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Purification and comparison properties of crude enzyme with purified α-amylase from *Bacillus licheniformis* ATCC 6346

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ABSTRACT

The α-amylase from Bacillus licheniformis ATCC 6346 was purified by ion-exchange chromatography (DEAE-Sepharose). The spent medium contained 37.5 UmL^{-1} α-amylase activity and 1.77 mgL^{-1} protein. Highest specific activity (65.54Umg^{-1}) was obtained at 50% $(NH_4)_2SO_4$ saturation and 66.6% recovered. The precipitated and dialyzed enzyme was purified using DEAE-Sephorose at pH 8.0, and eluted with the 0.01M Tris buffer containing 0-0.8 M NaCl. The recovery of α-amylase by ion-exchange chromatography was 7.5%, with 8.2 fold purification, showing the specific activity of 173.8Umg^{-1} protein. The purified α-amylase was tested for purity by SDS-PAGE. The purified enzyme showed a single band with an apparent molecular weight of 55.54 kDa. Crude α-amylases showed zero order kinetics for 10min while purified α-amylase showed zero order kinetics for 8min. The optimum temperature for the activities of crude and purified enzymes was 85^oC. The optimum pH was 7.0 for the crude and purified at 85^oC. When the crude enzyme was pre-incubated at 85^oC and at pH 7.0, it lost 40% of its initial activity at 10min while the purified enzyme lost 75% of its initial activity at 10min. Crude and purified enzymes showed 119, 77.7 & 20.3 and 107, 60, & 20% of relative activities respectively with amylose, amylopectin, and maltose when compared to soluble starch at 85^oC and pH 7.0. Both crude and purified enzymes showed no activity with cellulose, sucrose and pullulan. Therefore substrate specificity indicated, that both purified and crude α-amylases were able to hydrolyse mainly starch, amylose and amylopectin.

Key words: Purification**,** α-Amylase, *Bacillus licheniformis*, Enzyme stability, DEAE-Sepharose.

INTRODUCTION

α-Amylase is an important industrial bulk enzyme for the food processing industry. (EC 3.2.1.1, 1,4-α-Dglucanohydrolase, endoamylase) hydrolyses starch, glycogen and related polysaccharides

by randomly cleaving internal α-1,4-glucosidic linkages to produce different sizes of oligosaccharides. It is widely distributed in various bacteria, fungi, plants and animals and has a major role in the utilization of polysaccharides [1]. α-Amylases are glycoside hydrolases and have been classified in family 13 [2]. A number of amylases have been reported with different molecular weights, optimum pH and temperatures [3].

α-Amylase is an impotent industrial enzyme [4]. Among the various extracellular enzymes, α-amylase ranks first in terms of commercial uses [5]. Spectrum of applications of α-amylase has widened in many sectors such as clinical, medicinal and analytical chemistry. Besides their use in starch saccharification, they also find applications in baking, brewing, detergent, textile, paper and distilling industry [6]. All known α-amylases contain a conserved calcium binding site [7], [8], [9]. Enzyme production, purification and characterization are a growing field of biotechnology. The purification of α-amylase from the fermented broth is essential for stability and characterization [10]. α-Amylase with desirable properties such as thermostability, metal ion dependence, pH spectra and others can be very useful in related industries. Our research deal with purification and comparison kinetic properties of crude with purified α-amylase from *Bacillus licheniformis* ATCC 6346.

