Isolation, Purification and Characterization of Amylase Enzyme from *Bacillus atrophaeus.*

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BIOTECHNOLOGY

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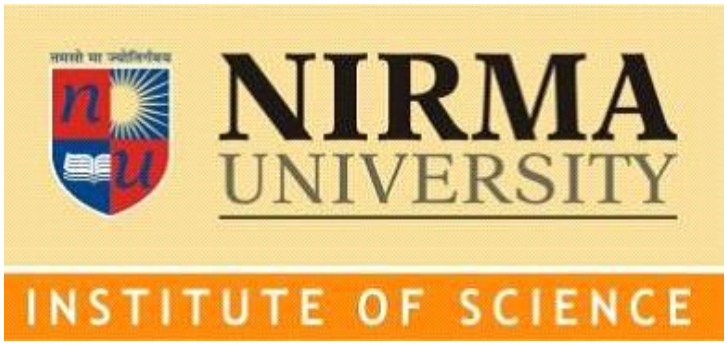

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Nikita Patel *Jay Soni*

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1. INTRODUCTION:

1.1. Protein stability:

The rise in interest of using enzymes in industry has increased the search for biocatalysts with improved properties (Kirk *et al.*, 2002 and Turner, 2003). Because of the unusual ability of enzymes to catalyse reactions with high velocity and unique specificity under a variety of conditions, the production of new biocatalytic enzymes are mainly useful and beneficial. The use of biotransformation in industry is increasing and it has been proved that there will be doubling of industrial production of biocatalytic enzyme every decade (Straathof *et al.,* 2002; Schmid *et al*., 2002; Panke *et al* 2004).

Industrial applications of microbial enzymes are well established and growing day by day (Chand *et al.,* 2003). Enzymes are ideal candidates for catalyzing the new synthetic routes in industrial processes due to their specificity, mild reaction condition and both eco and consumer friendly (Schmid *et al*., 2001). The uses of enzyme at industrial scale also reduce side product formation and in turn, downstream processing cost. The enzymes are only alternative for bio-catalytic use in food industry.

Unfortunately, naturally available enzymes are mainly not suitable for industrial applications. This often relates to stability of enzymes under process conditions. However it is possible to adapt mild and environmentally benign industrial process conditions which are in favor of enzyme but the uses of extreme conditions are often required.

The stability of enzyme is influence by many factors, like temperature, pH, the solvent, binding of metal ions or co-factors, oxidative stress and the present of surfactants. Since the detergent area is the huge application area of industrial enzyme, the effect of surfactants is very important from an industrial point of view. During the use of enzymes for the production of chemicals, the effect of organic solvent is mainly considered.

Among all possible factors which can affect the stability of enzymes, temperature is the most studied factor. When temperature increases to some extent, many enzymes become partially inactivated or unfolded; it means they are no longer capable to perform the required activity. This can be due to the incompatibility of the optimum temperature for activity and related to the intrinsic stability of enzymes (Danson *et al*., 1996; Daniel *et al*., 2001; Peterson *et al*., 2004). It has been seen in some cases that enzymes with improved thermal stability also become more resistant to

other denaturing factors. Although this correlation is not reliable, mainly when it comes to denaturation processes which do not or to a minor extent depend on folding stability e.g. oxidation of surface residues, temperature – induced denaturation of Asn and Gln. (Van den *et al*., 1998; D'Amico *et al*., 2003; Wang *et al*., 2002).

There are three major and different ways to obtain enzyme variants with improved stability properties: (1) Directed evolution (Tuner, 2003; Cherry and Fidantsef, 2003; Zhao *et al*., 2002; Lutz and Patrick, 2004; Robertson and Steer, 2004; Arnold *et al*., 2001). (2) Isolating enzyme variants from organisms living in appropriate extreme environments (Van den, 2003; Schiraldi and De Rosa, 2002; Vieille and Zeikus, 2002). (3) Rationale- based mutagenesis (Eijsink *et al*., 2004; O'Fagain, 2003; van den and Eijsink, 2002; Matthews, 1995).

Nature is evolving polypeptides over billions of years; proteins, which are designed by evolutionary mimicry, are progressing at a far rapid pace. The mutation, selection, and amplification steps of the evolutionary cycle can be imitated in the laboratory by using existing proteins, or molecules created from random sequence space, as starting templates. However, the astronomically large number of possible polypeptide sequences remains as an obstacle to identifying and isolating functionally interesting variants. Intelligently designed libraries and improved search techniques are consequently important for future advances. In this regard, combining experimental and computational methods holds particular promise for the creation of tailored protein receptors and catalysts which is unimagined by nature (Jackel *et al*., 2008).

1.2. Bacillus Species

The name *Bacillus* was given in 1835 which is genus of the family Bacillaceae. *Bacillus* is bacteria and a genus of Gram-positive, rod-shaped related to division of phylum Firmicutes. *Bacillus* species can be obligate aerobes which is oxygen reliant, or facultative anaerobes which have ability to be aerobic or anaerobic. Bacillus species give positive test when oxygen is used (Turnbull *et al*, 1996). They are common in nature which includes both non-parasitic and parasitic pathogenic species. The bacteria can produce endospore and bacteria remain in dormant state for very long periods. These characteristics are originally describing the genus, but some species are not closely related to these characteristics, many have been moving to other genera of Firmicutes (Madigan and Martinko, 2005). Some species of Bacillus produces plentiful amount of enzymes which are useful in different industrial application like detergent, production of antibiotic proteins and etc. In terms of molecular biology and cell biology, *Bacillus subtilis* is one of the best understood prokaryote. *Bacillus anthracis*

and *Bacillus cereus* are considered medically significants because they cause anthrax and food poisoning (Ryan and Ray, 2004).

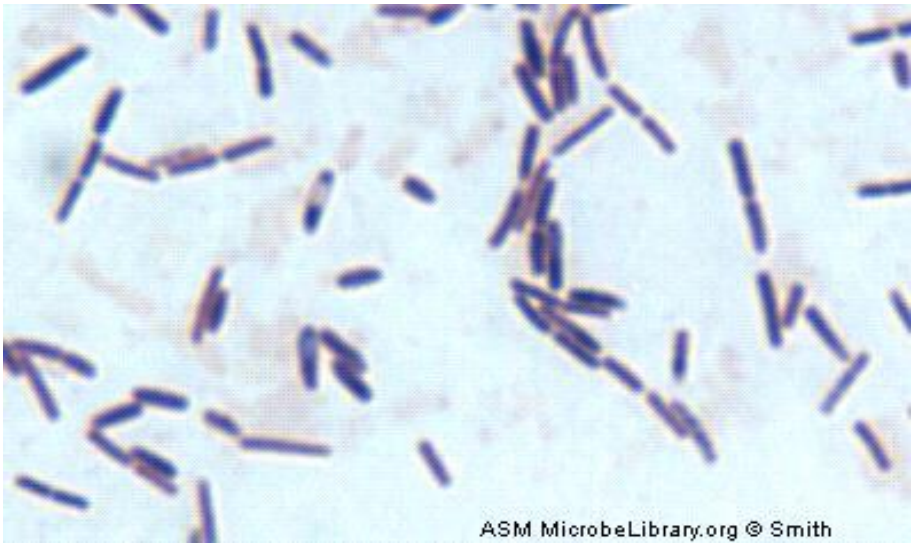

Figure 1 *Bacillus subtilis*

Different Bacillus species such as *Bacillus subtilis, Bacillus licheniformis, Bacillus stearothermophilus and Bacillus amyloliquefaciens* are known for good amylase production and therefore they are useful in various commercial applications. For higher production of alpha amylase by Bacillus and their mutants, UV and various chemical agents such as nitrous acid, ethyl methyl sulphonate (EMS), N-methyl-N-nitro-N-nitrosomethylguanidine (NTG) are used (Demirkan., 2011; Sidhu *et al*., 1997; Konishi, 1990; Haq., 2002). Most amylases are metal dependent such as bivalent metal ions like Ca^{+2}, Mg^{+2}, Zn^{+2}, Mn^{+2} (Demirkan., 2011; Pandey., 2000; Polaina., 2007).

1.3. Amylase

Amylases have useful applications in a huge number of industrial processes such as fermentation, food and pharmaceutical industries, beverages industries, paper and pulp industries etc. Amylase can be obtained from plants, animal, microorganisms. Yet, enzymes from fungal and bacterial sources have subordinated application in industrial sectors.

Amylase hydrolyses starch to different extents. Starch is common and is an easily accessible source of energy and composed of α-glucopyranose units that are

linked to each other by α-1, 4 or α-1, 6 glycosidic bonds. In starch, α- amylose and amylopectin are two high molecular weight components being 15 to 25% and 75 to 85% weight fraction of starch respectively, which is also an 1,4 linked glucopyranose polymer but in addition contains 1,6 glycosidic linkages representing branch points occurring at every 17 to 26 residues (Rayan *et* al., 2006). Enzymes used in starch breaking process are α-amylase (EC 3.2.1.1), β- amylase (EC 3.2.1.2), amyloglucoside or glucoamylase or γ-amylase (EC 3.2.1.3), pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68). In the hydrolysis of starch, enzyme should specifically hydrolyze α-1, 4 and β-1, 6 linked glucose molecules in starch (Sengupta and Dasgupta, 2006).

