ISSN : 0974 - 7435 Volume 6 Issue 3

Trade Science Inc.

BioTechnology

An Indian Journal

Full Paper

BTAIJ, 6(3), 2012 [78-86]

Purification and properties of fungal alkaline α-amylase from *Aspergillus oryzae* (Ah1b.) Cohn.

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Received: 4th April, 2012 ; Accepted: 4th May, 2012

ABSTRACT

The fungal strain *Aspergillus oryzae* (Ah1b.) Cohn. was isolated from nearby potato grown soil sample. The isolated strain was cultivated for α-Amylase production in Czapeck dox broth at room temperature for 7 days. For α-Amylase production, it was determined that the best carbon source was soluble starch and the best nitrogen source was peptone. The optimum pH and temperature for enzyme activity was found to be 8.5 and 45°C respectively. The enzyme was stable for 2 hours at pH 8.5 and 45°C. α-Amylase was purified by Acetone Precipitation and Lectin agarose affinity chromatography. The purified amylase was a monomer showed a molecular mass of 44± 1 kDa as estimated by SDS-PAGE with 4.18 fold purification with 14.19% yield. The enzyme was found to have good activity in the presence of $Cu^{2+}$and Fe^{2+}ions. The group specific reagents PMSF, TLCK, NEM, IAA and EDTA substantially decrease the activity of the enzyme, indicating the presence of Serine and Cysteine at the active site of the enzyme. The enzyme was found to be Ca^{2+} dependent. The K_m and V_{max}values of the enzyme for soluble starch hydrolysis was found to be 11.11mg/ml and 2.0µmol/min/ml respectively and it was an endoacting enzyme which was confirmed by its product analysis by thin layer chromatography.

KEYWORDS

Aspergillus oryzae (Ah1b.) Cohn.;
Extracellular α-amylase;
Enzyme characterization;
Purification.

INTRODUCTION

Amylase is an enzyme that catalyses the breakdown of starch into sugars. The amylases, which act on starch and polysaccharides to hydrolyze the glycoside bonds α -1,4 and α -1,6 can be divided into α- amylase which disrupt the inner bonds of the substrate (endoamylases); β-amylases which hydrolyze units from the non-reducing ends of the substrate (exoamylases) and glucoamylases (amyloglucosidases) which release glucose units from the non-reducing end of starch molecules. Amylase is present in human saliva, where it begins the chemical process of digestion. The pancreas also makes amylase[1].

α-Amylase is known as 1,4α- glucan-4-

glucanohydrolase [EC 3.2.1.1]. The α-amylases are metalloenzymes. They act at random locations along the starch chain, α-amylase breaks down long-chain carbohydrates, ultimately yielding maltotriose and maltose from amylose, or maltose, glucose and "limit dextrin" from amylopectin. As diastase amylase is the first enzyme to be discovered and isolated[2].

Plants, Fungi and some bacteria also produce amylase. All amylases are glycoside hydrolases and act on α-1, 4-glycosidic bonds. Studies on fungal amylase especially in the developing countries have concentrated mainly on Rhizopus sp. and *Aspergillus* sp. probably because of the ubiquitous nature and non-fastidious nutritional requirements of these organisms[3]. It has been reported that while a strain of A.niger produced 19 types of enzymes[4].

Considering all living forms, the diversity of soil organisms in general is more extensive than any other environment in the world. It has been found that more number of genera and species of fungi exist in soil than in any other environment,as soil is exposed to various conditions and basically receives all microorganisms present on this planet.

Amylase enzymes find use in bread making and to break down complex sugars such as starch (found in flour) into simple sugars[5]. In molecular biology, the presence of amylase can serve as an additional method of selecting for successful integration of a reporter construct in addition to antibiotic resistance[6]. α -amylase is used in ethanol production to break starches in grains into fermentable sugars. Alkaline α –amylase have a potential application in the industries such as textile industry, paper and cellulose, leather, beer, liquor, bread, and children cereals, fermentation industry (vitamins, amino acids, antibiotics), chemical and pharmaceutical industries[7,8]. Therefore the objective of this study was to isolate and purify the alkaline α –amylase from a new alkolaphilic fungal strain and its biochemical characterization.