MATERIALS AND METHODS

Strain of α-amylase producer and enzyme production

Bacillus licheniformis ATCC 6346 from Heriot-Watt University U.K was used in this study. The nutrient agar medium contained (gL^{-1}) nutrient agar, 25.0 and soluble starch, 3.0 and the activation medium contained (gL^{-1}) Nutrient broth, 25.0 and soluble starch 3.0 at pH 7.0. The fermentation medium contained (gL^{-1}) soluble starch, 4.0; $(NH_4)_2SO_4$, 5.0; peptone, 6.0; $FeCl_3$, 0.01; $MgCl_2.6H_2O$, 0.01; $CaCl_2.2H_2O$, 0.01; KH_2PO_4, 4.0 and K_2HPO_4, 7.5 at pH 7.0. A loopful of *Bacillus licheniformis* ATCC 6346 from nutrient agar slants with 0.3% soluble starch (grown at 37^oC for 24h) was transferred to 10mL activation medium which was incubated at 42^oC in a rotary shaker (100rpm) for 12 hours and used as inoculum. The fermentation medium was inoculated with 20% (v/v) inoculum and the inoculated flasks were incubated for 48h at 42^oC with shaking at 100rpm. The culture filtrate was used as source of α-amylase.

Measurement of α-amylase activity

Enzyme was diluted with 0.01M phosphate buffer (pH 7.0). The diluted enzyme and soluble starch ($2gL^{-1}$) in 0.0lM phosphate buffer (pH 7.0) were pre incubated for 3 min at 85^oC. Then 0.5mL of the enzyme was mixed with 0.5mL substrate and incubated for 5min at 85^oC. Reducing sugar was measured by the DNS method [11]. One unit of α-amylase activity is defined as the amount of enzyme that produces one μmole of reducing sugar in one minute at 85^oC, and pH 7.0 from soluble starch ($20gL^{-1}$) as substrate.

Purification of α-amylase

Effect of ammonium sulphate on the precipitation of α-amylase

To crude α-amylase, solid $(NH_4)_2SO_4$ was added to bring the $(NH_4)_2SO_4$ saturation to 10% [12]. The solution was mixed well for 2 hour and allowed to settle. Then it was centrifuged (8000rpm at 4^oC) for 30 minutes. The precipitate was dissolved distilled water and dialyzed overnight against distilled water and the α-amylase activity and protein content were measured. The supernatant was dialyzed as said above and analyzed for α-amylase activity and protein content. Similar procedure was repeated with 20, 30, 40, 50, 60 and 70% of $(NH_4)_2SO_4$ saturations. The α-amylase activities and protein contents of both supernatants and the precipitates were analyzed.

Application of enzyme to activated and equilibrated DEAE-Sepharose

To the activated and equilibrated (with 0.01M Tris buffer, pH 8.0) DEAE-Sepharose^{TM} CL-6B containing column (Bed volume 5x1cm (3.92ml)), the α-amylase precipitated with 50% ammonium sulphate and dialyzed against distilled water (7.0mL) was loaded through the 7mL loop. To the column first 20ml of 0.01M Tris buffer (pH 8.0) was added at a flow rate of 1mLmin^{-1} to remove the unbound proteins. After washing the unbound proteins the bound proteins were eluted with a linear gradient (automatic system) of 0-0.8 M NaCl in 0.01M Tris buffer, pH 8.0 (50mL) at a flow rate of 1mLmin^{-1}. Fractions each of 1mL volume were collected using a fraction collector. The samples were analyzed for α-amylase activity and protein contents.

Electrophoresis

The purity of the enzyme was checked by SDS-PAGE (12.5%) by the Laemmli method [13].

Molecular weight determination

Method of Weber and Osborn [14] was used.

Effect of time

Soluble starch (0.5mL, 20gL^{-1} in phosphate buffer pH 7.0) was allowed to react with crude and purified α-amylases (0.5mL in 0.01M phosphate buffer pH 7.0) at 85^{o}C and the amount of glucose produced was monitored. The time suitable for the incubation was optimized.

