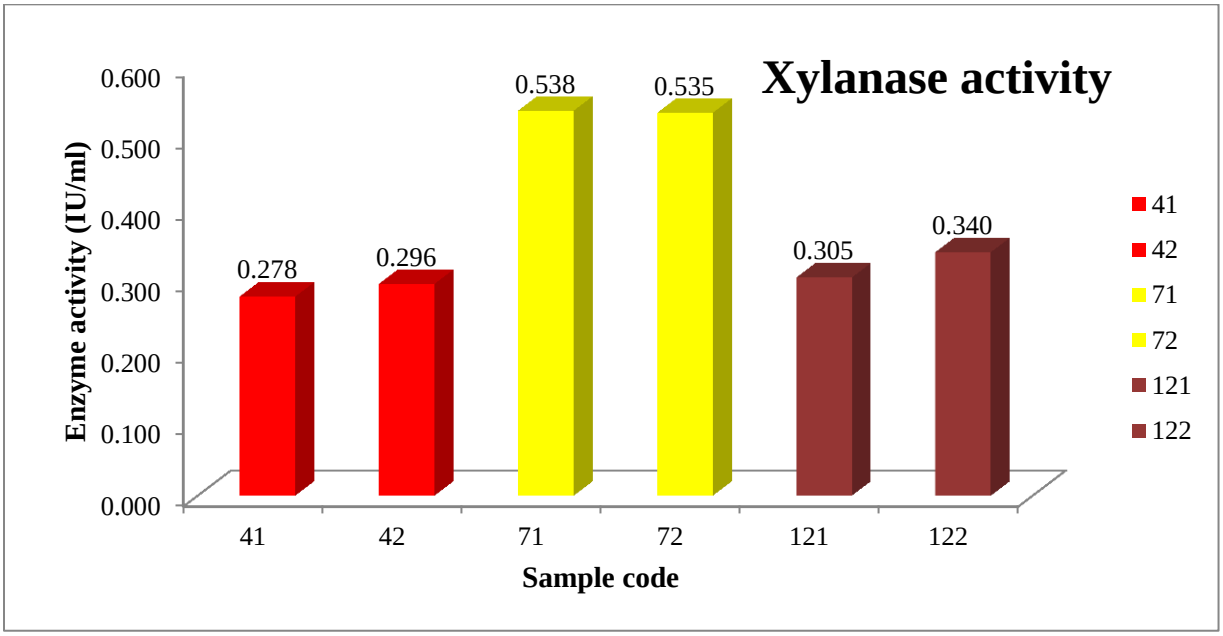


Figure 16: xylanase activity of different organisms using DNS method

c. Cellulase enzyme activity: Activity of Cellulase was very low in the CMC media; it may be due to any experimental error or low production of Cellulase in media.

The activity of 4 and 7 culture was high in case of amylase and 7 and 12 was high in case of xylanase.

3.4. QUANTITATIVE ANALYSIS ENZYME ACTIVITY BY DNS METHOD USING SPECIFIC SUBSTRATE.

Same 3 cultures 4, 7 and 12 were used to check the ability of these organisms to degrade a specific substrate like pine needles.

A. **Preparation of seed culture for enzyme production.**

- Cultures were grown successfully in LB media at 45° C for 24 hrs.
- OD for the strains 4, 7, 12 in the pellet at 600 nm is given in table 24.

Table 24: OD at which inoculum was taken for culture

Organism Tag.	OD at 600 nm
4	0.801
7	0.360
12	0.656

At this OD the cells were taken and cultured in the substrate specific pine media

B. The activity of amylase in substrate specific media.

Enzyme activity: O.D of samples were taken at 540nm and Enzyme activity was calculated as,

- First Final O.D of the sample was calculated as,
- *Final O.D: O.D of sample – (O.D of Substrate control – O.D of Enzyme Control)*
- Unknown concentration of glucose was calculated from the standard plot, and using that the Enzyme activity was calculated by the formulae,

Enzyme activity (IU/ml) = (Concentration of Glucose (mg/ml)*1000)/ (Incubation time (mins)*Volume of Enzyme (ml)*molecular weight of glucose)

- The process was followed for 96 hours by taking OD periodically at 24 hours interval.