MATERIALS AND METHODS

Micro organisms

The various strains of amylase producing fungi were isolated from soil. Soil samples were collected from Kerala. The organism showed positive was further characterized and identified as *Aspergillus oryzae* (Ah1b.) Cohn. By Agarkar Research Institute, Pune)[9,10].

Culture conditions and optimization

The selected starch hydrolyzing organism was inoculated into 250ml of culture medium (g/L) containing 10.0g soluble starch, 35.0g Czapeckdox broth, 10.0g peptone, 0.2g tetracycline in 500ml conical flask and incubated for 7 days. The effects of various temperature and pH and with different sources of carbon and nitrogen were estimated in relation to enzyme activity. The initial pH of the media was adjusted to 10.0 using 1M Na_2CO_3.

Crude amylase preparation

In order to remove fungus cells and spores, the culture broth was filtered and the mycelia mat was removed. The filtrate was centrifuged at 10,000 rpm for 15 minutes at 40^0C to obtain the cell free extract. The resulting supernatant was taken directly as source of extracellular crude enzyme. The crude extract was stored at –20 °C until used[9,11].

α –amylase enzyme activity

α- amylase was assayed according to the method proposed by Bernfeld (1995)[12]. 3,5-Dinitrosalicylic acid (DNS or DNSA, IUPAC name 2-hydroxy-3,5-dinitrobenzoic acid) is an aromatic compound that reacts with reducing sugars and other reducing molecules to form 3-amino -5-nitrosalicylic acid, which absorbs light strongly at 540 nm.. One unit (U) of amylase was defined as the amount of enzyme releasing 1.0 µg of maltose equivalent per minute under the assay conditions. The protein quantity of the crude enzyme extract was determined by the Lowry method[13] using bovine serum albumin as standard.

First step of purification of α-amylase

The cell free extract was further partially purified by the conventional methods like ammonium sulfate, ethanol and acetone precipitation[14]. As the ammonium sulfate, ethanol precipitation did

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not yield an enzyme with good activity; the acetone precipitate enzyme was preferred for further experiments.

Second step of purification (Affinity chromatography)

Lectin affinity chromatography is a form of affinity chromatography where lectins are used to separate components within the sample. Lectins, such as Concanavalin A is a protein which can bind specific carbohydrate (sugar) molecule. The most common application is to separate glycoproteins from non-glycosylated proteins, or one glycoform from another glycoform.

Hydrolysis of different types of starch (Slide test) (Freer,1993)[15]

The hydrolysis of different types of starch by isolated partially purified amylase was studied by preparing various starch agar plates. The starch agar plates were prepared by adding 1ml of 1% samples of potato, corn, wheat, amylopectin and soluble- starches which were mixed with liquid agar and poured on a clean glass slide and allowed to solidify. After solidifying the plates, a well was created in the plate using gel punch and 20 µl of partially purified enzyme was added to it. Then slides were placed over moistened filter paper which was kept in different petriplates and incubated for overnight. After overnight incubation one ml of iodine solution was poured.

Effect of ph on activity and stability of α-amylase

Amylase activity of the enzyme was determined using 1% soluble starch as substrate at a pH range of 3.0- 10.0. The measurement of the amylase activity was performed using buffers such as glycine-HCl (2.5-3.5), acetate buffer (4.0 – 5.5), citrate buffer (6 - 6.5), Phosphate buffer (7 - 8), Tris buffer (8.5 – 9.0), carbonate buffer (9.2 – 10.7) and glycine buffer (9.5 - 10) of 0.2M strength each. Stability of the enzyme at optimum pH value was also studied by incubating the enzyme at that pH value and residual enzyme activity was measured at different time intervals (0, 30, 45, 60, 120 minutes and overnight).

Effect of temperature on activity and stability of α-amylase

A temperature gradient was employed in order to determine the amylolytic activity of the enzyme. The buffer mixture, substrate solution, and crude enzyme extract were incubated over range temperatures between 0°C to 80°C. Thermo stability was determined by incubating the partially purified enzyme at optimum temperature at different time intervals and the residual activity was determined.