The α-amylases are the most important enzymes among the all commercial enzymes. The spectrum of amylase applications has widened in many other fields, such as clinical, medical analytical chemistry, and pharmaceutical aid for the treatment of digestive disorders. Thermostable amylases have wide range of applications in a number of industrial productions, most prominently glucose and beer production. The high cost of malting has demonstrated the need for replacement of indigenous barley enzymes with microbial amylases and proteases (Obi and Odibo, 1983). The production of sweetener from corn starch by microbial saccharidases is an important application of enzyme technology in the food industry. The current process for high-fructose corn syrup production involves several separate enzymatic steps including liquefaction by α-amylase, saccharification by glucoamylase, and isomerization by glucose isomerase (Lee *et al*., 1990).

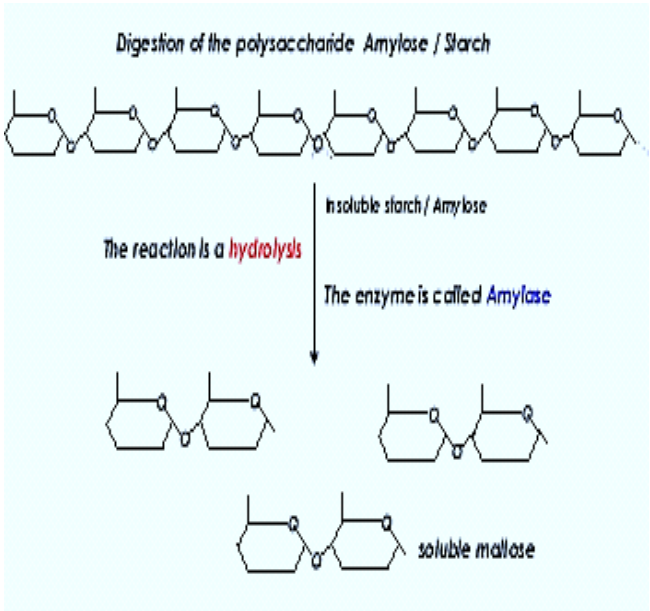

Figure 2 Action of Starch Degrading Enzyme**.**

In addition to the increasing potential applications in biotechnology, enzymes from extremophiles are of great interest to investigate the structural requirements that are beyond their superior thermodynamic stability (Zeikus *et al*., 1998; Pantazaki *et al*., 2002).

Industry segment	Enzymes	Chemical process replaced
Detergents	Lipases, proteases, cellulases, amylases	Phosphates, silicates, high temperature
Textile	Amylases, cellulases, catalases	Acid, alkali, oxidizing agents, reducing agen water, pumis, energy, new garment manufacture
Starch	Amylases, pullulanases	Acids, high temperatures
Backing	Amylases, proteases, xylanases	Emulsifying agents, sodium bisulfate
Pulp and paper	Xylanases, mannanases	Chlorine, toxic waste
Leather	Proteases, lipases	Sulfides, high temperature
Biocatalyst	Isomerases, lipases, reductases, acylases	Acids, organic solvents, high temperature

Table 1Enzymes contributing to sustainable industrial development (Acharya and Chaudhary, 2003).

Structure of amylase:

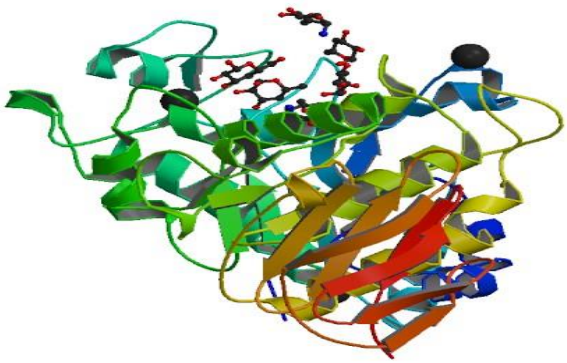

Figure 3 Structure of Amylase from *Bacillus subtilis* (PDB ID: 1UA7)

2. Materials and Methods:

2.1. Materials:

Glycine, Starch, Dipotassium hydrogen phosphate, Potassium dihydrogen phosphate, and Sodium chloride were bought from Merck. Calcium chloride and Coomassie brilliant blue were bought from S.D Fine Cure Ltd. Acrylamide, Bis-Acrylamide, Sodium Dodecyl Sulphate and Tris-Base was bought from Himedia, Ammonium persulphate and Triton-x 100 were bought from Central Drug House, Dialysis bag was bought from Sigma Aldrich. *Bacillus atrophaeus* was bought from ATCC (ATCC NO: 1942).

2.2. Bacillus atrophaeus:

Bacillus atrophaeus is Gram positive bacteria having short rod morphology. Optimum temperature for growth of organism is 30°C and pH is 7.0. *Bacillus atrophaeus* is a mesophilic organism and the enzyme taken under study was amylase. The medium used for growth of the organism contained 1.3% nutrient broth having pH 7.0. The organism takes 24 hours to grow in the medium at 30^{o}C.

2.2.1. Production Media for Amylase from *Bacillus atrophaeus*:

The following media were checked for amylase production by *Bacillus atrophaeus.*

Table 2: Media Compositions for Enzyme Production

Media A	**Media B**	**Media C**	**Media D**	**Media E**	**Bushnell-Hash Medium**	**M-9 Medium**
Maltose-3.0%	Maltose- 3.0%	Maltose- 3.0%	$(NH_4)_2SO_4$-0.2%	Maltose-1.0%	0.327%	5.64%
Citrate-0.1%	Citrate- 0.1%	Citrate- 0.1%	Na_2HPO_4-0.25%	$(NH_4)_2SO_4$-0.2%	-	-
Glycine-0.5%	Glycine-0.5%	Glycine-0.5%	NaCl-0.1%	K_2HPO_4-1.74%	-	-
KH_2PO_4-1.3%	KH_2PO_4- 1.3%	KH_2PO_4-1.3%	KH_2PO_4-0.1%	$MgCl_2.6H_2O$-0.02%	-	-
CaCl2-0.01%	CaCl2- 0.01%	$CaCl_2$- 0.01%	$CaCl_2$-0.05%	$CaCl_2$- 0.001%	-	-
$FeSO_4.7H_2O$-0.001%	$FeSO_4.7H_2O$-0.001%	$FeSO_4.7H_2O$-0.001%	Tryptone-0.2%	-	-	-

Media 1	**Media 2**	**Media 3**	**Media 4**	**Nutrient Broth**	**N.Broth+Starch**
Starch- 1 %	Starch- 1.0%	Starch- 0.5%	Starch- 1.0%	N-broth-1.3%	N-broth- 1.3%
Yeast extract-0.04%	Peptone- 0.4%	Peptone- 2.0%	Peptone- 0.5%		Starch- 1%
NH_4Cl- 0.2%	NH_4Cl- 0.2%	$MgSO_4.7H_20$-0.1%	$MgSO_4.7H_2O$- 0.5%		
K_2HPO_4 - 0.2%	K_2HPO_4 – 0.2%	K_2HPO_4 – 0.3%	Yeast extract- 0.2%		
KCl- 0.1%	KCl- 1.0%	-	NaCl- 0.05%		
$MgSO_4.7H_2O$-0.05%	$NH_4H_2PO_4$-0.4%	-	$CaCl_2$-0.015%		

All the components were weighed, dissolved in distilled water and autoclaved at 15 psi for 15 minutes. Microorganism was grown in Nutrient broth for 24 hours and then transferred to production medium containing 1% Starch and grown at 30°C for 12 hours for Amylase production.

2.3. Checking and Confirming of Enzyme:

2.3.1. Checking Amylase Activity on Starch agar Plate:

The Amylase activity was checked by observing the clearance zone in starch agar plates (Atlas *et al*., 1995). The composition of starch agar plate was 2.8% nutrient agar with 1% starch. Wells were bored on the starch agar plates and cell free supernatant of *Bacillus atrophaeus* grown in different media were poured into these wells. Plates were incubated at 35℃ for 24 hours. The zone of hydrolysis of starch was detected by flooding the plates with iodine solution.

2.3.2. Detection of Amylase by Starch-Iodine test:

Screening for amylase in liquid medium was carried out through starch iodine test. In this process starch reacts with iodine to give blue color. The sample was initially incubated with the starch solution so that amylase activity in the sample hydrolyzed the starch. Hence when iodine was added to it, there was no blue color formation as compared to the blank which contains all the intact starch and turns blue.

0.20 ml sample (cell free supernatant) was taken in test tubes and the volume was made up to 1 ml with phosphate buffer, pH 7.2. Starch was added into it to a final concentration of 1% and the mixture was incubated at 35˚C for 2 hours. 0.5 ml of 0.1% iodine solution was then added in to it followed by addition 9.15 ml of distilled water. The intensity of the blue colour developed was observed in all the tubes turn blue.