Table 25: Activity of amylase in substrate degradation media per day				
Sample ID	**Enzyme activity (IU/ml) Day 1**	**Enzyme activity (IU/ml) Day 2**	**Enzyme activity (IU/ml) Day 3**	**Enzyme activity (IU/ml) Day 4**
4	1.056	0.999	1.048	1.158
7	1.117	1.190	1.158	1.280
12	0.670	0.755	0.812	0.771

(**Note:** 4, 7, 12 are codes for organisms Annexure)

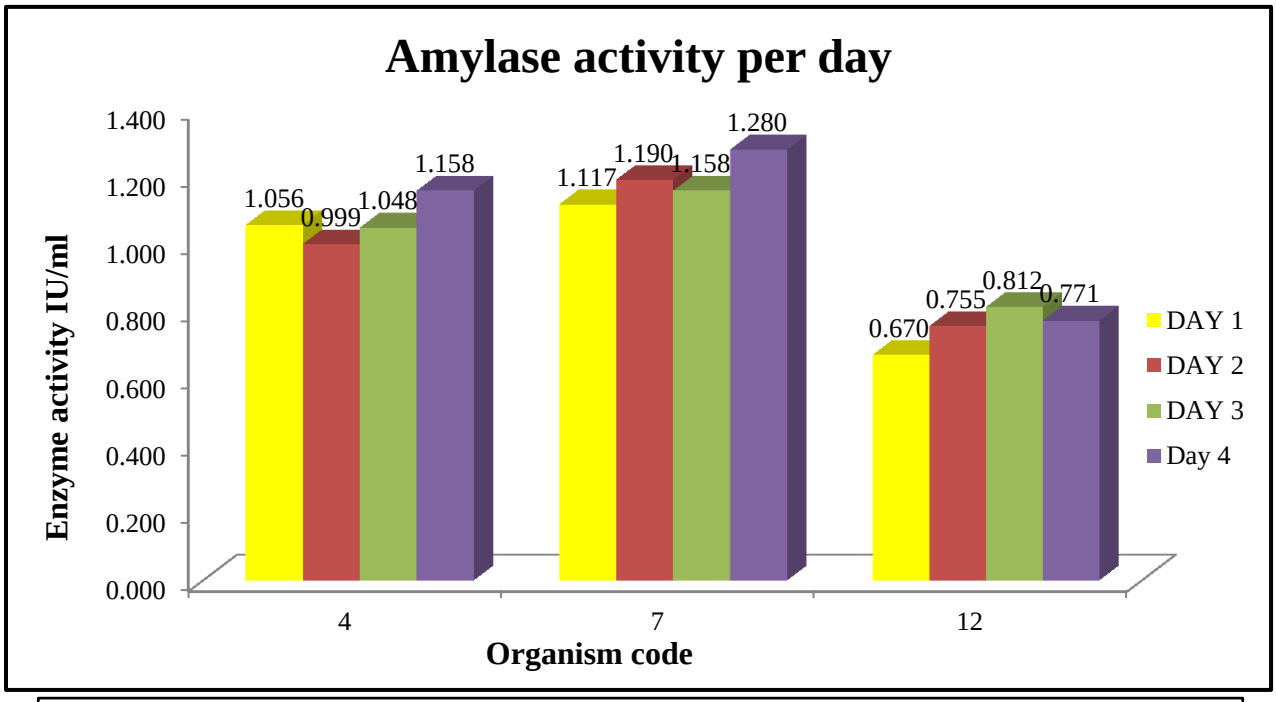


Figure 17: Comparative analysis of amylase activity in different organisms each day

Maximum activity was seen for 4 and 7th strain with increasing activity each day. Thus amylase activity was confirmed by quantitative analysis in specific substrate media.

C. The activity of Xylanase in substrate specific media.

Enzyme activity: O.D of samples were taken at 540nm and Enzyme activity was calculated as,

- First Final O.D of the sample was calculated as,
- *Final O.D: O.D of sample – (O.D of Substrate control – O.D of Enzyme Control)*

- Unknown concentration of xylose was calculated from the standard plot, and using that the Enzyme activity was calculated by the formulae,

Enzyme activity (IU/ml) = (Concentration of Xylose (mg/ml)*1000)/ (Incubation time (mins)*Volume of Enzyme (ml)*molecular weight of Xylose)

- The process was followed for 96 hours by taking OD periodically at 24 hours interval.

Table 26: Activity of xylanase in substrate degradation media per day

Sample ID	Enzyme activity (IU/ml) Day 1	Enzyme activity (IU/ml) Day 2	Enzyme activity (IU/ml) Day 3	Enzyme activity (IU/ml) Day 4
4	1.402	1.977	2.155	1.997
7	1.654	2.127	2.159	1.974
12	2.088	2.064	2.025	1.725

(**Note:** 4, 7, 12 are codes for organisms Annexure)

