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# Partial Purification of Alpha-Amylase Produced by *Brevibacillus Borstelensis* R1

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## Abstract

Ideal purification should optimize both the purity and the concentration of the metabolite. Alpha-amylase is an extracellular enzyme. The precipitate collected from 70% $(NH_4)_2SO_4$ salting-out was dissolved in required amount of 0.1M phosphate buffer (pH 6.8). The dialysis was conducted to get rid of the ions in the protein. After dialysis in the buffer for 24hrs, the sample was subjected to gel filtration. The fraction number 38$^{th}$ had shown highest α-amylase activity (3793±12U/ml) with protein concentration (4.8mg/ml). The fold purification obtained (3.9) when the sample was subjected to sephadex G-100 gel filtration. The specific activity increased from 202.6U/mg protein to 790.21U/mg protein. In ascending paper chromatography and thin layer chromatography the $R_f$ value of test (maltose formed by the action of α-amylase on starch) was similar to standard maltose. Native polyacrylamide gel electrophoresis (PAGE) was conducted to determine the homogeneity of α-amylase at pH 8.3 in 12% slab gel. Specific staining was conducted to confirm the protein band as α-amylase.

**Keywords:** *Brevibacillus borstelensis* R1, Alpha-amylase, sephadex G-100, Native polyacrylamide gel electrophoresis, Specific staining.

## Introduction

Marine bacteria have diverse range of novel enzymes, secondary metabolites and therapeutics [1]. Amylases are divided basically into four groups: endoamylases, exoamylases, debranching enzymes and transferases [2]. Alpha-amylase (E.C:3.2.1.1, alternate names: 1, 4-α-D-glucan glucanohydrolase): Alpha-amylases randomly break down amylose, yielding maltotriose and maltose and also break amylopectin yielding maltose, glucose and limit dextrin. Alpha-amylases are metalloenzymes. They require calcium ions for their stability, activity and structural integrity. They belong to family 13 (GH-13) of the glycoside hydrolase group of enzymes [3]. Amylases derived from bacterial sources have economically dominated applications in industrial sectors [4].The literature survey revealed that bacterial amylases are widely used than fungal amylases. Species of *Bacillus* are ubiquitous in terrestrial, fresh water and marine habitats [5].

Ideal purification or recovery process (Downstream process) should optimize both the purity and the concentration of the metabolite. The desired product is a metabolite, which may be extracellular (amylases, proteases, alcohol, citric acid, penicillin and streptomycin), intracellular (nucleic acids, vitamins, sisomycin and griseofulvin) and both intra and extra cellular (vitamin-$B_{12}$ and flavomycin) [6].

There is no exact procedure that is sure to work for the purification of all proteins and each have steps that are specific to the properties of the protein. Protein purification is a series of processes intended to isolate a single type of protein from a complex mixture. The various steps in the purification process may free the protein from a matrix that confines it, separate the protein and non-protein parts of the mixture and finally separate the desired protein from all other proteins. Separation of one protein from all others is typically the most laborious aspect of protein purification. Separation steps may exploit differences in protein size, physico-chemical properties, binding affinity and biological activity [7].

The first step in product recovery is the separation of cell biomass and the insoluble nutrient ingredients from the supernatant by centrifugation method. A very common preliminary separation comes from adding ammonium sulfate in stages. Different proteins precipitate at different salt

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concentrations and this divides the enzymes and other proteins into fractions.

Ammonium sulfate is an inorganic salt. It is the best, first-choice salt for initial development of a salting-out program to precipitate sought-for proteins because of sulfate's kosmotropic and protein-molecule exclusionary powers. If precipitation of proteins does not occur quickly, it may be best to wait for few hours, to allow time for precipitation to form. This becomes necessary for dilute protein solutions if the overall concentration is less than 1%. Alpha-amylase purification by $(NH_4)_2SO_4$ precipitation is reported in *Bacillus spp.* [8-11].
Dialysis helps in separation of small molecules from larger ones by diffusion through a semi permeable membrane. It increases the volume of the enzyme solution, because of the initial osmotic effect of the $(NH_4)_2SO_4$. Dialysis in purification of α-amylase from the fermented broth is very essential for its stability as well as its activity [12&13].