Table 3: Detection of Amylase Activity in Liquid medium

	Sample tube	**Positive control**	**Negative control**
Sample	200 µl	-	-
Phosphate buffer (1M)	100 µl	100 µl	100 µl
NaCl (4M)	25 µl	25 µl	25 µl
Standard amylase (1 mg/ml)	-	200 µl	-
Starch (1%)	200 µl	200 µl	200µl
Water	475 µl	475 µl	675 µl
Total volume	1.0 ml	1.0 ml	1.0 ml

2.4. Enzyme Production & Isolation:

2.4.1. Ammonium Sulphate precipitation:

Basic principle of ammonium sulfate is salting out process. Ammonium sulphate precipitates out protein by salting out process. The total amount of ammonium sulfate required is depending upon the volume of cell free supernatant and percent of saturation required; ammonium sulphate was added in small amount into the cell free supernatant. The whole process was carried out on ice. Further 30 minutes of stirring was done after saturation on ice for complete saturation. Then Centrifugation of pellets were done at 7500 rpm for 35 minutes. For obtaining tight pellets and retain the activity of enzyme, centrifugation was done at 4°C. The obtained pellets were dissolved in minimum amount of 25 mM phosphate buffer (pH 7.2) containing 25mM NaCl. After this, dissolved pellets were dialyzed against the same strength of buffer at 4°C (Englard and Seifter, 1990).

2.4.2. Dialysis:

To separate the salts and other impurities from protein sample, dialysis was performed so that they do not interfere in process like SDS-PAGE and further purification process. Dialysis is also help to remove some contaminant of protein that is present and non-covalently bound to protein (Cussler, 1997).

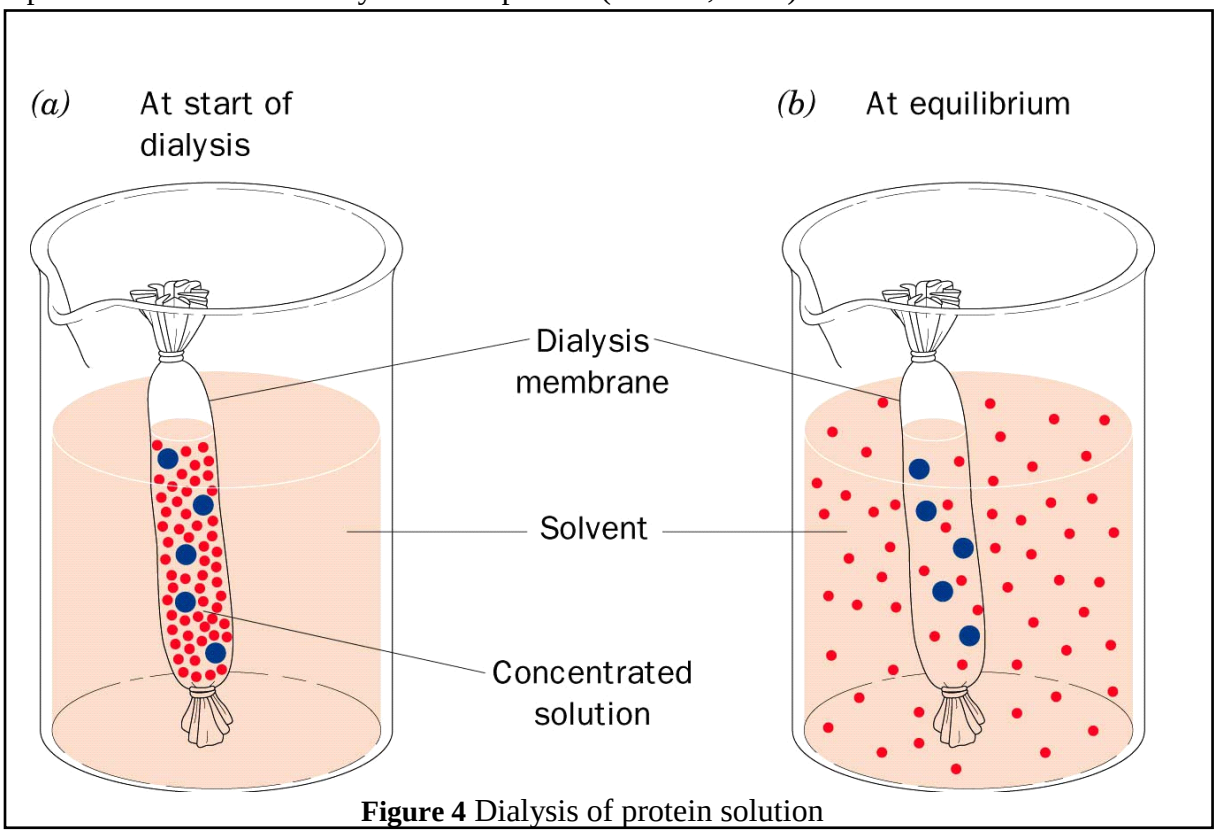

Figure 4 Dialysis of protein solution

Dialysis was performed with the dialysis bag which is made up of cellulose. And have molecular weight cut off of 11 KD. Dialyzing buffer should be of same strength and pH in which the protein is suspended i.e. 25 mM phosphate buffer. Process is carried out at 4^0C. For the process of dialysis, Dialyzing bag was first washed with distilled water followed by boiling in distilled water. Dialyzing bag then allowed to cool to room temperature. After it was cool, the bag was sealed at one end with rubber band and protein sample to be dialyzed was filled in the bag and it was sealed at other end. After that dialysis bag was tied to the glass rod with rubber band and dipped in dialysis buffer. Buffer was changed at regular time interval (initially after 3 hours and later after 7 to 8 hours). Dialysis was allowed to continue for 12 hours. The protein sample was collected from dialysis tube and centrifuged at 7500 rpm for 35 minutes at 4^0C.

To verify the amylase activity in ammonium sulphate precipitated sample after dialysis, the samples were loaded into bored wells in starch agar plates. These agar plates were prepared as mentioned above.

2.4.3. Protein Sample Preparation:

50 ml seed culture of *Bacillus atrophaeus* was prepared in nutrient broth which was incubated for 24 hours, from that 1% inoculum was inoculated in to 2500 ml media containing 1% starch as sole source of carbon followed by incubation at 35°C for 12 hours on shaker. After 12 hours of incubation, the culture was harvested and cell free supernatant was centrifuged at 7500 rpm for 35 minutes. Protein pellets were obtained by ammonium sulfate fractionation and dissolved in minimum volume of 25mM phosphate buffer having pH 7.2. At the end of this process protein was dialyzed against same buffer so as to remove ammonium sulphate and other impurities from protein.

2.5. Quantitative Estimation of Enzyme Activity:

2.5.1. Estimation of Amylase Activity by DNSA assays Method:

Quantitative estimation of amylase activity was carried out by DNSA (dinitro salicylic Acid) assay method (Miller *et al*., 1959). Method quantifies the free maltose that is released as a result of starch hydrolysis. Assay requires various reagents such as 25mM phosphate buffer pH 7.2, soluble starch solution 1%; DNSA reagent (was prepared according to standard protocol). All assays were performed in 25 mM phosphate buffer pH 7.2. For the enzyme samples, 1% starch was made in 50 mM phosphate buffer, pH 7.2. 0.5 ml starch was taken with 50 µl enzyme sample and final

volume was made 1 ml with distilled water. The mixture was incubated at 35°C for 30 minutes. The reaction was stopped by addition of 0.5 ml 2 N NaOH. After that 1 ml DNSA (prepared by standard method) is added into reaction mixture. The content was mixed well and then incubated in boiling water bath for 5 minutes. Absorbance was measured at 540 nm after cooling tubes at room temperature.

$$\text{Enzyme activity (IU)} = \frac{\text{(net amount of sugar produced)}}{\text{(Molecular weight} \times \text{Incubation time} \times \text{volume of substrate)}}$$

2.5.2 Construction of Standard curve of Maltose:

Different aliquots were taken from 180 mg/ml standard maltose stock solution so as to obtain standard concentrations ranging from 0.2 mg/ml to 1.0 mg/ml. Final volume was adjusted to 2 ml using distilled water. 1.0 ml DNSA solution was added to each tube and kept in boiling water bath for 5 min. The tubes were then cooled to room temperature and 9 ml of distilled water was added to each tube. The optimal density of the solution in each tube was measured at 540 nm.

2.6. SDS-PAGE

The protein sample was loaded into SDS polyacrylamide gels of different porosity ranging from 5 to 15% to get separation of all proteins on the basis of their molecular weight. The protocol followed according to Laemmli (Allen *et al*., 1984; Andrews, 1986; Blackshear and Hames, 1981; Laemmli, 1970). The gradient gel electrophoresis was also performed to have idea about the % of gel to be prepared for good separation of proteins.