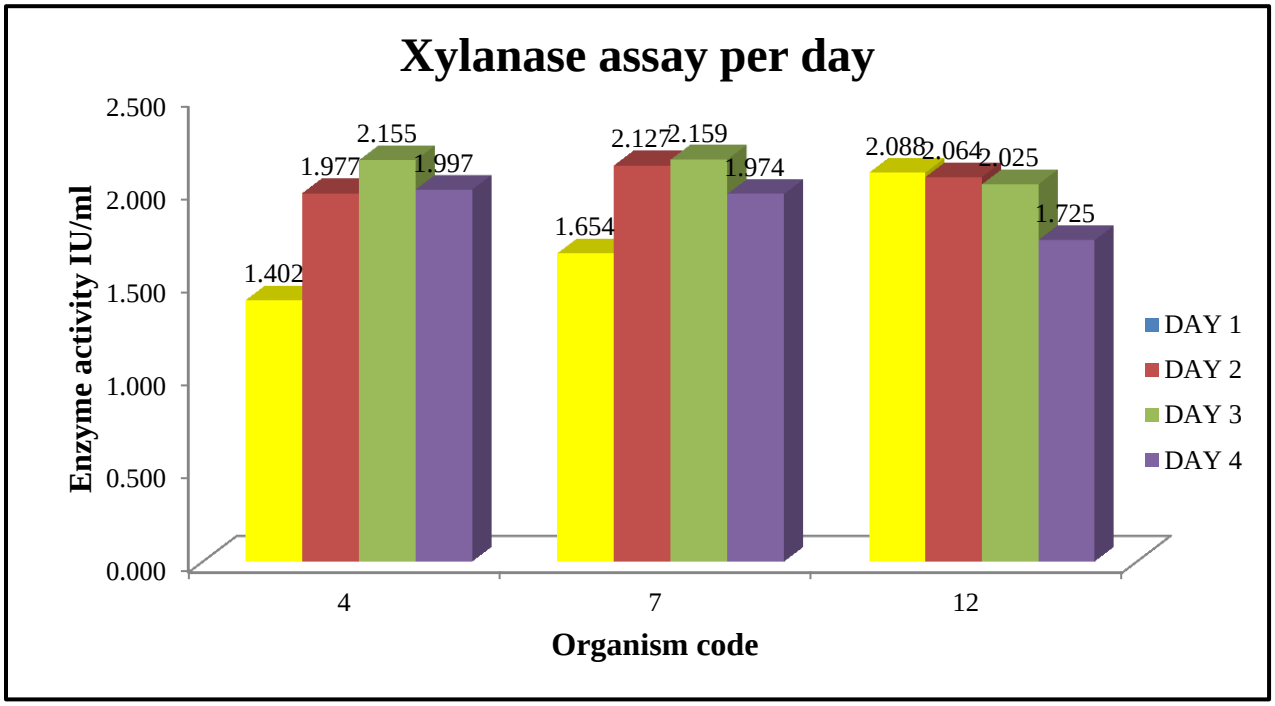


Figure 18: Comparative analysis of xylanase activity in different organisms each day

Maximum activity was seen for 4 and 7th strain with increasing activity each day. Thus Xylanase activity was confirmed by quantitative analysis. The highest activity was seen for xylanase enzyme, Also after 4th-day activity degraded as the media volume depleted.

D. Cellulase substrate degradation and activity check

Enzyme activity: O.D of samples were taken at 540nm and Enzyme activity was calculated as,

- First Final O.D of the sample was calculated as,
- *Final O.D: O.D of sample – (O.D of Substrate control – O.D of Enzyme Control)*
- Unknown concentration of glucose was calculated from the standard plot, and using that the Enzyme activity was calculated by the formulae,

Enzyme activity (IU/ml) = (Concentration of Glucose (mg/ml)*1000)/ (Incubation time (mins)*Volume of Enzyme (ml)*molecular weight of Glucose)

- The process was followed for 96 hours by taking OD periodically at 24 hours interval.

Table 27: Activity of cellulase in substrate degradation media per day

Sample ID	Enzyme activity (IU/ml) Day 1	Enzyme activity (IU/ml) Day 2	Enzyme activity (IU/ml) Day 3	Enzyme activity (IU/ml) Day 4
4	0.288	0.316	0.306	0.251
7	0.304	0.394	0.398	0.461
12	0.339	0.392	0.400	0.272

(**Note:** 4, 7, 12 are codes for organisms Annexure)