Gel filtration is also known as molecular sieve or exclusion chromatography. Matrix is a cross linked polymer which swells in water forming a gel of a three dimensional net work of pores. The size of pore is determined by degree of cross linking of polymeric chains. Large molecules are excluded through interstitial space; small molecules enter into the porous interior of the gel bead and are capable of diffusing in and out of the gel beads [14].

Ascending paper chromatography is a process where the paper is saturated with water vapors to form a thin film of water around cellulose fibers of the paper acts as a stationary phase and the solvent as mobile phase. The separation of different components occurs on the basis of differences in their partition coefficients. The separated compounds are identified on the basis of their $R_f$ values. Relative factor is equal to distance travelled by the component from the base line/distance travelled by the solvent from base line [14].

Thin layer Chromatography (TLC) helps in separation based on differential adsorption as well as partitioning of the analytes between the liquid stationary phase and mobile solvent phase. The $R_f$ value of an analyte depends upon the solvent system, degree of saturation of the mobile phase, particle size of the adsorbent, type of adsorbent, temperature and humidity [14]. The reducing sugar released by the α-amylase activity was maltose under assay conditions in TLC was reported by Gashaw Mamo & Amare Gessesse [15] and Ashabil Aygan *et al.* [16]. Vaseekaran *et al.* [17] reported that the α-amylase activity released glucose, under assay conditions.

Native Polyacrylamide gel electrophoresis (Native PAGE): The separation of proteins in native polyacrylamide gel electrophoresis is based on both charge density and size of the molecules. In native PAGE, same buffer ions are present throughout the sample, gel and electrode vessel reservoirs at constant pH such that proteins retain their native conformation and biological activity and hence it is known as native PAGE. In the present study, native PAGE is used to determine the homogeneity of α-amylase [18].

Specific Staining (Iodine staining): Alpha-amylase is an exoenzyme that hydrolysis starch. The ability to degrade starch is used as a criterion for the determination of α-amylase production. Starch test is used to determine the absence or presence of starch in the gel by using iodine solution as an indicatior. Starch in the presence of iodine produces a dark-blue colouration of the gel, and a halo zone around the band indicates amylolytic activity [19].

## Materials and Methods

**Ammonium sulphate precipitation:**

The amylase produced by *Brevibacillus borstelensis* R1 under standardized conditions was centrifuged at 5000rpm for 15minutes. The supernatant was subjected to ammonium sulfate precipitation. Ammonium sulfate crystals were added to the supernatant to bring the saturation to 70% in an ice bath. After 2hrs, the precipitate was collected by centrifugation at 5000 rpm, at +4ºC, for 15 min. The enzyme was recovered by resuspending the precipitate in 1.0 ml of phosphate buffer (0.1M) at pH 6.8 [20]. Then the precipitate was estimated for protein (mg/ml) and amylase activity (µg/ml). Specific activity (U/mg protein), total activity, recovery and fold purification were calculated.

**Dialysis**:

Dialysis tube was procured from Himedia, India. The precipitate formed by 70% ammonium sulfate was diluted with 0.1M phosphate buffer (pH 6.8). Five milliliters of the protein salt solution was taken and one end was clamped and the other end of the dialysis tube was gently tagged. Then the tube was suspended in a 2L glass beaker containing buffer. The contents were gently stirred using a magnetic stirrer. The process of dialysis was continued for one day during which the buffer was changed for every 2hours for desalting. The buffer was tested for sulfate ions by Barium chloride test (Barium chloride solution 5%w/v). The positive test gives white turbidity of barium sulphate indicates

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sulphate ions. The negative test indicates the complete separation of salt from protein solution.