Effect of temperature

The effect of temperature on crude and purified α-amylase activities were determined by incubating the appropriately diluted enzyme (in 0.01M phosphate buffer, pH 7.0) for optimized time with 0.5mL of soluble starch (20gL^{-1}) at different temperatures, varied from 40 to 95^{o}C. Then activities of the enzyme samples were measured and relative activities were calculated

Effect of pH

The effect of pH on activities of crude and purified α-amylase were measured by preparing 20gL^{-1} soluble starch in buffers of different pH values ranging from 3.0 to 10.0 (for pH from 3.0 to 6.0 citrate phosphate buffer, for pH 8.0 Tris buffer, for pH 9.0 glycine NaOH buffer and for pH 10.0 carbonate, bicarbonate buffer were used). Enzymes were incubated at optimized temperatures for optimized period at 85^{o}C.

Stability of enzymes with temperature

Crude and purified α-amylases were pre-incubated at 85^{o}C and at pH 7.0 and the activities of the enzymes were monitored.

Substrate specificity of the enzymes

Starch, amylose, amylopectin, pectin, chitin, xylan, cellulose, pullulan, sucrose and maltose of 20gL^{-1} concentration in 0.01M phosphate buffer (pH 7.0) were prepared and were used as substrates. The activities of the crude and purified enzymes were determined at 85^{o}C.

RESULTS AND DISCUSSION

To crude α-amylase (activity 37.5UmL^{-1}, protein content 1.77mgmL^{-1}) solid $(NH_4)_2SO_4$ was added to α-amylase to bring the saturation from 10 to 70% separately. When the saturation percentage of $(NH_4)_2SO_4$ was increased from 10 to 70%, the precipitation of protein was increased but the enzyme activity increased up to 50% of $(NH_4)_2SO_4$. This is because, above 50%

of $(NH_4)_2SO_4$ saturation non-enzyme-protein could be precipitated. The crude enzyme sample precipitated with 50% $(NH_4)_2SO_4$ saturation and showed highest specific activity (65 Umg^{-1} protein) than the samples precipitated with different concentration of $(NH_4)_2SO_4$ (Table I). By these $(NH_4)_2SO_4$ precipitation, the specific activity of α-amylase has increased by 3 times than that of crude enzyme 66.6% yield. In supernatant, when the saturation percentage of $(NH_4)_2SO_4$ was increased from 10 to 70%, the protein content and enzyme activity were reduced. α-Amylase from *Bacillus* sp.WN11, purified with 60% $(NH_4)_2SO_4$ saturation showed the specific activity of 205 Umg^{-1}, which was 9.0 fold higher than that of the crude enzyme with 67% yield [15]. α-Amylase from *Bacillus licheniformis* CUMC 305 precipitated with 30-60% ammonium sulphate, showed 28 Umg^{-1} specific activity, with 6.7 fold purification and 51.6% yield [16]. When α-Amylase from *Bacillus subtilis* AX20 was saturated with 70% $(NH_4)_2SO_4$, 119.8 Umg^{-1} specific activity was obtained, which was 1.1 fold higher than the crude enzyme in the 94.2% yield [17]. Ammonium sulphte (50%) precipitation increased the specific activity of α-amylase from *B. licheniformis* ATCC 6346 by 3 times than that of the crude enzyme.

Table I : Effect of ammonium sulphate saturation (in percentage) on the precipitation of α-amylase from spent medium

$(NH_4)_2SO_4$ (%)	Precipitate				Supernatant			
	α-Amylase activity (UmL^{-1})	Protein ($mgmL^{-1}$)	Specific activity (UmL^{-1})	Activity (%)	α-Amylase activity (UmL^{-1})	Protein ($mgmL^{-1}$)	Specific activity (UmL^{-1})	Activity (%)
10	17.03	0.563	30.2	14.98	8.8	0.758	11.6	11.73
20	29.74	0.741	40.1	27.75	7.2	0.642	11.2	10.75
30	33.56	0.696	48.2	33.11	6.3	0.626	10.0	10.08
40	40.56	0.712	56.9	43.26	5.7	0.585	9.7	10.03
50	58.86	0.905	65.0	67.49	4.5	0.552	8.1	8.52
60	57.14	1.397	40.9	76.18	3.3	0.462	7.1	7.04
70	56.01	2.154	26.0	77.66	0.0	0.316	0.0	0.00