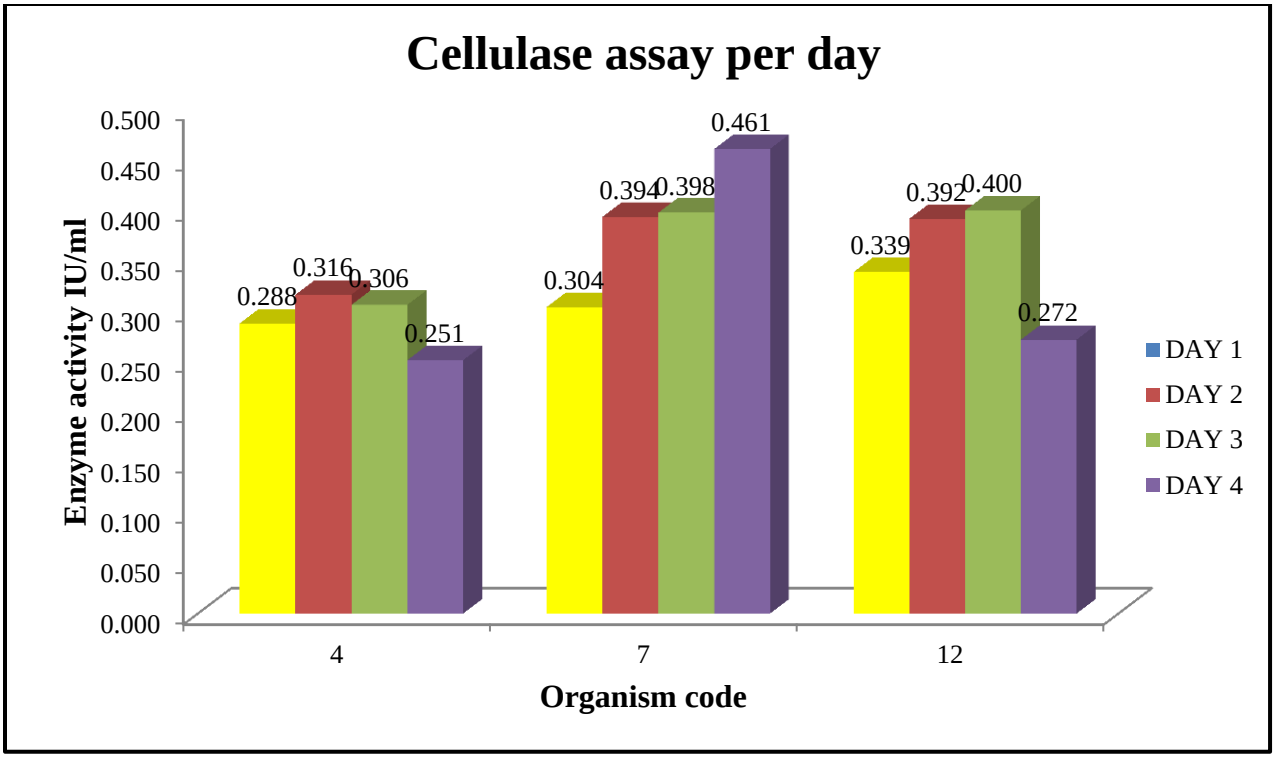


Figure 19: Comparative analysis of Cellulase activity in different organisms each day

Maximum activity was seen for 4 and 7th strain with increasing activity each day. Thus Cellulase activity was confirmed by quantitative analysis. Further processing will be done using the strain 4.

3.5. PROTEIN PURIFICATION BY AMMONIUM PRECIPITATION AND DIALYSIS

The purification table for xylanase is shown in table 25. Total protein content in calculated using Bradford method discussed in **(3.6)**

For the 4th culture better purification was seen at 60 % concentration of ammonium sulphate both maximum activity and purification fold. Other parameters like yield and specific activity were good at this time. Also, at 30% concentration the results were good so maybe most of the enzyme got purified at 30% cut and then complete purification was achieved at the 60% cut.

Table 28: Purification table for xylanase						
Steps	**Total Enzyme activity (IU/ml)**	**Total protein content (mg/ml)**	**Specific activity (IU/ml)**	**Purification fold**	**Purification yield (%)**	**Ammonium precipitation cut (%)**
Crude	1.664	0.1264	13.16	0	100	
Ammonium precipitation	1.179	0.013	90.69	6.889	70.853	**30**
	1.173	0.0146	80.34	6.103	70.492	**60**
	1.3	0.0322	40.37	3.067	78.125	**90**
Dialysis	1.253	0.0083	150.96	11.467	75.300	**30**
	1.208	0.0124	97.42	7.400	72.596	**60**
	1.418	0.0307	46.19	3.508	85.216	**90**

The above parameters are calculated as,

Specific activity = Total Enzyme activity (IU/ml)/ Total protein content (mg/ml)

Purification fold = Specific activity at each step (IU/ml)/ Specific activity in crude sample (IU/ml)

Yield % = Enzyme activity at each step (IU/ml)/ Enzyme activity in crude sample (IU/ml)