**Gel filtration:**

Five grams of Sephadex G-100 (dry bed diameter about 200 microns) were swollen at $25^0$C in sufficient distilled water for three days. Then the gel was repeatedly washed with 0.1M phosphate buffer, pH 6.8. Fairly thick slurry was degassed under vacuum and packed into column of dimensions (20x1.5cm) with a bed volume of 25ml. The flow rate of 20ml per hour was fixed. This was done to stabilize the bed and to equilibrate with the eluent buffer. After packing 3.0 ml of the sample was loaded on the column. Three milliliter fractions were collected and subjected to protein assay by UV (Kalcker method for quantifying proteins by Kalcker *et al.*, [21] and amylase assay by DNS method.

**Estimation of protein by Lowry method:**

The blue color developed in Lowry method is a result of reaction of the peptide bonds of the protein with cupric ions under alkaline conditions and reduction of phosphomolybdic acid by tyrosine and tryptophan (Aromatic amino acids) residues of the protein. The color exhibits absorbance at 720nm. The protein concentration was measured with bovine serum albumin as standard [22].

To 1.0ml of standard Bovine serum albumin (BSA) protein solution (100µg/ml) containing 10-100µg of protein, 4.0ml of alkaline copper reagent [2%W/V $Na_2CO_3$ in 0.1N NaOH solution), 2.0ml of reagent B (1% w/v cupper sulphate solution, freshly prepared), and reagent C (2% w/v sodium potassium tartrate solution freshly prepared)] were added to the contents and mixed well. After 10minutes of incubation at room temperature, 0.4ml of Folin's reagent (1N) was added and the contents were immediately vortexed. Reagent blank was run with 1.0 ml of distilled $H_2O$ along with the standards and the sample. After 30minutes of incubation at room temperature, the blue color developed was recorded at 720nm absorbance in a colorimeter. Calibration curve on a graph was constructed by plotting the protein concentration (10-100µg) on X-axis and absorbance at 720nm on Y-axis.

**Estimation of amylase by DNS method:**

Maltose produced by the hydrolytic activity of α-amylase on α-1, 4 linkages present in polysaccharides, reduce 3, 5 dinitro salicylate to an orange red colored 5-nitro 3-amino salicylate which can be measured at 520nm. The starch substrate [0.5ml of 0.5% in 0.1M phosphate buffer (pH 6.8)] was mixed with 1% (0.2ml) NaCl in a test tube and pre incubated at $37^0$C for 10 minutes. The supernatant collected from the centrifugation of the production media was used as enzyme source, 0.5ml of this was added to the reaction mixture. The reaction was terminated by the addition of 1.0 ml of 3, 5-dinitrosalicylic acid reagent [1.0 gm DNS in 0.8% NaOH, 60% Na K tartrate] after incubation at $37^0$C for 15 minutes. The contents were mixed well and kept in boiling water bath for 10 minutes. Then they were cooled and diluted with 10 ml of distilled $H_2O$. The color developed was read at 520nm. One unit of enzyme activity is defined as the amount of enzyme that releases 1.0 mmol of reducing sugar (maltose) per minute under the assay conditions [23].

## Analysis of the Hydrolysis Pattern of Soluble Starch Digested by the PURIFIed α-Amylase

**Ascending paper chromatography:**

Whatman No.1 filter paper (20x20cm) was taken and a line was drawn below at 3cm away from the border. The sample was prepared as follows [1% starch, glucose, maltose and Test [0.5 ml soluble starch (0.5% in phosphate buffer at pH 6.8)] was added to partially purified amylase (0.5 ml) and was incubated for 15minutes at $37^0$C in the incubator. The activity was inhibited by adding 1.0ml DNS reagent]. The samples were spotted with capillary tube (10µl). The paper was saturated for 1hr with solvent (Isopropanol and water in 4:1 ratio) system in chromatography chamber. The paper was allowed to run for 4hours. After that the paper was air dried and sprayed with aniline diphenylamine reagent (5 volumes of 1% aniline and 5 volumes of 1% diphenyl amine in acetone with 1 volume of 85% phosphoric acid). The paper was allowed to dry for few minutes and subjected to heat at $100^0$C for 10minutes in a hot air oven. The $R_f$ values were calculated for the brown spots developed. $R_f$ = Distance (cm) moved by the solute from the origin /distance (cm) moved by the solvent from the origin.