Effect of divalent cations on the activity of α-amylase

The effect of metal ions on the activity of α-amylase was determined by adding different known concentrations of metal ions in the enzyme substrate reaction system. The metal ions selected for the present study were $CuSO_4$, $FeSO_4$, $CaCl_2$, $MgSO_4$, $ZnSO_4$ and $MnSO_4$. Each of these divalent cations were added to the enzyme substrate reaction and incubated for 5 minutes. The residual enzyme activity was determined by standard assay procedure.

Effect of different group specific reagents on the activity of α-amylase

The group specific reagents used were PMSF (Phenyl methane sulphonyl fluoride), DTT (Dithiothreitol), IAA (Indole acetic acid), NAI (N -acetyl imidazole), NEM (N-ethyl malamide), NaN_3 (Sodium azide), DAN, SDS (Sodium dodecyl sulphate), Urea, TLCK (Tocyl chloride methyl ketone), TPCK (Tosylphenylalanylchloromethyl ketone), $HgCl_2$ and EDTA (Ethylene diamine tetra acetic acid). All the inhibitors are added in the reaction mixture and incubate for 5 minutes and enzyme assay was carried out to determine the active site amino acids.

Determination of Km and Vmax using different types of starch as substrates

Initial rates of starch hydrolysis was determined at various substrate concentrations (1-10 mg/ml) using different substrates (corn, potato, and soluble starches). The reactions contain 0.5ml of partially purified enzyme. The assay was performed according to the standard enzyme assay method. The kinetic constants Km and Vmax

were estimated by the method of Line-weaver and Burk plot[16]. Initial rates of starch hydrolysis was determined at various substrate concentrations (1-10 mg/ml) using soluble starch as substrate and $HgCl_2$ as inhibitor. The reactions contain 0.5 ml of partially purified enzyme. The assay was performed according to the standard enzyme assay method. The kinetic constants Km and Vmax were estimated by the method of Line-weaver and Burk plot.

Chromatography of hydrolysis products. (Balkan et. al. 2005)[17]

The hydrolysis of soluble starch with crude enzyme was determined by thin-layer chromatography (TLC) using 20 cm plates (Merck, TLC aluminium sheets 20 x 20 cm, silica gel 60 F254). Crude enzyme- 0.5 ml will be incubated overnight with 0.5ml of 2% starch dissolved in 0.2 M phosphate buffer (pH 7.0). This was compared with standards such as maltose, glucose and starch.

Native page & activity staining (Davis, 1964)[18]

In native PAGE, the SDS is absent and the proteins are not denatured prior to loading. Since all the proteins in the sample being analyzed carry native charge at the pH of the gel, proteins separate according to their charge /mass ratio. Native PAGE was performed at room temperature with 12% gel and Tris-glycine buffer (pH 8.3) at 50 V for 3hrs.

SDS page (Laemmli, 1970)[19] with silver staining

SDS-PAGE was performed at room temperature with 12% gels and Tris-glycine buffer (pH 8.3) at 100 V. After electrophoresis, the proteins were visualized by silver staining[20] for determining homogeneity and relative molecular mass using standard molecular weight markers.

RESULTS

Screening for amylase producing fungi

Screening for α amylase production from fungi, isolated from the soil of Kerala was performed by plate culture method. Several fungi were isolated and screened for maximum production of α-amylase by observing clear zone of starch hydrolysis on starch agar plates. After 5 days incubation, many fungal colonies were seen and from that positive amylase producing strain was identified by iodine staining method.

After iodine staining the ability of starch degrading activity of fungi were estimated in terms of the clear zone. The positive isolated culture was identified as *Aspergillus oryzae* (Ah1b.) Cohn. and further confirmed by National Fungal Culture collection of India, Agarkar Research Institute Pune.

Culture conditions and optimization:

Different types of carbon and nitrogen sources were tested in order to determine their effects on the growth of the fungus and production of α-amylase. The best growth was obtained with carbon and nitrogen sources soluble starch and peptone respectively. Maximum α-amylase activity was determined as 63.15μmoles/min/ml and 68.77μmoles/min/ml on the 7th day in a soluble starch and peptone medium.