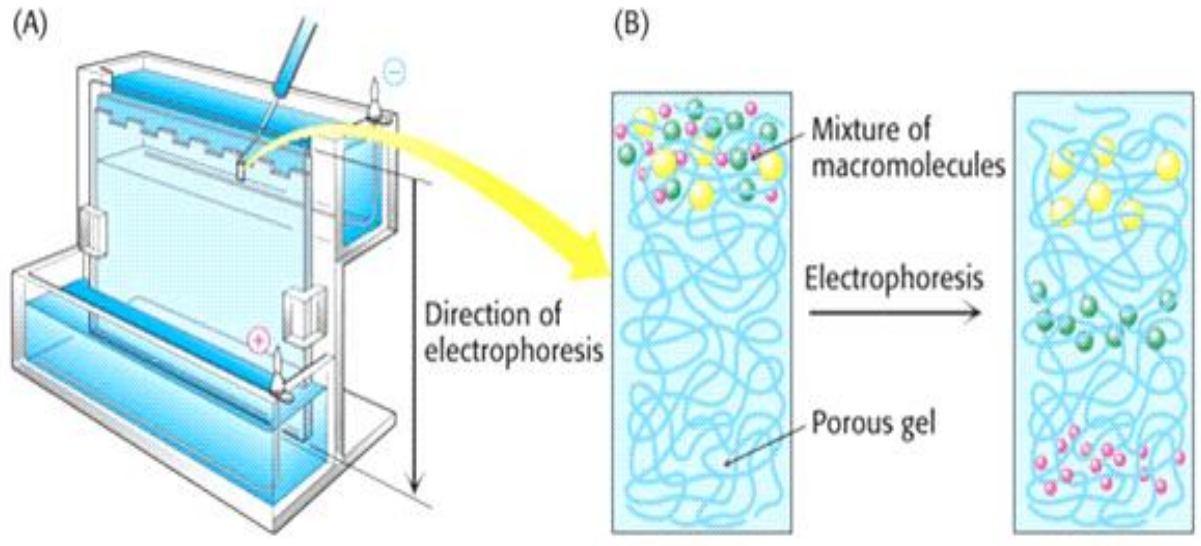

Figure 5 Polyacrylamide Gel Electrophoresis.

(A) Gel electrophoresis apparatus. Typically, several samples undergo electrophoresis on one flat Polyacrylamide gel. A micro liter pipette is used to place solutions of proteins in the wells of the slab. A cover is then placed over the gel chamber and voltage is applied. The negatively charged SDS (sodium dodecyl sulfate)-protein complexes migrate in the direction of the anode, at the bottom of the gel. (B) The sieving action of a porous Polyacrylamide gel separates proteins according to size, with the smallest moving most rapidly (Berg *et al*., 2002).

To load more amount of sample into well, the proteins in samples were ammonium sulphate precipitated, followed by dialysis. The pellets were dissolved in SDS sample loading buffer and boiled at 95^{o}C for 20 minutes and loaded into gel.

2.7. Zymography:

2.7.1. Activity Staining on Gel for Amylase:

To run Zymography for amylase, 2% starch solution in milli Q water was prepared. From this final 1.0% starch was added and copolymerized with SDS PAGE.

For sample preparation 15µl of sample was taken in a clean microfuge vial. 5 µl of gel loading dye containing SDS but not β-mercaptoethanol (so that enzymes are reversibly denatured) was added. Sample was loaded into SDS gel. The gel was run as for SDS electrophoresis. When run was complete the gel was removed and washed with 2.5% Triton-x 100 for one hour. To remove Triton-x 100, gel washing with distilled water was done until foam formation was stopped. The gel was then soaked in refolding buffer overnight at 35^{o}C. Next day gel was treated with iodine for around 1 minute.

2.8 Gel filtration:

Gel filtration is performed using porous beads as the chromatographic support. It is also known as size exclusion chromatography because the separation is based on the size of molecules. The dimensions important to gel filtration are the diameter of the pores that access the internal volume and the hydrodynamic diameter of the protein molecules. A mixture of proteins is applied in a discrete volume or zone at the top of a gel filtration column and allowed to percolate through the column. In this chromatography, large molecules will elute first because they cannot go into the beads and passes in between the beads and elute out. While small molecule goes into the internal space of the beads and takes longer time to travel and elute after the bigger molecules (Guide to protein purification, 2nd edition.2009**. (463)**373-374).

First beads of column were equilibrated with 25 mM buffer to maintain the pH and remove the impurities. For the purification of crude amylase sample, gel filtration was carried out. The ammonium sulphate precipitated sample of cell free supernatant, after dialysis, was loaded on to Sephadex G-75 column, having an exclusion limit of 80 kDa. Eluted fractions from the column were collected and checked for amylase activity by the starch agar plate assay, DNSA assay and zymography.

2.9. Characterization of Enzyme:

2.9.1 Effect of Temperature:

The optimum temperature for the partial purified amylase was determined over a temperature range of 35^0C to 90^0C. The reaction mixture containing enzyme and substrate starch was incubated at temperatures ranging from 35^0C to 80^0C for half an hour and then enzymatic activity was stopped by addition of 2N NaOH (sodium hydroxide). Absorbance at 540nm was measured. The assay was performed in triplicates to get reliable results. The graph of optical density vs. temperature was plotted. From the graph optimum temperature of amylase was determined.

2.9.2 Effect of pH:

The optimum temperature for the purified amylase was determined over a pH range of 3.0 to 10.0. The reaction mixture containing enzyme and substrate starch was incubated with different pH ranging from 3.0 to 10.0 and at optimum temperature for half an hour and then enzymatic activity was stopped by addition of 2N NaOH. Absorbance at 540nm was measured. The assay was performed in triplicates to get reliable results. The graph of optical density vs. pH range was plotted. From the graph optimum pH of amylase was determined.

3. Result and Discussion:

3.1. Gram Staining of *Bacillus atrophaeus:*

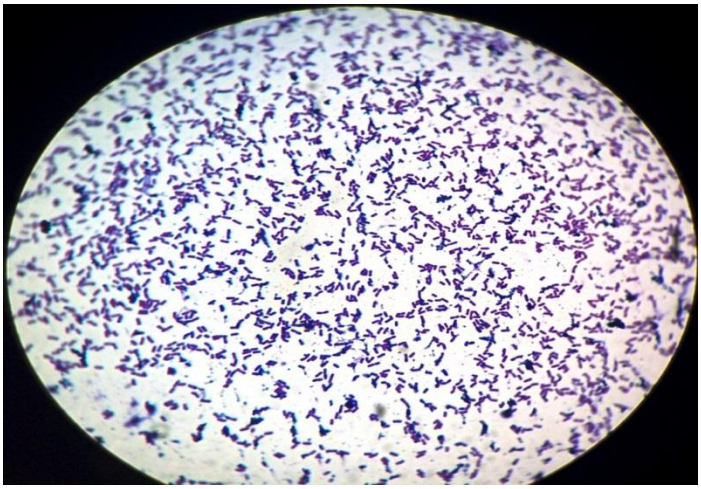

Figure 6 *Bacillus atrophaeus*, Gram Stained, 100 X Microscopic Observation.

To observe the morphology of *Bacillus atrophaeus* Gram's staining was done. As shown in **Figure 6,** gram staining images give blue colored, gram positive and rod like characteristics of *Bacillus atrophaeus*.

3.2. Monitoring Enzyme Activity:

3. 2.1. Monitoring Amylase Activity on Starch-Agar plates:

Wells were bored on starch agar plate with cup borer and cell free supernatant of *Bacillus atrophaeus* was loaded on to starch agar plate and incubated at 35°C for 24 hours. After incubation when plate was flooded with Iodine solution, as shown in **Figure 7** starch hydrolysis zone was obtained which indicates positive amylase activity.

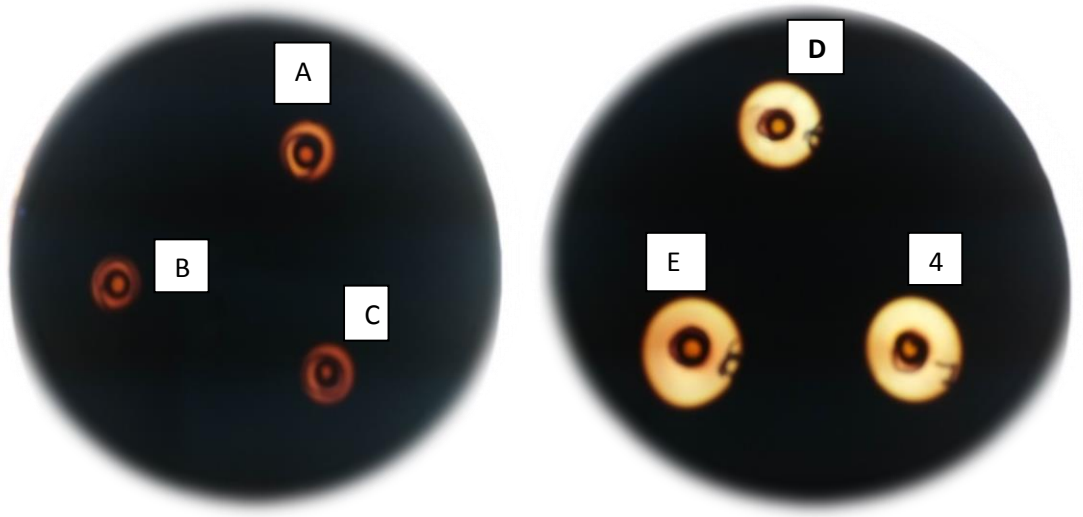

Figure 7 Zone of clearance showing positive Amylase activity on starch agar plate for different media

Above figure indicate Bacillus atrophaeus grown in different media A, media B, media C, media D, media E, media 4. Among all different media, media 4 and media E gave higher zone of starch.