**Thin layer Chromatography (TLC):**

The TLC silica gel 60 $F_{254}$ (20x20cm, MERCK, Germany) sheet was taken and a line was drawn below at 3cm away from the border. After the samples [S=Starch, G=Glucose, M= Maltose and T=Test [(0.5 ml soluble starch (0.5% in phosphate buffer at pH 6.8)] was added to partially purified amylase (0.5 ml), and incubated for 15minutes at $37^0$C in the incubator. The activity was inhibited by adding 1.0ml DNS reagent and spotted on TLC with capillary tube (10µl). The sheet was kept for 1hr in saturated solvent (Ethylacetate: Isopropanol: water:

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pyridine in ratio 26:14:7:2) in chromatography chamber. The silica sheet was allowed to run for 40minutes. Later, the sheet was allowed to air dry and sprayed with resorcinol reagent [1% ethanolic solution of resorcinol and 0.2N HCl in 1:1 ratio]. The silica plate was allowed to dry for 10 minutes and heated at $100^0$C for 10minutes in hot air oven. The $R_f$ value was calculated for the brown spots developed. The dark brown sugar spots appeared were identified by comparing with the standards.

## Determination of the homogeneity of α-amylase by Native PAGE

**Sample preparation:**

To 38th fraction (0.2ml) obtained by gel filtration was added with 0.1ml sucrose (40% w/v) and 0.1ml bromophenol blue (0.01% w/v) solution prior to loading of the sample.

**Preparation of slab gels:**

The glass plates and spacers were thoroughly cleaned and assembled with bulldog clips in upright position. Agar (2%) was applied around the spacers to avoid the leakage of the solution. All the components of the 30.0ml resolving gel (12%) [12.0ml acrylamide-bis-acrylamide stock solution (30:0.8), 7.5ml resolving gel buffer stock solution (1.5M Tris pH 8.8), 9.9ml $H_2O$, 0.012ml TEMED and 0.3ml 10.0% ammonium persulfate (freshly prepared)] were mixed well and poured gently without air bubbles with pasteur pipette in the chamber between the glass plates.The gel was allowed to stand for 30min for polymerization. Water drops overlayed to ensure flat meniscus were removed and rinsed the surface with reservoir buffer. The components of 20.0ml stacking gel (5%) [3.4ml acrylamide-bis-acrylamide stock solution (30:0.8), 2.5ml stacking gel buffer stock solution (1.0M Tris pH 6.8), 13.6ml $H_2O$, 0.02ml N,N,N′,N″-Tetramethyl ethylene diamine (TEMED) and 0.2ml 10% ammonium persulfate (APS)] were mixed thoroughly and poured over the resolving gel in slab with pasteur pipette. Immediately insert the comb in the stacking gel and allowed to set for 20min. After complete polymerizeation the comb was removed gently without distorting the wells. The polymerized slab was installed in the electrophoresis apparatus after removing the agar from the base of the slab. The upper and lower chambers were filled with reservoir buffer without air bubbles.

**Coomassie blue staining:**

After the run was completed, the gel was removed from the slab and immersed in 0.1% coomassie blue R-250 [24&25] staining solution [0.1g coomassie brilliant blue, 40ml methanol, 10ml acetic acid, 50ml water] for overnight. Protein bands were detected by destaining the gel in methanol-acetic acid-water solution (4:1:5 by volume). Further destaining was carried out with several changes of deionized water.

**Silver staining:**

Silver staining is 100 to 1000 fold more sensitive than staining with Coomassie brilliant blue R250 and is capable of detecting as little as 0.1- 1.0 ng of polypeptide in a single band.