Plate assay method

The α-amylase activity of the *Aspergillus oryzae* (Ah1b.) Cohn. crude extract was determined by plate assay method by hydrolysis of different substrates such as soluble starch, corn starch, potato starch, wheat starch, and amylopectin were studied by appearance of clear zone around the centre of various slides indicating the degree of substrate specificity of the amylase enzyme. At optimal temperatures, enzyme hydrolysis activity was highest towards soluble starch. For the slides containing corn starch, soluble starch and potato starch, clear zones were obtained by staining with I-KI solution which shows that it was hydrolyzed by the enzyme, to varying degrees.

Purification of enzyme

Purification of Extracellular α-amylase. The extracellular α-amylase from *Aspergillus oryzae (Ah1b.)* was purified to 4.18-fold with a yield of 14.19% (TABLE 1), using a series of purification steps that included acetone precipitation followed by affinity purification using lectin agarose column chromatography. The purified

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enzyme was homogenous showing a single-protein band on SDS-PAGE with a molecular mass of 44± 1 kDa when compared to authentic standards (Figure 1). Activity staining of crude and purified enzyme showed that only one extra cellular amylase is secreted (Figure 2). The Purified α-amylase showed 118.60U/ml of specific activity. Detailed summary report is given in TABLE 1.

TABLE 1: Summary of the purification of α-amylase from Aspergillus oryzae (Ah1b.) Cohn.

Step	Volume (ml)	Total Activity (µmol/min)	Total Protein (mg)	Specific activity (units/mg of protein)	Purification fold	Yield (%)
Crude enzyme	500	2339.00	53.00	44.13	1.0	100
Purified enzyme by chilled acetone precipitation method	4.1	1054.93	12.67	83.26	1.88	45.00
Affinity Chromatography eluate	1.0	332.1	1.875	118.60	4.18	14.19

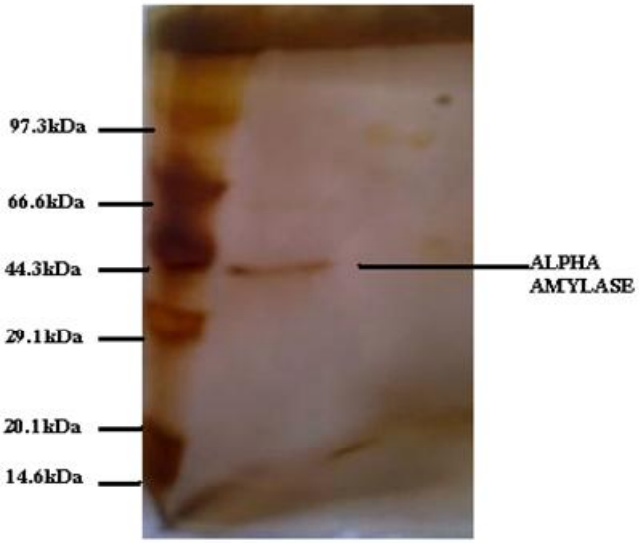

Figure 1 : SDS PAGE pattern for purified α-amylase.

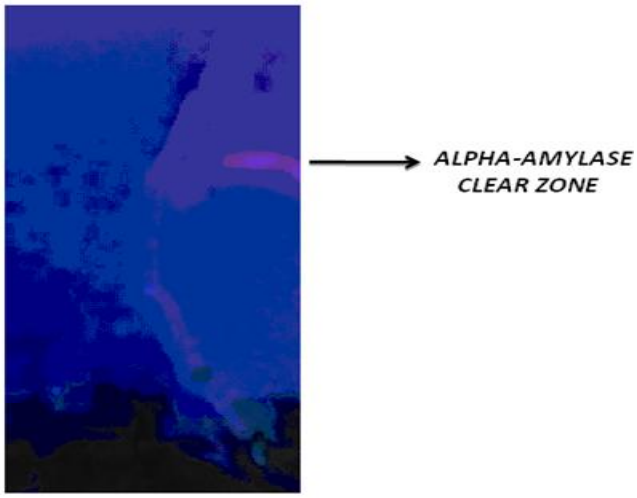

Figure 2 : Native PAGE pattern for α-amylase and activity staining.