3.2.2. Starch-Iodine Assay for Amylase Activity:

Starch iodine test was carried out with the cell free supernatant after the growth of *Bacillus atrophaeus* after the growth of *Bacillus atrophaeus* in media 4 at 35°C for 12 hours. In negative control, having no starch degrading enzyme developed a dark color upon addition of enzyme, while the positive control tube, where known amylase was added, remained colorless as all the starch had been degraded by the enzyme. Similarly, the sample tube, which contains our cell free supernatant, remained colorless, indicating the presence of amylase activity in sample, which had degraded all the available starch.

Figure 8 Starch-Iodine Assay for Amylase Activity from Bacillus atrophaeus. 1- Negative control tube, 2- Positive control tube, 3- Sample tube

After checking amylase activity on starch agar plates, we conduct at 35^{o}C for 24 hours. The highest activity is obtained in media E and media 4. But, we were unable to get a well formed pellet in media E; hence we carried out all further experiment in media 4. Media 4 was giving higher activity after 12 hours.

3.2.3. Growth curve and Amylase production for *Bacillus atrophaeus*

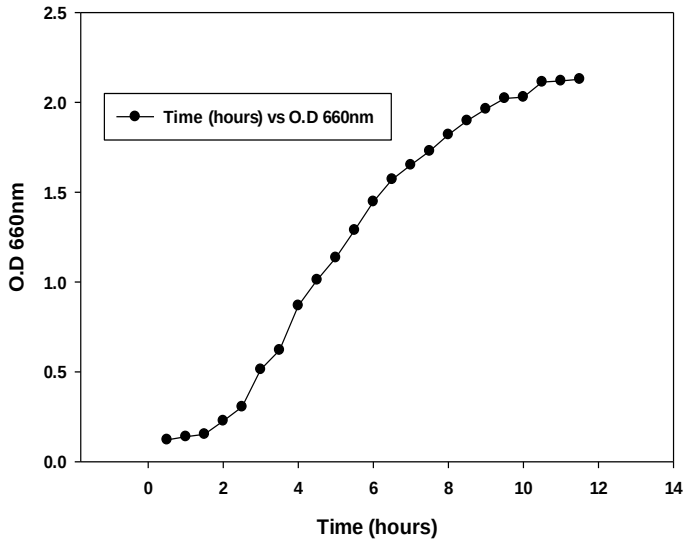

Figure 9 Monitoring Growth of *Bacillus atrophaeus*.

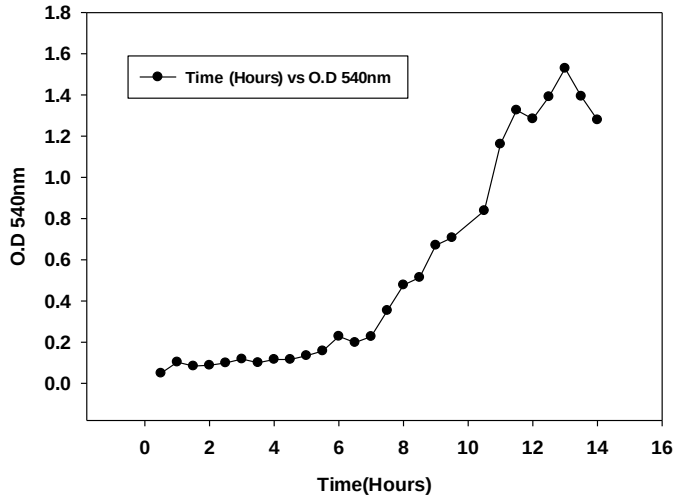

Figure 10 Monitoring amylase activity by DNSA assay

As shown in the **figure 9**, *Bacillus atrophaeus* achieved its stationary phase in 12 hours and as per **figure 10**, maximum amylase production was achieved in 13 hours. So, the harvesting period for the cell was kept 12 hours.

3.3 Quantitative Enzyme Activity Assay:

3.3.1 Standard maltose curve for Amylase:

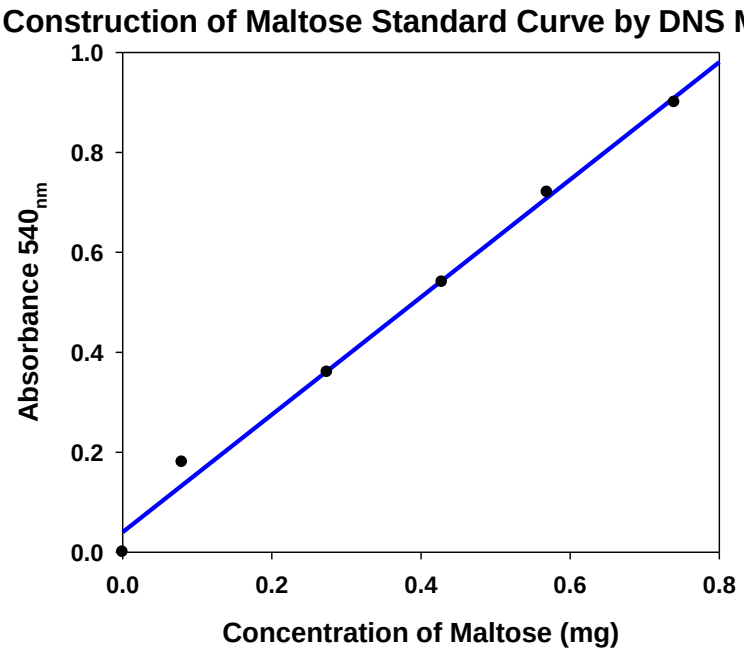

Figure 11: Standard maltose curve by DNSA method (Miller et al., 1959)

The activity of crude amylase from cell free supernatant of *Bacillus atrophaeus* obtained by following equation y = 0.0004x-0.0315,

y = optical density

x = net amount of sugar produce

$$\text{Enzyme activity} = \frac{\text{(net amount of sugar produced)}}{\text{(Molecular weight} \times \text{Incubation time} \times \text{volume of substrate)}}$$

3.4 12% SDS PAGE and Zymography:

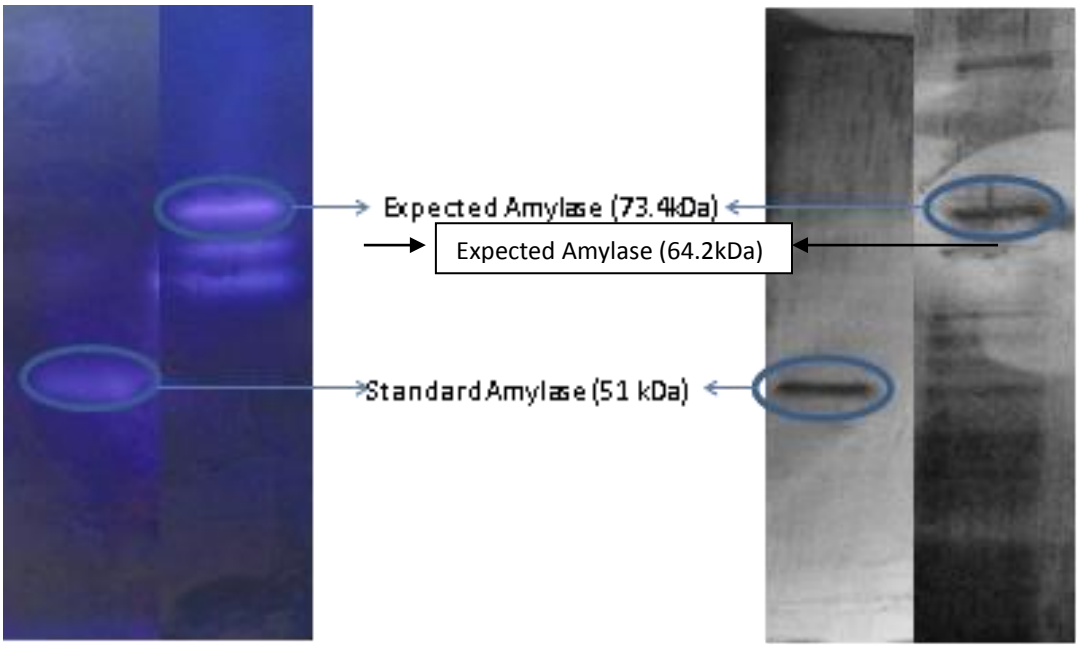

Figure 12 12% SDS PAGE and Zymography; Lane 1 was loaded with standard amylase (1mg/ml), Lane 2 was loaded with Ammonium sulphate precipitated crude protein sample

12% SDS-PAGE of standard amylase and Ammonium sulphate precipitated Crude protein of *Bacillus atrophaeus* isolated from 1% starch containing medium as well from normal growth medium was loaded on gel and bands were obtained as shown As shown in **Figure 12**.

12% Zymography with starch as substrate shows two bands after staining with Iodine as described in whole genome sequence. Clear bands were obtained as shown in the **figure 12**. Due to hydrolysis of starch clear bands were visualized indicating positive amylase activity. The bands on zymography were parallel to the SDS PAGE. Due to presence of two amylases, to separate them gel filtration chromatography was done.

3.5 Gel filtration chromatography:

3.5.1 Fractions of gel filtration chromatography showing Amylase activity:

Wells were bored on starch agar plate with cup borer and fractions of gel filtration chromatography were loaded on to starch agar plate and incubated at 35°C for

24 hours. After incubation when plate was flooded with Iodine solution and amylase activity was checked in different fractions.