To the activated and equilibrated DEAE-Sepharose containing column [column size 11.5x1cm and bed volume 5x1cm (3.92mL)] the $(NH_4)_2SO_4$ precipitated sample was loaded through 7mL loop. The $(NH_4)_2SO_4$ precipitated sample contained a total protein of 57.21mg and the total α-amylase activity of 3750Units. When the gel was washed first with 0.01M Tris buffer (pH 8.0, 20mL) at a flow rate of $1mLmin^{-1}$, the unbound proteins in the $(NH_4)_2SO_4$ precipitated and dialyzed sample was washed away. The bound α-amylase was eluted at a flow rate of $1mLmin^{-1}$ by using linear gradient of 0-0.8M NaCl in 0.01M Tris buffer.

The fractions from 6 to 29 eluted with NaCl buffer (0.01M Tris buffer, pH 8.0) solution contained proteins without α-amylase activity. The protein content was increased again in the fractions from 36 to 47. The fractions from 37 to 47 (11fractions) contained α-amylase activity. Among the fractions, fraction 41 had the highest enzyme activity (Fig. 1). The fractions from 37 to 47 were pooled and this pooled enzyme (11fractions) showed 38.33 UmL^{-1} enzyme activitiy, containing 0.2205 $mgml^{-1}$ protein. Other fractions from 47 to 77 contained non enzyme protein. By this ion exchange purification, the specific activity of α-amylase was increased from 65.54 to173.8 Umg^{-1} protein (Table II), which was 8.2 fold higher than that of the crude α-amylase with 7.5% yield.

Figure 1: Speration of α-amylase from 50% ammonium sulphate precipitated and dialyzed sample by Ion-exchange chromatography on DEAE-Sepharose fast flow column. (▲) α-amylase activity (UmL^{-1}) and (●) Proteins (absorbance at 280nm) eluted with 0-0.8M NaCl containing 0.01M Tris buffer (pH 8.0) at flow rate of $1mlmin^{-1}$