Effect of pH on the activity and stability of α-amylase

The activity of α-amylase was determined by using soluble starch as a substrate and the optimum pH was found to be 8.5 (Figure 3). The residual activity of α-amylase from *Aspergillus oryzae (Ah1b.) Cohn* was determined after 24 h of incubation at various pH values ranging from 3.0 to 10.5 at room temperature. The *Aspergillus oryzae* (Ah1b.) Cohn. α-amylase was extremely stable at an optimal pH and retained at least 70% of its original activity at a pH range of 8.5, after 1 hour of incubation at room temperature (Figure 4).

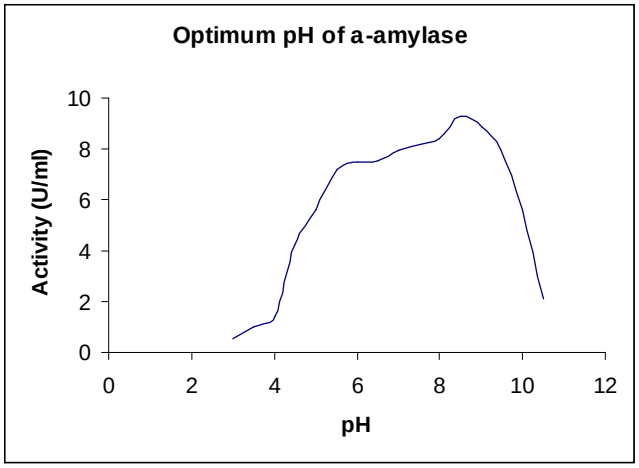

Figure 3 : Determination of optimum pH of α-amylase.

Effect of temperature on the activity and stability of α-amylase

The optimum temperature of *Aspergillus oryzae* (Ah1b.) Cohn. *α*- amylase was found to be

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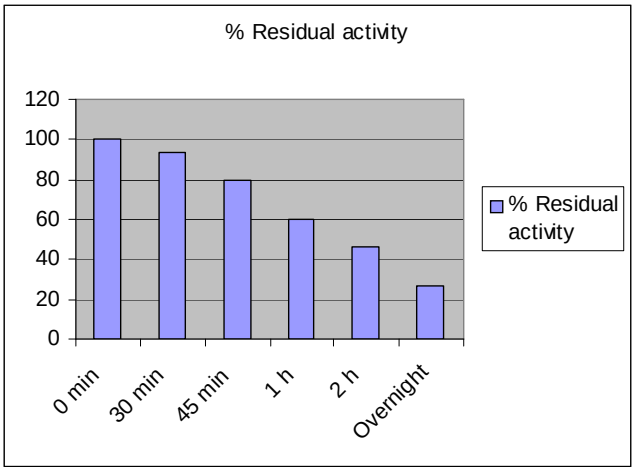

Figure 4 : Stability of α-amylase at optimum pH .

45 °C (Figure 5). A reduction in enzyme activity of α-amylase of *Aspergillus oryzae (*Ah1b.) Cohn. was identified after 1 hr incubation at optimum temperature 45^0C. The enzyme was stable for 2 hrs at 45^0C but loses its activity after overnight incubation (Figure 6).

Effect of divalent cations on the α-amylase activity

The results showed that Cu^{2+},Fe^{2+},Ca^{2+} and Mn^{2+}ions produce no change in α-amylase activity, but the enzyme lost 50% of its activity when treated with Mg^{++} and Zn^{++} ions at concentration 10mM (Figure 7).