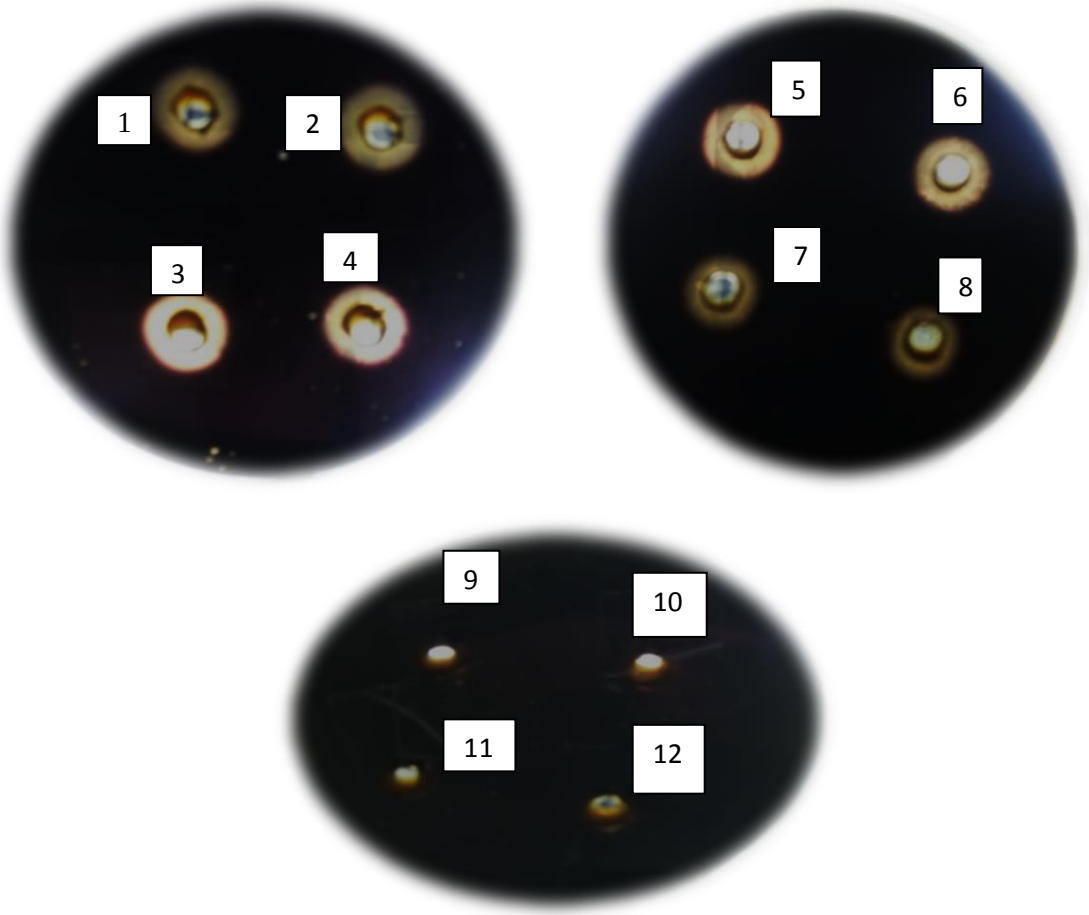

Figure 13 Zone of clearance showing amylase activity on starch agar plates of gel filtration fractions. Wells 1 to 12 contain fractions of gel filtration chromatography

The fractions eluted with 25 mM phosphate buffer containing 25 mM NaCl were added into wells of starch agar plates. As shown in figure, fraction number 3 to 8 showed maximum amylase activity. The fractions showed maximum amylase activity were checked by DNSA assay and loaded into Zymography.

3.5.2 Amylase activity in the gel filtration fractions by DNSA assay:

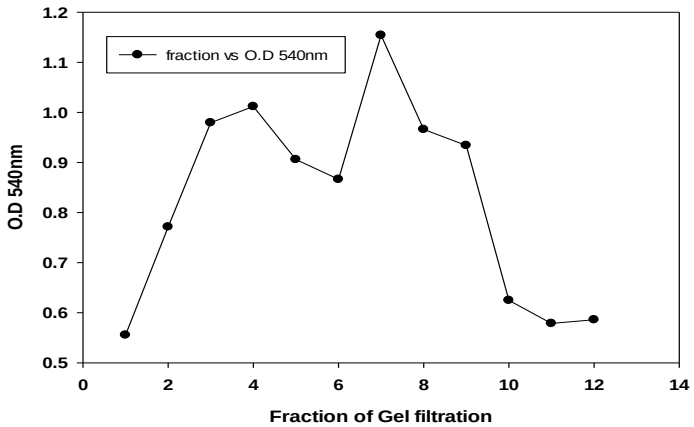

Figure 14 Amylase activities in the gel filtration fractions by DNSA assay

3.5.3. 12% Zymography for fractions eluted by Gel filtration chromatography:

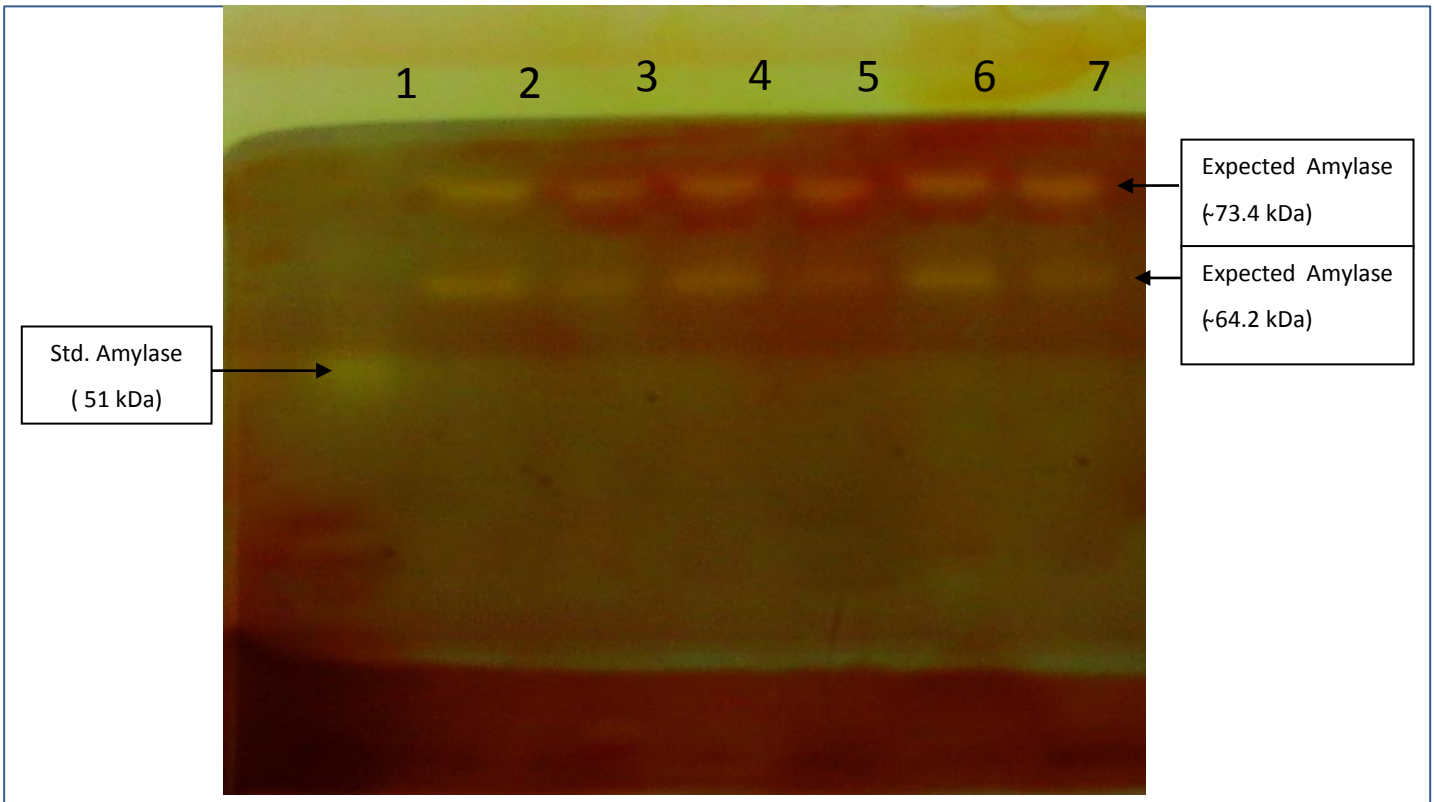

Figure 15 Zymography of fractions eluted from Gel filtration chromatography. Lane1- Standard amylase (10µl); Lane 2 to 7 contain fractions of gel filtration

3.6 Partial Purification of Amylase Enzyme:-

There was identification of two amylases produced by *Bacillus atrophaeus.* The identification *was* done by activity staining on zymography. On zymography, two bands were obtained which indicates presence of two amylases of two different molecular weights.