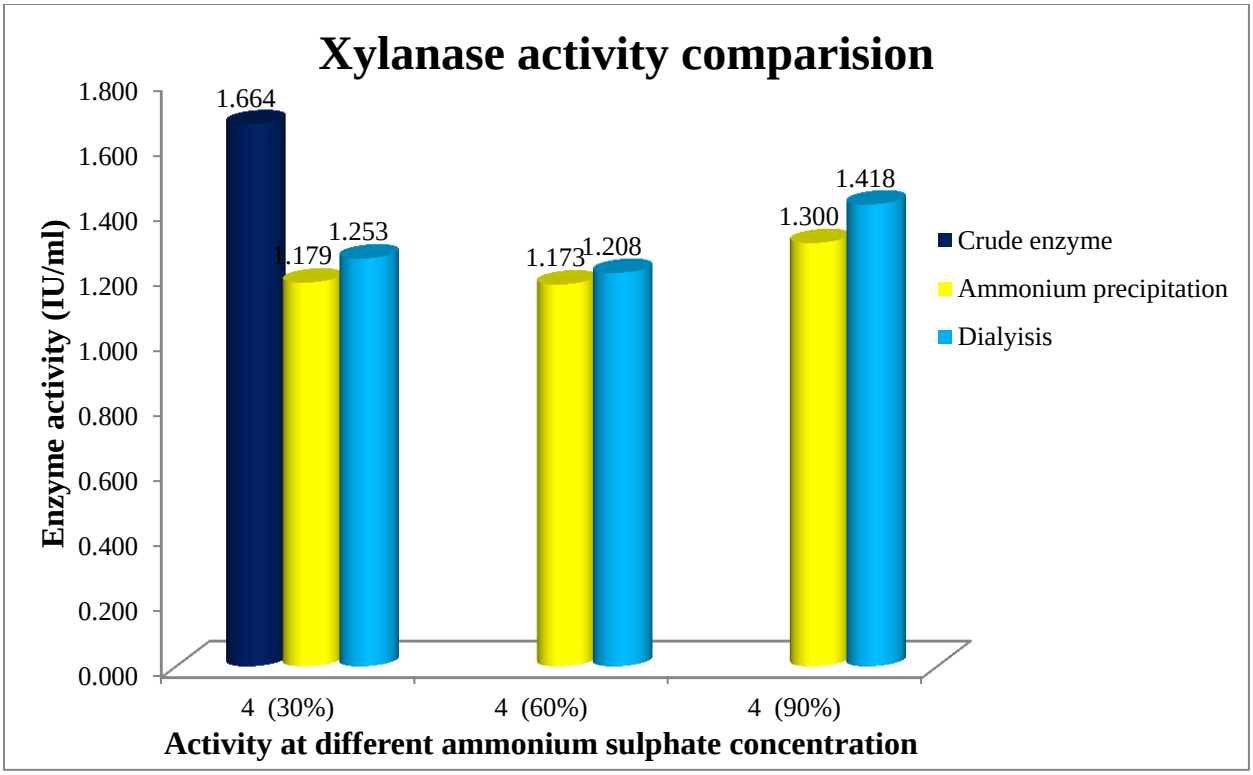


Figure 20: Comparative analysis of Xylanase activity at each step of purification

3.6. PROTEIN ESTIMATION USING BRADFORD METHOD

Standard Curve: Absorbance was plotted after blank reduction as shown in the table. Sample OD was taken in triplets and the average of all was calculated respectively and used.

Table 29: OD for standard curve of BSA

Tube	BSA concentration (mg/ml)	O.D at 595 nm
1	0.2	0.20
2	0.4	0.32
3	0.6	0.50
4	0.8	0.66
5	1	0.78