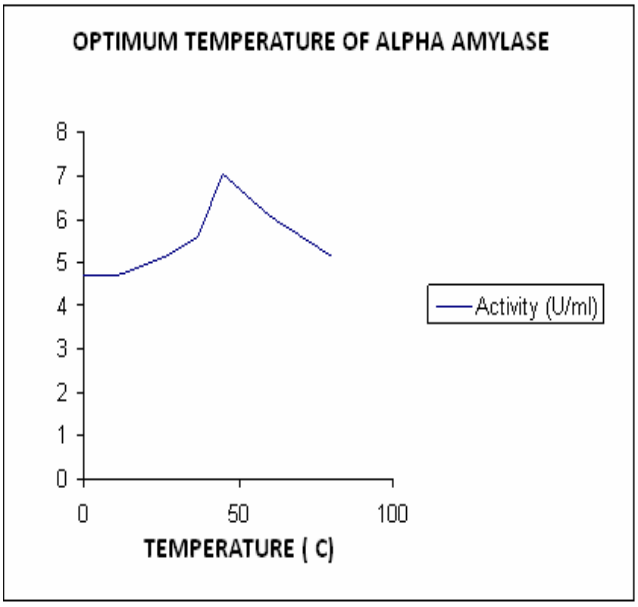

Figure 5 : Determination of optimum temperature of α -amylase.

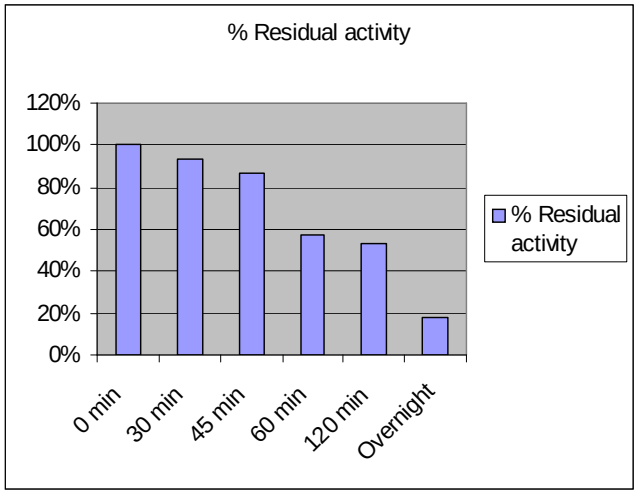

Figure 6 : Stability of α-amylase at optimum temperature.

Effect of group specific reagents on α-amylase

NEM modifies the amino acid cysteine, PMSF and TLCK modify the amino acid serine and there by inhibited the enzyme activity. This shows that amino acid like cysteine and serine play an important role in the catalytic activity of the enzyme. EDTA a metal chelator inhibits the enzyme which shows that the isolated enzyme was a metallo enzyme. It was found that N acetyl imidazole did not have much of an inhibitory effect on α-amylase indicating tyrosine or lysine

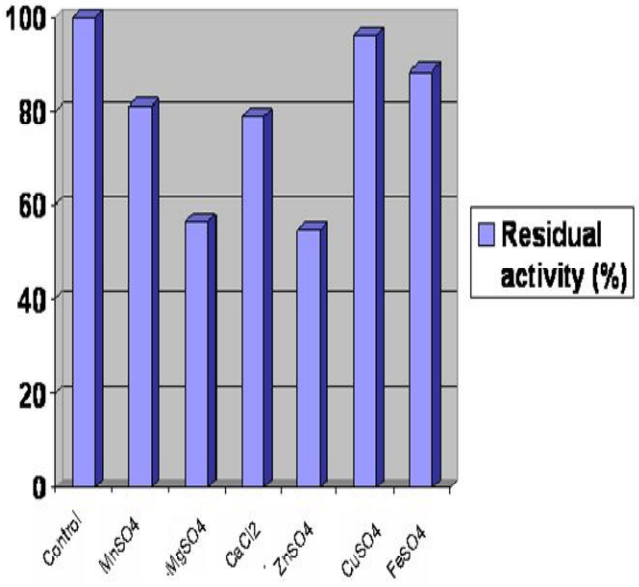

Figure 7 : Effect of divalent metal ions on activity of α-amylase.