After Native-PAGE the proteins were fixed by incubating the gel for 12 hours at $30^0$C with gentle shaking in the fixative solution of ethanol: glacial acetic acid: water (30:10:60). Later the fixative was discarded and the gel was incubated for 30 minutes at $30^0$C in 30% of ethanol with gentle shaking. Ethanol and deionized water were discarded. The gel was incubated for 10 minutes at $30^0$C with gentle shaking. This step was repeated twice and the water was discarded and 0.1% solution of $AgNO_3$ was added (freshly diluted from a 20% stock, stored in a tightly closed brown glass bottle at $30^0$C). The $AgNO_3$ solution was discarded and washed the gel gently under a stream of deionized water. Freshly prepared aqueous solution of 2.5% sodium carbonate, 0.02% formaldehyde were added and incubated the gel at $30^0$C until stained bands of protein appear after desired contrast. The reaction was stopped by washing the gel with 1% acetic acid.

**Iodine staining (Specific staining):**

After Native-PAGE the gel was incubated in 2% starch solution (Phosphate buffer at pH 6.8) at $37^0$C for 30 min, the solution was discarded and flooded the plates with Lugol solution (1% iodine in 2% potassium iodide w/v)[26]. The clear band after iodine solution treatment indicate α-amylolytic activity [27&16].

**Statistical analysis:**

All the experiments were conducted in triplicate. The results were given as mean value ± standard deviation.

## Result

**Ammonium sulphate precipitation:**

Ammonium sulphate precipitation was carried out to precipitate proteins by salting-out.

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Ammonium sulphate fraction of 70% resulted in 7.05 fold purification with specific activity (68.75U/mg protein) (Table 1). Protein was estimated by Lowry method.

The precipitate collected from 70% $(NH_4)_2SO_4$ salting-out was dissolved in required amount of 0.1M phosphate buffer (pH 6.8). The dialysis was conducted to get rid of the ions in the protein. After dialysis in buffer for 24hrs, the buffer was subjected to barium chloride test which gave white turbidity of barium sulfate. Negative test for sulphate indicates the complete separation of salt from protein solution.

**Gel filtration:**

Gel filtration is an exclusion column chromatography. The chromatographic media used are porous, polymeric organic compounds with molecular sieving properties. The size of pore is determined by degree of cross linking of polymeric chains. Sephadex G-100 was used in the present experiment.

Each fraction of Gel filtration collected was assayed for protein (mg/ml) by spectrophotometric method and amylase activity by DNS method. The fraction number ($38^{th}$) had shown highest amylase activity (3793±12U/ml) with protein concentration (4.8mg/ml) as shown in figure 1.

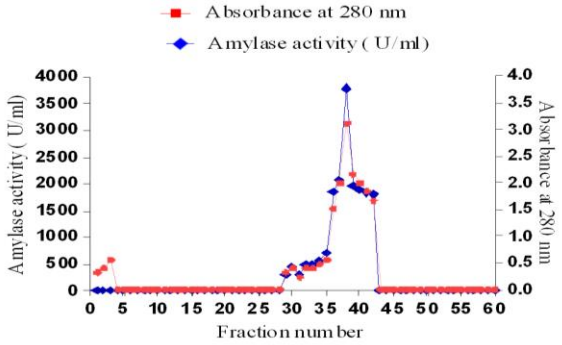


**Figure 1. Gel filtration of α-amylase on sephadex G-100**

Table 1. **Step wise purification profile of α-amylase produced by *Brevibacillus borstelensis* R1**

| Purification steps | Total Volume (ml) | Total units (U) | Total protein (mg) | Specific activity (U/mg protein) | % yield | Fold purification |
|---|---|---|---|---|---|---|
| Crude broth | 100 | 3,12,000 | 1540 | 202.6 | 100 | 1.0 |
| Ammonium sulfate fractionation (70 %) | 50 | 1,65,000 | 420 | 392.9 | 52.9 | 1.94 |
| Sephadex G-100 | 25 | 94,825 | 120 | 790.21 | 30.4 | 3.9 |

Purification Table was constructed to monitor the progress of the amylase purification. The specific activity increases as the purification progresses. Fold purification for crude broth was taken as one and the fold purification increased to 3.9 when the sample was subjected to $(NH_4)_2SO_4$ precipitation and sephadex G-100 gel filtration. The specific activity was increased from 202.6U/mg to 790.21U/mg protein as shown in Table 1.