Final O.D = O.D of Sample – OD of Blank

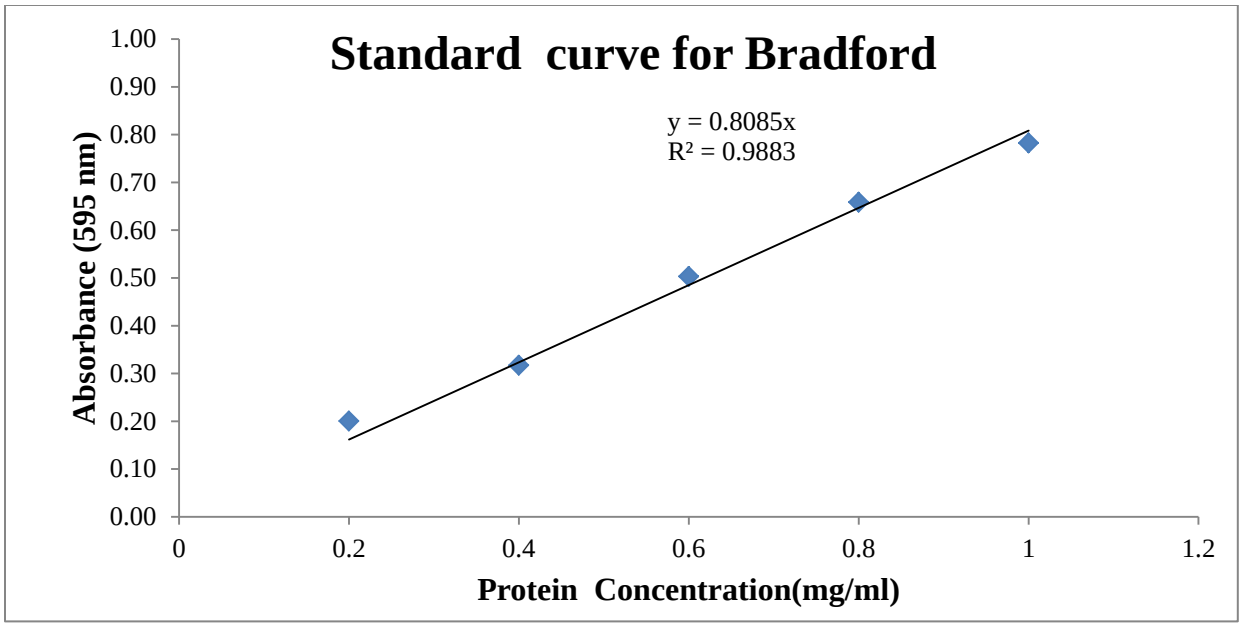


Figure 21: Standard plot of BSA

Protein concentration:

- O.D of samples was taken at 595nm.
- Final O.D of the sample was calculated from the graph and showed in table 25.

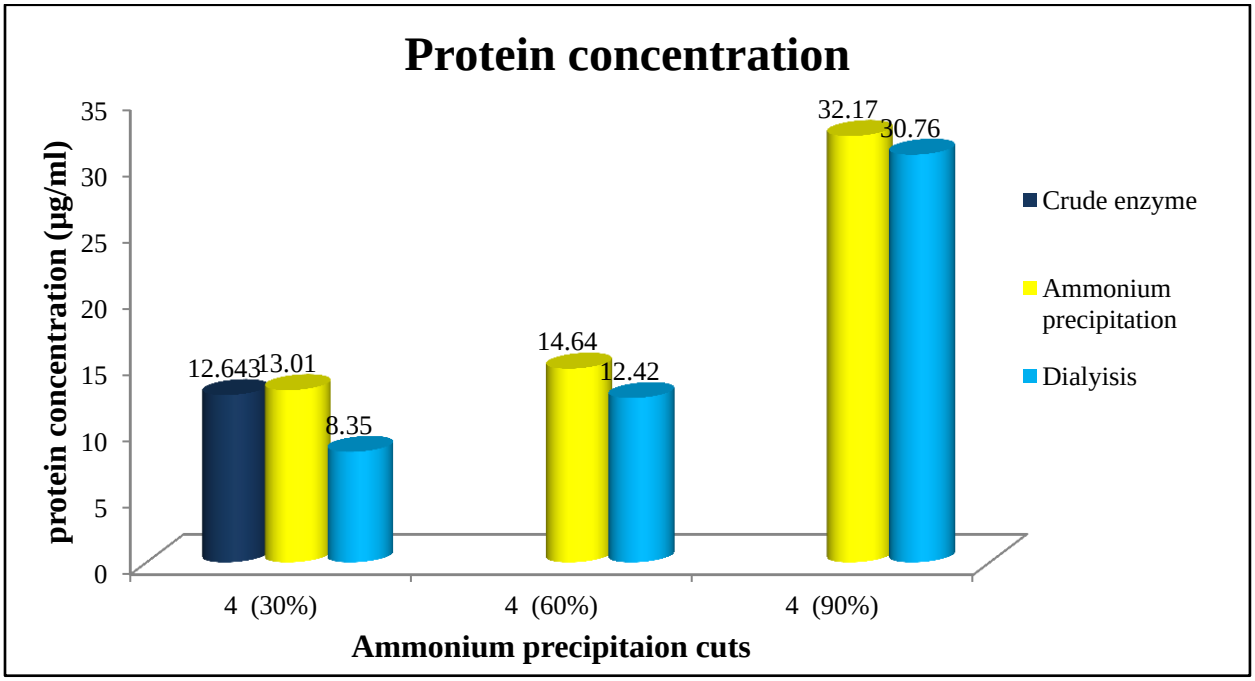


Figure 22: Protein concentration at different steps of purification

3.7.CONCLUSION

Initially, 20 noble anaerobic strains of thermophilic bacteria were isolated from the biogas digester sample. Initial growth showed 7 facultative anaerobic strains growing under aerobic condition. These cultures were further subcultured and used for qualitative analysis of enzyme activity. 3 strains 4, 7 and 12 showed the good activity of Cellulase, Xylanase, Pullulanase, Amylase and Laccase. After this, the enzymes were analysed by quantitative activity assay in specific media. This showed the better activity of 4^{th} and 7^{th} cultures for xylanases and amylase enzyme. Further substrate degradation was performed and enzyme activity was checked. The above three strains showed activity which increased each day. In pine needle media the activity of all three enzymes i.e. amylase cellulase and xylanase was seen which showed that, organism are efficiently degrading pine needles. All three organisms showed good activity, out of which strain 4 was selected for purification purpose due to its good activity in substrate degrading media for all 3 enzymes. In the purification steps, activity of xylanase was calculated in each step and purification table was formed which showed, for the 4^{th} culture better purification was seen at 30 % concentration of ammonium sulphate both maximum activity and purification fold. Other parameters like yield and specific activity were good at this time. Also, at 60% concentration the results were good so maybe most of the enzyme got purified at 30% cut and then complete purification was achieved at the 60% cut.