To separate these two amylases, ammonium sulphate fractionation was done. Cell free supernatant was precipitated from 20 % to 80%. All fraction pellets were dissolved in 25mM phosphate buffer and dialyzed against the same strength of phosphate buffer. The sample was loaded on to 12% zymography.

The activity of amylase enzymes in ammonium sulphate fractions were determined by DNSA method.

3.6.1 Activity of Amylase Enzyme in Ammonium Sulphate fractionations:

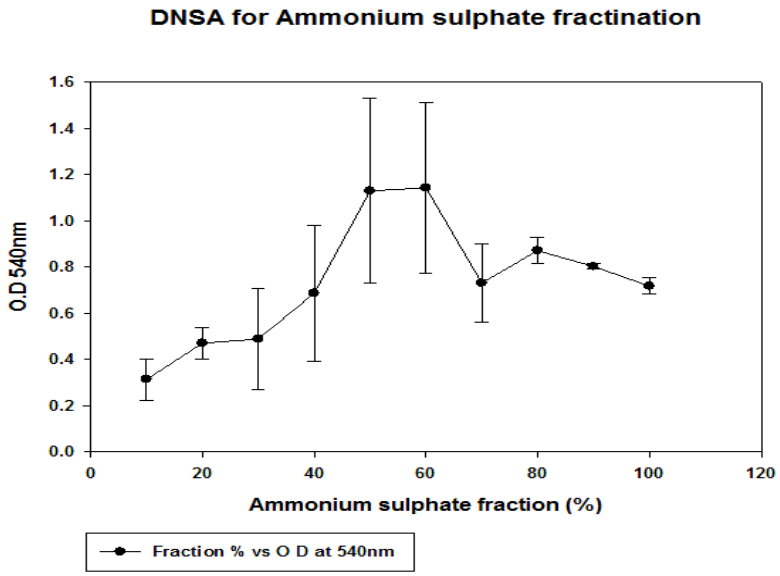

Figure 16 Activity of Amylase in Ammonium Sulphate Fractionation by DNSA assay

As shown in figure, DNSA assay of various fractions ranging from 20% to 100% was done. The highest activity was obtained in 50% and 60% cut off. The fractions showing high amylase activity were checked by zymography to see if the amylases had been separated by this fractionation process.

3.6.2 Activity staining of partially purified Amylase on Zymography:

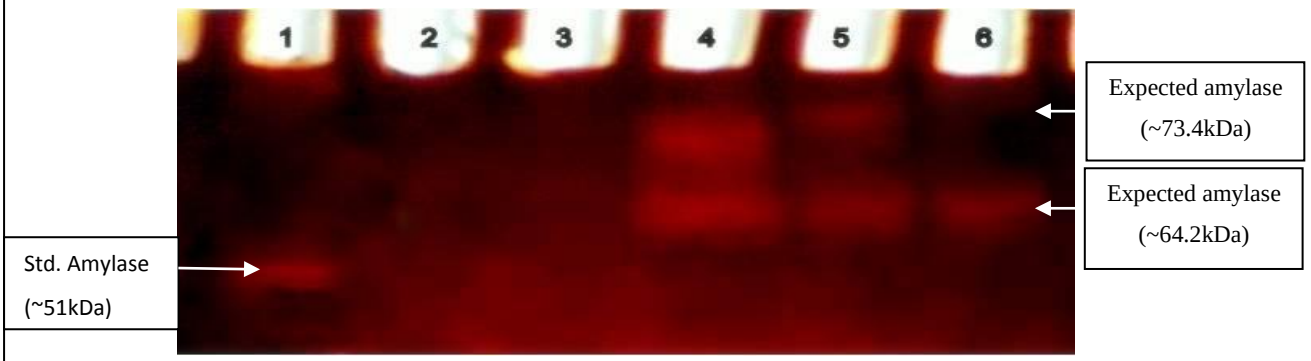

Figure 17 12% zymogram for Amylase. Lane 1 loaded with standard amylase; Lane 2 loaded with 20% (NH4)2SO4 fraction; Lane 3 loaded with 40% fraction; Lane 4 loaded with 50% fraction; Lane 5 loaded with 60% fraction; Lane 6 loaded with 80% fraction.

The separate band of low molecular weight amylase (~64.2kDa) got in 70%-80% cut off, so, further characterization of amylase was done with this partially purified amylase.

3.7 Characterization of Amylase:

3.7.1 Temperature profile of Partially Purified Amylase Enzyme

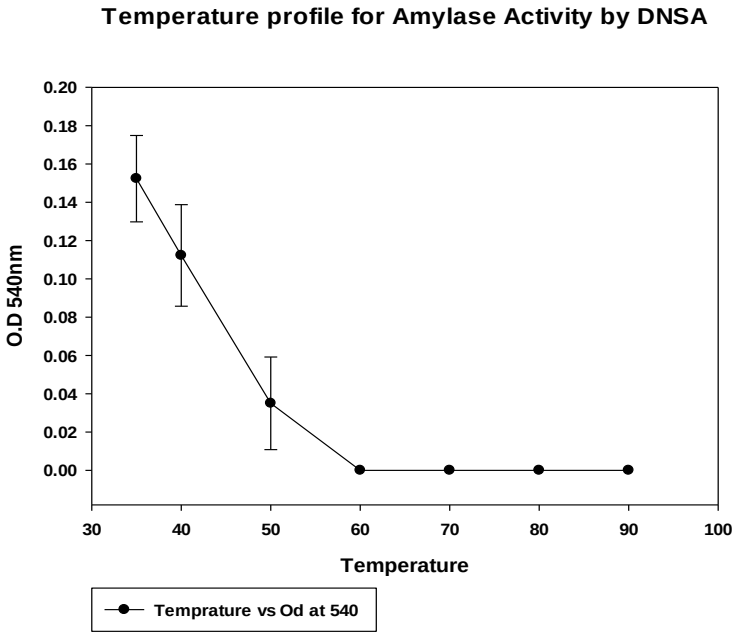

Figure 18 Optimum Temperature of Amylase by DNSA assay

The optimum temperature for the partial purified Amylase was determined over a temperature range of 35- 90° C. As shown in **figure 18** different temperatures from 35° C to 90° C was taken and activity of enzyme was measured. As the graph is showing that, at 35 ° C, there is maximum activity of enzyme and after that there were gradually decreasing activity of enzyme with increasing temperature. The plot of temperature as a function of Amylase activity showed that optimum temperature of partial purified Amylase is 35° C or less. This is considering that it is isolated from a mesophilic microorganism.

3.7.2 pH profile of Partially Purified Amylase Enzyme:

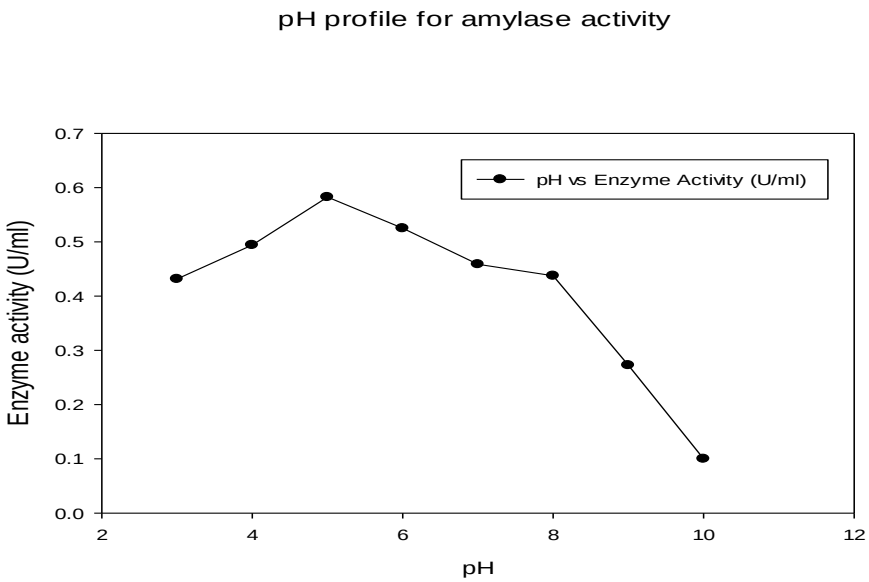

Figure 19 Optimum pH of Amylase enzyme.

The optimum pH for the partial purified Amylase was determined over a pH range of 3.0 to 10.0. As shown in **figure 19,** different pH from 3.0 to 10.0 was taken and activity of enzyme was measured. As the graph is showing that, at pH 5.0, there is maximum activity of enzyme and after that there were gradually decreasing activity of enzyme with increasing pH. The plot of pH as a function of Amylase activity showed that optimum pH of partial purified Amylase is 5.0.

4. Conclusion:

As the Amylase is very important enzyme, it has wide range of application in industry. Amylase has wide spread application in food industries, biosciences, pharmaceuticals etc. Amylase from the *Bacillus atrophaeus* was partially purified and characterized. By zymography, it was identified that, *Bacillus atrophaeus* is produced two amylases with two different molecular weights. With the help of ammonium sulphate fractionation, low molecular weight amylase could be separated and its optimum temperature and pH was determined. The optimum temperature of amylase obtained is around 35°C, which shows its mesophilic character. The optimum pH of amylase obtained is 5.0. The further characterization is under process.