## Analysis of the hydrolysis pattern of soluble starch digested by the purified α-amylase

**Ascending paper chromatography:**

In ascending paper chromatography (Figure 2a) the distance travelled by the solvent front was 13.4cm. The distance travelled by starch, glucose, maltose and test were 0.0, 8.5cm, 7cm and 7cm respectively from base line. The $R_f$ value of the starch, glucose, maltose and test were 0.0, 0.63, 0.52 and 0.52 respectively. The $R_f$ value of test was similar to standard maltose.

**Thin layer chromatography (TLC):**

In thin layer chromatography (Figure 2b) the distance travelled by the solvent front, starch, glucose, maltose and test were 16.9cm, 0.0, 7cm, 5.0cm and 5.0cm respectively from base line. The $R_f$ value of the starch, glucose, maltose and test were 0.0, 0.41, 0.30 and 0.30 respectively.The $R_f$ value of test was similar to standard maltose.

Analysis of the end products of the soluble starch hydrolysis. After 15 min of incubation of the enzyme with the substrate mixture, maltose was the shortest detectable sugar observed on ascending paper chromatography and thin layer chromatography (Figure 2), indicate that the enzyme was α-amylase E.C: 3.2.1.1 (1,4-α-D-Glucan glucanohyrolase

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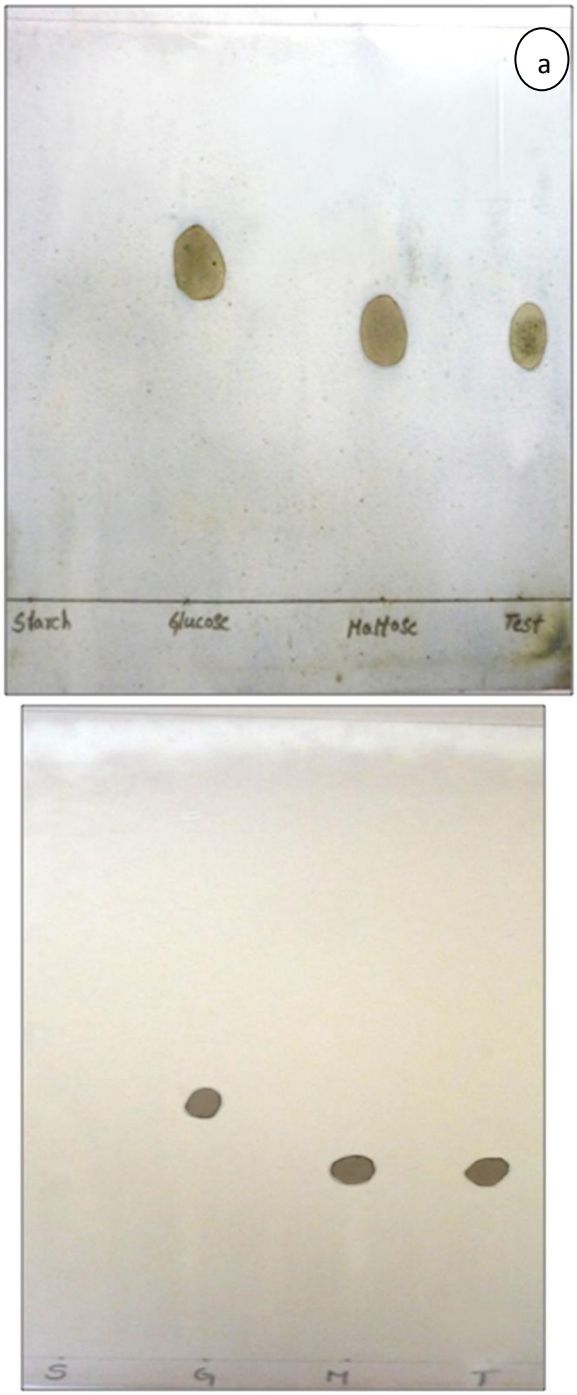