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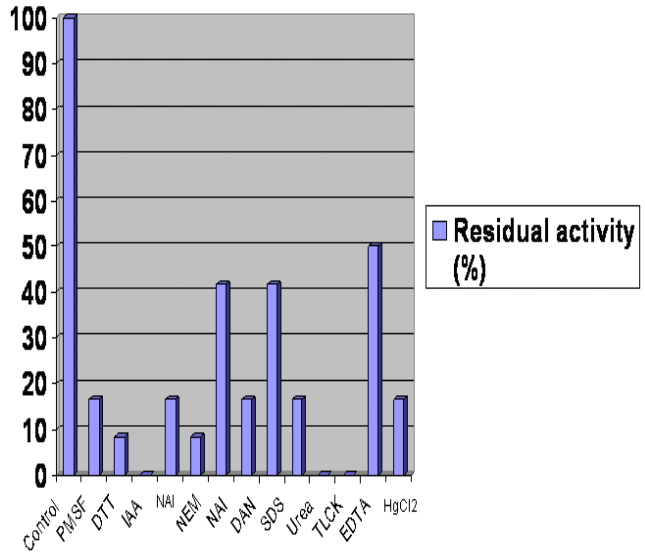

Figure 8 : Effect of group specific reagents on activity of α-amylase.

were not playing an important role at the active site of the enzyme. Hgcl2 inhibited alpha amylase enzyme activity in *Aspergillus oryzae* (Ah1b) Cohn (Figure 8).

Kinetic determination

The Km and Vmax value for the purified α-amylase was 11.11(mg/ml)and 2.0(µmoles/min/ml) respectively for soluble starch as substrate whereas for wheat starch the Km and Vmax was 20.0(mg/ml) and 5.0(µmoles/min/ml) respectively. This shows that soluble starch is a better substrate for purified α-amylase.(Figure 9). The Km and Vmax values were determined from the LB Plot using hgcl2 as inhibitor are 50(mg/ml) and 20(µmoles/min/ml).

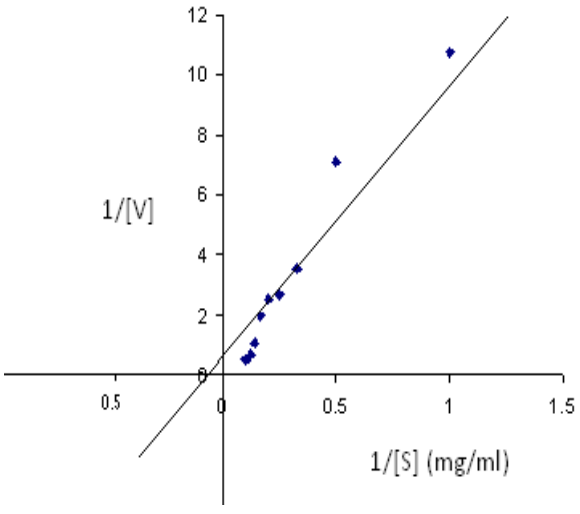

Figure 9 : LB Plot using soluble starch as substrate.

Chromatography of hydrolysis products.

Hydrolysis products of soluble starch are maltose, unidentified oligosaccharides, and trace amounts of glucose. This data from thin layer chromatography confirmed the endo-action of the *Aspergillus oryzae* (Ah1b.) Cohn. enzyme on starch substrates.(Figure 10).

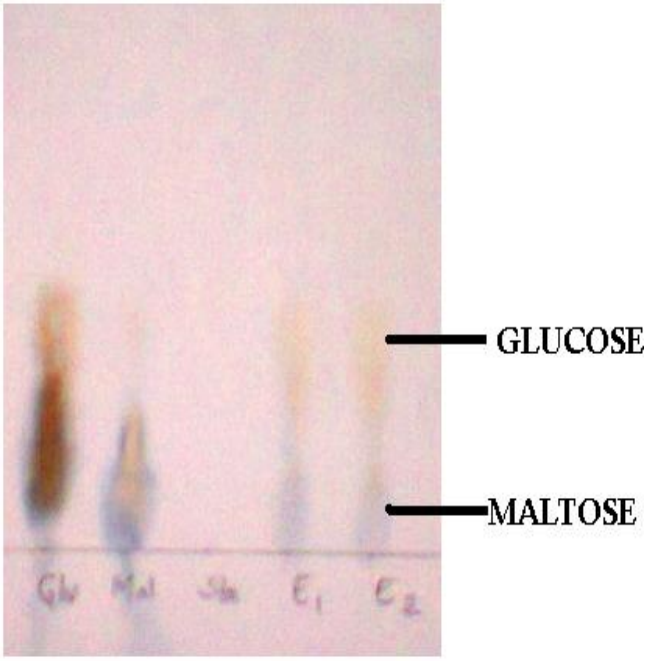

Figure 10 : Thin Layer Chromatography of enzyme hydrolysed product.