5. Future aspects:

Possible work can be done on this study is as follows:

- The stability studies and molecular studies of these enzymes can give some insight about molecular determinants of thermo tolerance.
- The cloning of desired gene of interest and its over expression in mesophilic host can be done to have large scale production of these enzymes for industrial purpose.
- The mutation studies on these enzymes can be done using site directed mutagenesis and it can be determined that if we can change stability of these enzymes logically.

6. Appendix:

SDS-PAGE:

Principle

The most popular electrophoretic method develop by Laemmli is the SDS-PAGE (Allen *et al*., 1984; Andrews, 1986; Blackshear, Hames, 1981; Laemmli, 1970). This system is discontinues system and having two contiguous, but distinct gels: a resolving or separating (lower) gel and a stacking (upper) gel. Two gels are cast with different porosities, pH and ionic strength. In addition, different mobile ions are used in the gel and electrode buffers. The buffer discontinuity acts to concentrate large volumes in the stacking gel, resulting in better resolution than is possible using the same sample volumes in gels without stackers. Proteins, once concentrated in the stacking gel, are separated in the resolving gel.

The SDS-PAGE system developed by Laemmli is made up of four components. From the top of the cell downward are the electrode buffer, the sample, the stacking gel and the resolving gel. Sample prepared in low- conductivity buffer (0.06M Tris-Cl, pH 6.8) are loaded between the higher conductivity electrode (0.025M Tris-Cl, 0.192M glycine, pH 8.3) and staking gel (0.125M Tris-Cl, pH 6.8) buffers. When system is connected to the power supply, a voltage drop develops across the sample solution which drives the proteins into the stacking gel. A moving boundary region is rapidly formed with the highly mobile chloride ions in the front and the relative slow glycinate ions in the rear (Allen *et al*., 1984; Andrews, 1986; Blackshear, Bury, 1981; Hames. 1981; Wyckoff *et al*., 1977). Localized high-voltage gradient forms between the leading and trailing ion fronts, causing the SDS- chloride and glycinate phases, within broad limits, regardless of the height of the applied sample, all SDS-proteins condense into a very narrow region and enter the resolving gel as a well-defined, thin zone of high protein density. The large-pore stacking gel (4% T) does not retarded the migration of most proteins and servers mainly as an opposite medium. At the interface of the stacking and resolving gels, the proteins experience a shape increase in retardation due to the restrictive pore size of resolving gel. The glycine ions overtake the proteins, which then move in a space of uniform pH 9.5 formed by the Tris Cl and glysine. Molecular sieving causes the SDS polypeptide complexes to seprate on the basis of their molecular weights.

SDS PAGE applied on samples which were ammonium sulphate precipitated fractions. Separation was carried out in thermo scientific electrophoresis device. 12% poly acrylamide gel prepared according to protocol table which consist of 30%

acrylamide mixture, 1.5 M Tris-CL buffer, pH 8.8, 10% SDS (w/v), 10% (w/v) ammonium per sulphate and TEMED. Glass plate's sizes are 10x10cm and have ten wells for sample loading.

For resolving gels, 12% polyacrylamide gel was prepared by mixing 3.35 ml of water, 2.5 ml of 1.5 M Tris- Cl (pH 8.8), 100 µl of 10 % SDS 4.0 ml of 30 % acrylamide mixture, 50 µl of 10% ammonium per sulphate and lastly 5 .0 µl of TEMED to final volume of 10ml. Polymerization started immediately after adding TEMED to mixture. The mixture was poured into the thin gap between two glass plates and allowed to get polymerized for 30 minutes. The iso propanol was layered onto the resolving gel to prevent oxidation.

For stacking gels, 6% gel was prepared by mixing 2.75 ml of water, 1.25 of 0.5 M Tris-HCl (pH 6.8), 50 µl of 10 % SDS, 0.9 ml of 30% Acrylamide, 2 µl of 10 % ammonium per sulphate and lastly 5 µl of TEMED to final volume of 5 ml. The mixture was poured into the polymerized resolving gel. The comb containing 10 wells placed on the gel before casting the gel.

After polymerization of stacking gel, the drag removed from the top of gel. The gel was placed in a buffer tank and the reservoirs were filled until it is reach the filling line level with 1X Tris –Glycine-SDS (TGS) running buffer. The samples were mixed with sample buffer (non-reducing buffer). The sample was heated at 95°C in water bath for 5 minutes. Sample buffer make the sample's colour blue.

The sample was loaded into the wells in the presence of running buffer. Electrophoresis run at Constance voltage at 75 volts for required time until the blue dye reached the bottom of the gel. After electrophoresis was finished, the gel was taken out from the two glass plates by the help of the distilled water and place into the staining tank. Coomassie brilliant blue staining solution was applied on gel for 12 hours with slow shaking. After staining process, the gel was washed with water and then treated with destaining solution.

After each process, gel was washed with water. After staining and destaining steps, the photograph of the gel was taken. Gel was stored in 5% acetic acid solution (v/v) for months.

- Preparation of 1X Tris-Glycine-SDS (TGS) running buffer: 0.9 g of Tris –base and 0.4 g of glycine were weighed and dissolved in 250 ml distilled of water. Then, 3 of 10% (w/v)

- SDS solution were added and the final volume was adjusted to 300 ml with distilled water. It was store at room temperature.

- Preparation of 30% acrylamide mixture: 29.2 g of acrylamide and 0.8 g of N, N'- methylenebisacrylamide were dissolved in total volume of 70 ml of distilled water. The total volume was adjusted to 100 ml distilled with water. This solution was stored at 4°C in dark bottles for one month.

- Preparation of 0.5M, 1.5 M Tris –HCl, pH 8.8, and pH 6.8: The desired amount of Tris-base was weighted and dissolved in 60ml of distilled water. pH was adjusted to 8.8 for resolving gel and pH was adjusted to 6.8 for stacking gel with HCl. Then the volume was adjusted to 100 ml distilled water. It was stored at 4°C.

- Preparation of sample buffer: 3.8 ml of distilled, 1ml of 0.5 M tris-HCL buffer, 0.8 ml of glycerol, 1.6 ml of 10% (w/v) SDS, and 0.4 ml of 1% (w/v) bromophenol blue were mixed to final volume of 8 ml. It was stored at room temperature.

- Preparation of staining solution: the 0.25 g CBB dye was added into methanol: glacial acetic acid which in ratio of 40:10.

- Preparation of destaining solution: Mixed methanol and glacial acetic acid in ratio of 40:10.

Zymography for Amylase:

Various reagents required for Zymography for amylase which are listed below:

1. 1% soluble starch,
2. Iodine solution,
3. 2.5% Triton X 100,
4. Refolding buffer (50mM phosphate buffer pH 7.2, 200mM NaCl, 5mM $CaCl_2$),
5. The entire reagent required for SDSPAGE.

Chemical Used:

All the chemicals denoted here were purchased from Sigma-Aldrich, High media, CDH and Merck.

- **Chemical Used in Gram's Staining Procedure**
    1. Absolute alcohol
    2. Crystal violet
    3. Iodine
    4. Potassium iodine

5. Safranine

- **Chemical and Media Used in Experiment for Biochemical Characterization**
    1. Bacteriological agar
    2. Starch
    3. Bacteriological peptone
    4. Yeast extract powder
    5. Dipotassium hydrogen phosphate
    6. Potassium dihydrogen phosphate
    7. Glacial acetic acid
    8. Glycerol
    9. Potassium iodide
    10. Sodium chloride
    11. Sodium potassium tartrate tetra hydrate
    12. Dinitro salicyclic acid
    13. Sodium hydroxide
    14. Maltose
    15. Peptone
    16. pH strips

- ## Stains and Indicator :

- ## Solutions for Gram staining:

Crystal violet	2g
Ethanol (95%)	20mL
1% Ammonium oxalate	80mL

1% Ammonium oxalate solution was prepared and crystal violet was dissolved in it. Finally add 20mL absolute alcohol and mix.

- ## Gram's iodine:

Iodine	1g
Potassium iodide	2g
Distilled water	300mL

Iodine and potassium iodide were grinded. Water was then added slowly and the solution was stirred until the iodine was dissolved.

- ## Safranine solution:

Safranine	0.25g
95% alcohol	10mL
Distilled water	100mL

Safranine was dissolved in alcohol and then distilled water was added, finally filtered.

- **Buffer and Stock Solutions**
  - **1M Phosphate Buffer:** 17.18 g of dipotassium hydrogen sulphate dissolve in 70 ml of disstiled water then make the volume 100 ml by adding distilled water. Then dissolve potassium dihydrogen phosphate in 70 ml of distilled water and make up volume 100 ml by adding distilled water. Then take 72 ml of dipotassium hydrogen phosphate solution and take 28 ml of potassium dihydrogen and mix it well filtered it by watman filter paper no 1 and autoclave it. Stored at 4^0C, pH 7.2.
  - **4M Sodium Chloride:** 23.37 g sodium chloride dissolves in 70 ml of distilled water and make up volume 100 ml. stored at 4^0C.
  - **2N Sodium Hydroxide**: 8.0 g sodium hydroxide dissolves in 70 ml of distilled water and make up the volume 100 ml. stored at room temperature.

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