**Figure 3. Identification of the end products of partially purified α-amylase on soluble starch by a) Paper chromatography and b) Thin Layer chromatography.**

**Determination of the homogeneity of α-amylase by Native PAGE:**

Native PAGE (Polyacrylamide gel electrophorosis) was conducted to determine the homogeneity of α-amylase at pH 8.3 in 12% slab gel (Figure 3). The first lane was loaded with 38th fraction of Sephadex G-100, which showed highest amylase activity. The second lane was loaded with 70% ammonium sulphate dialyzed fraction and the third lane was loaded with the crude enzyme produced by *Brevibacillus borstelensis* R1 in Pikovskaya's production medium by submerged fermentation.

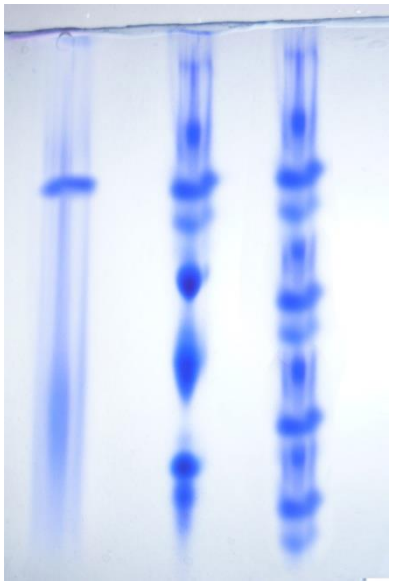


**Figure 3. Determination of the homogeneity of α-amylase by Native PAGE (Polyacrylamide gel electrophorosis) pattern at pH 8.3 in 12% slab gel.**
Lane 1. Sephadex G-100 fraction
Lane 2. Ammonium sulphate dialyzed fraction
Lane 3. Crude enzyme preparation

**Different staining methods to identify α-amylase:**

The protein band obtained in Native PAGE was found to be α-amylase by conducting specific staining method (Iodine staining) in polyacrylamide gel electrophoretic pattern at pH 8.3 in 12% slab gel. Single band was obtained when silver staining technique employed (Figure 4).

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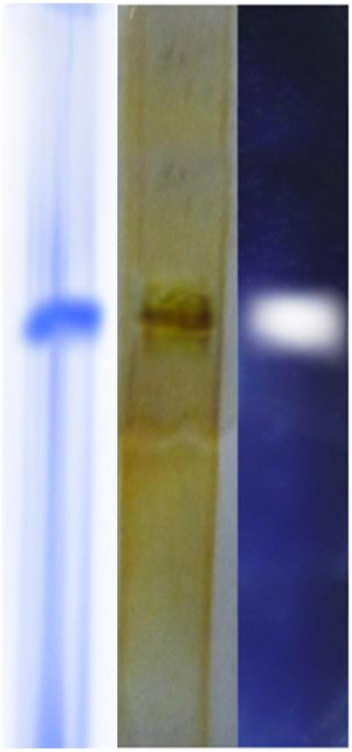

1 2 3

**Figure 4. Different staining methods to identify α-amylase**
Lane 1. Coomassie blue staining
Lane 2. Silver staining
Lane 3. Iodine staining (Specific staining)

Partially purified α-amylase from *Brevibacillus borostelensis* R1 proved its potency in many fields: in bakery industry, food preparations, automation dishwashing, ethyl alcohol dual fermentation, treatment of effluents from sago and rice industries, sewage water treatment, fodder production, laundry and textile industries.