DISCUSSION

Alpha amylase (endo-1,4-Dglucose- D glucohydrolase 3.2.1.1.) belongs to the family of endo amylases. They are important enzymes employed in the starch processing industries for the hydrolysis of polysaccharides[21].

In the present study, the optimum pH of *Aspergillus oryzae (*Ah1b.) Cohn. α-amylase was found to be 8.5. This is in agreement with the report that optimum pH of α-amylase from *Aspergillus flavus* was observed at 7[22]. Kundu and Das (1970) reported that *Aspergillus* oryzae prefers optimum pH of 4.5-5.0. Thus, the crude α-amylase of *Aspergillus oryzae* (Ah1b.) Cohn. seems to be active at alkaline pH 8.5 and stable

over a wide range of pH. α amylases are generally stable over a wide range of pH from 4 to 11[23]. α-amylases examined in this study had an optimum temperature of 45^0C which is similar to *Aspergillus fortidus* (24). Optimum yield of alpha amylase were achieved at 30-37^o C for A.oryzae[25,26].

Various divalent metal ions effects the production of amylase[27,28,29,30,31,32,33]. In the present study the enzyme lost 50% of its activity when treated with Mg^{++} and Zn^{++}. It was found that alpha-amylase enzyme isolated from *Aspergillus flavus* was found to be strongly inhibited by Mn^{2+}, Zn^{2+}, Cu^{2+} and Fe^{3+} ions[34] and that of alpha-amylase from *Aspergillus oryzae* ATCC 76080 was inhibited by NAI (6 mM)[35].

The enzyme isolated from *Aspergillus oryzae (*Ah1b.) Cohn. was a glycoprotein like that of enzyme from *Aspergillus tamari*[36]. In the present study, α-amylase had wide range of substrate specificity which was similar to the α-amylase isolated from Streptococcus bovis JB1[15].

The molecular mass of enzyme from *Aspergillus oryzae* (Ah1b.) Cohn. was estimated to be 44±1kDa. The result is in well accordance with studies on ectomycorrhizal fungus Tricholoma matsutake where in the molecular mass of α-amylase was demonstrated as 46 kDa by SDS-page[37]. Enzyme from *Aspergillus flavus* had a molar mass of 52.5 ±2.5 kDa.(38).

The kinetic parameter K_m and Vmax for amylase from *Aspergillus oryzae* (Ah1b.) Cohn. was calculated as 11.11 g/mL and *2.0(*μmoles/min/ml*)* respectively when soluble starch was used as the substrate.

CONCLUSION

Aspergillus oryzae (Ah1b.) Cohn. was isolated from the soil samples and partially purified by chilled acetone precipitation method with 1.88 fold purification and a total yield of 45.0%, with a specific activity of 83.26 U/mg. The enzyme was further purified by Affinity Chromatography, the fold purification was 4.18, yield decreased to 14.19% and the specific activity increased to 118.60 U/mg. Enzyme was subsequently characterized against pH 8.5 and temperature 45^0C and amylase was stable at 45ºC for 2hrs. Enzyme activity was unaltered for most of the ions except Mg^{++} and Zn^{++} with 50% reduction. The molecular mass was estimated as 44±1kDa by SDS-PAGE. The purified enzyme had a Km and Vmax of 11.11 g/mL and 2.0(μmoles/min/ml) respectively for soluble starch as the substrate. According to the present studies, *Aspergillus oryzae* (Ah1b.) Cohn. was found to be an alkolophilic fungal strain which can therefore be utilize in starch processing, detergent industry.

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