The organism *Bacillus sonorensis* and *Bacillus thermocopriae* stain 4 can be used in degradation of different substrates like pine needles studied above. The benefit of using these cultures are, a) they are facultative anaerobes, b) they are thermophilic and grow better at relatively high temperatures, c) the secrete a combination of different enzymes like Cellulase, xylanase, amylase and laccase which gives it a cutting edge over other organisms and their combination used in present.

3.8.FUTURE WORK:

There is a lot of potential in the study of these organisms as they are of high industrial value based on,

- They are facultative anaerobes and thermophilic in nature
- They produce a combination of enzymes perfect for lignocellulosic and polysaccharide degradation.

Further study of the specific strains 4^{th}, 7^{th} and 12^{th} can be done to check their efficiency on different substrates and also in scaling up processes.

CHAPTER 4

REFERENCES AND ANNEXURES

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4.2. ANNEXURE

1. LB media composition

Composition	Quantity
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Distilled water	1 L
pH	7.0

2. Starch agar media composition

Composition	Quantity
Beef extract	3g
Soluble starch	10g
Agar	12g
Distilled water	1 L

3. Pullulan agar media composition

Composition	Quantity(g/l)
Pullulan	10
NaCl	2
$MgSO_4.7H_2O$	0.1
K_2HPO_4	0.17
$KH_2PO_4.7H_2O$	0.12
Agar	15
pH	7.5±0.2

4. CMC Agar Composition

Composition	Quantity(g)
CMC	0.5
$NaNO_3$	0.1
K_2HPO_4	0.1
KCl	0.1
$MgSO_4$	0.05
Yeast extract	0.05
Glucose	0.1
Water	100 ml

5. Xylanase Agar Composition

Composition	Quantity(g/l)
Xylan	0.80
KNO_3	2
K_2HPO_4	1
$CaCO_3$	3
$FeSO_4$	0.01
$MgSO_4$	0.5
Agar	20

6. Guaiacol Agar composition

Composition	Quantity (%)
Guaiacol	0.01
Yeast extract	1
Agar	2

7. Pectin Agar Composition

Composition	Quantity(g/l)
$NaNO_3$	3
Sucrose	30
K_2HPO_4	1
KCl	0.5
$FeSO_4$	0.01
$MgSO_4$	0.5
Agar	20
Pectin	15

8. DNS Reagent composition

Composition	Quantity
DNS reagent	1g
2 M Sodium hydroxide(NaOH)	20 ml
Sodium potassium tartrate	30 g
Distilled water	80 ml (approx.)

Made by adding 1 g of DNS in 20 ml 2M NaOH, add water to make volume of 60 ml and slowly add sodium potassium tartrate 30 g by heating and continuous mixing on hot plate magnetic stirrer, and dilute to final volume of 100 ml.

9. Sodium phosphate buffer

Composition	Concentration Needed	Added to final Buffer
Mono basic Dihydrogen Phosphate (1)	1 M	
Dibasic Mono Hydrogen Phosphate(2)	1 M	
Sodium Phosphate Buffer	1 M (*48 ml (1) +52 ml (2)*)	
Sodium Chloride (NaCl)	1 M	
Sodium phosphate	**0.02**	**2 ml**

concentration for final buffer		
NaCl concentration for final buffer	**0.006**	**0.6 ml**
Distilled water		**97.4 ml**

Above table shows the stepwise approach of making final buffer solution.

10. Citrate Buffer

A: 0.1M Citric acid Buffer

B: 0.1M Sodium citrate Buffer

pH	A(ml)	B(ml)
4.8	23	27
5.3	17	33

11. Amylase production media

Composition	Quantity
Beef extract	3g
Soluble starch	10g
Distilled water	1 L

12. Xylanase Production media

Composition	Quantity(g/l)
Xylan	0.80
KNO_3	2
K_2HPO_4	1
$CaCO_3$	3
$FeSO_4$	0.01
$MgSO_4$	0.5
Distilled water	1L

13. Cellulase production media

Composition	Quantity(g/l)
CMC	5
$NaNO_3$	1
K_2HPO_4	1
KCl	1
$MgSO_4$	0.5
Yeast extract	0.5
Glucose	1