The 70% $(NH_4)_2SO_4$ precipitation fraction with 1.94fold purification was selected for further purification. Haider (2004) reported the precipitation of the amylase by ammonium sulfate (40-60%) saturation in *Bacillus subtilis*. Ventosa *et al.* [28] precipitated amylase (halophilic *Bacillus spp.*) using 40 to 90% ammonium sulfate and the active enzyme fraction was recovered at 70-80%. Ikram-ul-haq *et al.* [29] recovered precipitated amylase with purification fold (1.815) in *Bacillus licheniformis* EMS-6. Varalakshmi *et al.* [10] reported 1.06fold purification fold with ammonium sulphate fractionation.

The 38$^{th}$ fraction of Sephadex G-100 with 3.9 fold purification, yielded (30.4%) with specific activity (790.21U/mg protein). Chung *et al.* [30] purified α-amylase (*Thermococcus profundus*) by gel filtration with sephadex G-200. Haider [31] purified *Bacillus subtilis* α-amylase by gel filtration chromatography on Sephadex column (G-100) and obtained purification fold and recovery 67.92 and 43.9% respectively. The specific activity of purified enzyme was 49.3 fold of the crude enzyme in *Bacillus amyloliquefaciens* strain [32].

In the present study, the reducing sugar released by the α-amylase activity was maltose under assay conditions. Similar studies were conducted on *Bacillus spp.* by Ashabil Aygan *et al.* [16] and Gashaw Mamo & Amare Gessesse [15]. Hansen [33] employed thin layer chromatography (TLC) for identification of maltose and oligosaccharides of starch hydrolysates. Jin & Run [34] reported that the concentration of the resulting sugars increased with increase of the incubation time. These hydrolysis patterns revealed that amylase functioned as a typical amylase to hydrolyze the α-(1, 4)-glycosidic linkage in *Bacillus* strain 7326. Vaseekaran *et al.* [17] reported that the α-amylase activity resulted in releasing glucose, under assay conditions.

Native PAGE (polyacrylamide gel electrophoresis) conducted to determine the homogeneity of α-amylase at pH 8.3 in 12% slab gel. The zymogram of native-PAGE result of partially purified sample was identified as single protein.

The protein band obtained by partial purified fraction was found to be α-amylase by conducting specific staining methods in polyacrylamide gel electrophoretic pattern at pH 8.3 in 12% slab gel: iodine staining. Silver staining was done to separate less amount of protein [35]. The α-amylolytic activity was confirmed by halo zone of starch hydrolysis [27&16]. Hmidet *et al.* [36] purified amylase with Sephadex G-100, with activity 178.5(U/mg protein), Yield 15.9% and purification (fold) 3.08 in *Bacillus licheniformis.*

## Conclusion

Ideal purification should optimize both the purity and the concentration of the metabolite. Alpha-amylase is an extracellular enzyme. The precipitate collected from 70% $(NH_4)_2SO_4$ salting-out was dissolved in required amount of 0.1M phosphate buffer (pH 6.8). The dialysis was conducted to get rid of the ions in the protein. After dialysis in the buffer for 24hrs, the sample was subjected to gel filtration. The fraction number 38$^{th}$ had shown highest α-amylase activity (3793±12U/ml) with protein

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concentration (4.8mg/ml). The fold purification obtained (3.9) when the sample was subjected to sephadex G-100 gel filtration. The specific activity increased from 202.6U/mg protein to 790.21U/mg protein. In ascending paper chromatography and thin layer chromatography the $R_f$ value of test (maltose formed by the action of α-amylase on starch) was similar to standard maltose. Native polyacrylamide gel electrophoresis (PAGE) was conducted to determine the homogeneity of α-amylase at pH 8.3 in 12% slab gel. Specific staining was conducted to confirm the protein band as α-amylase.

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