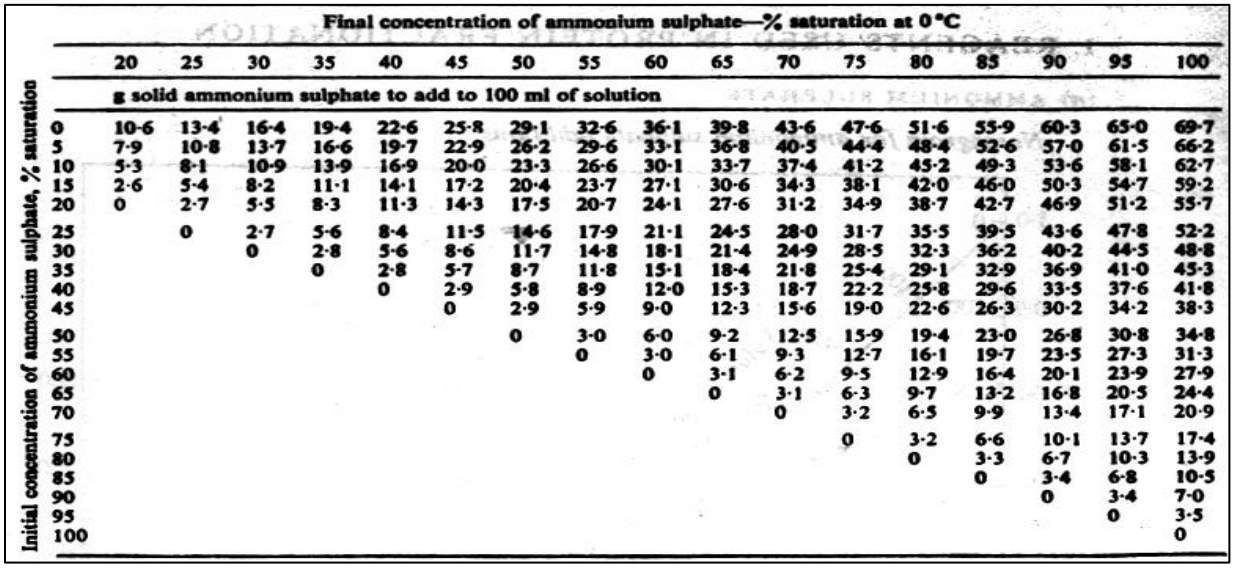
14. PBS Buffer

Composition	Quantity in 100 ml
NaCl	1g
KCl	20 ml
Na_2HPO_4	30 g
KH_2PO_4	80 ml (approx.)

15. Pine needle specific media

Composition	Quantity in 100 ml
LB media	For 100 ml
Pine needles	2%

16. Ammonium sulfate precipitation chart



Final concentration of ammonium sulphate—% saturation at 0 °C

Initial concentration of ammonium sulphate, % saturation	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	g solid ammonium sulphate to add to 100 ml of solution																
0	10·6	13·4	16·4	19·4	22·6	25·8	29·1	32·6	36·1	39·8	43·6	47·6	51·6	55·9	60·3	65·0	69·7
5	7·9	10·8	13·7	16·6	19·7	22·9	26·2	29·6	33·1	36·8	40·5	44·4	48·4	52·6	57·0	61·5	66·2
10	5·3	8·1	10·9	13·9	16·9	20·0	23·3	26·6	30·1	33·7	37·4	41·2	45·2	49·3	53·6	58·1	62·7
15	2·6	5·4	8·2	11·1	14·1	17·2	20·4	23·7	27·1	30·6	34·3	38·1	42·0	46·0	50·3	54·7	59·2
20	0	2·7	5·5	8·3	11·3	14·3	17·5	20·7	24·1	27·6	31·2	34·9	38·7	42·7	46·9	51·2	55·7
25		0	2·7	5·6	8·4	11·5	14·6	17·9	21·1	24·5	28·0	31·7	35·5	39·5	43·6	47·8	52·2
30			0	2·8	5·6	8·6	11·7	14·8	18·1	21·4	24·9	28·5	32·3	36·2	40·2	44·5	48·8
35				0	2·8	5·7	8·7	11·8	15·1	18·4	21·8	25·4	29·1	32·9	36·9	41·0	45·3
40					0	2·9	5·8	8·9	12·0	15·3	18·7	22·2	25·8	29·6	33·5	37·6	41·8
45						0	2·9	5·9	9·0	12·3	15·6	19·0	22·6	26·3	30·2	34·2	38·3
50							0	3·0	6·0	9·2	12·5	15·9	19·4	23·0	26·8	30·8	34·8
55								0	3·0	6·1	9·3	12·7	16·1	19·7	23·5	27·3	31·3
60									0	3·1	6·2	9·5	12·9	16·4	20·1	23·9	27·9
65										0	3·1	6·3	9·7	13·2	16·8	20·5	24·4
70											0	3·2	6·5	9·9	13·4	17·1	20·9
75												0	3·2	6·6	10·1	13·7	17·4
80													0	3·3	6·7	10·3	13·9
85														0	3·4	6·8	10·5
90															0	3·4	7·0
95																0	3·5
100																	0