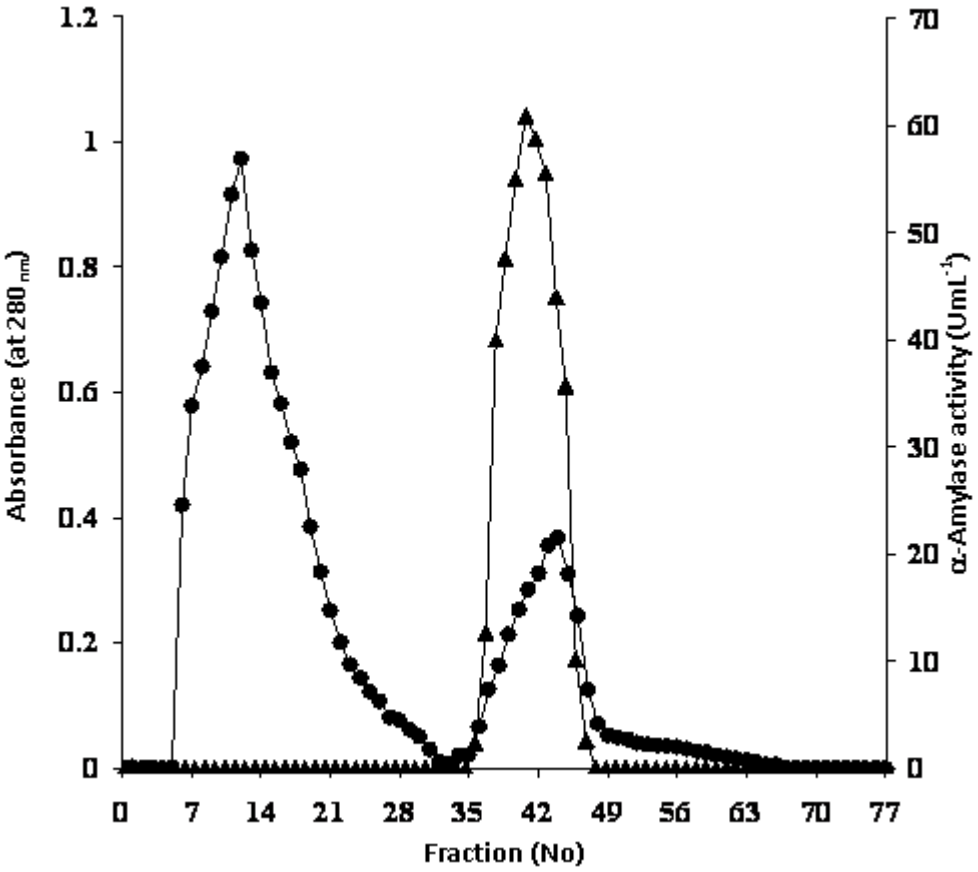
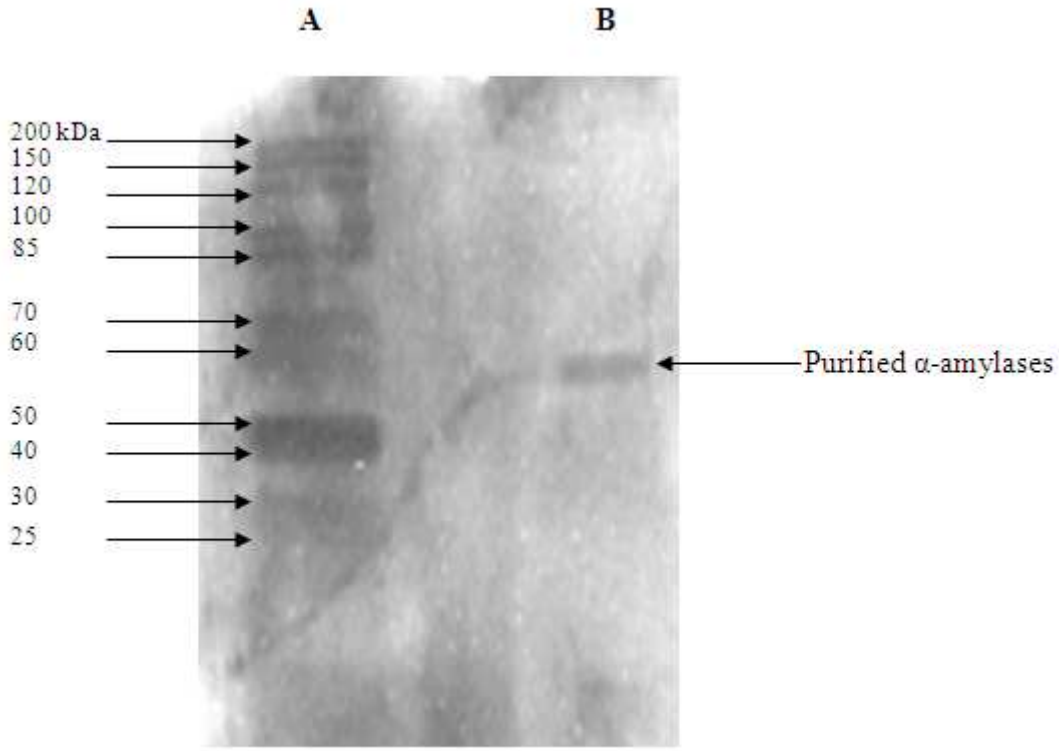


Table II: Purification of crude α-amylase by precipitation with 50% ammonium sulphate and ion exchange chromatography using DEAE-Sepharose

Purification step	Volume	Total activity (U)	Total protein (mg)	Specific activity (Umg^{-1})	Purification fold	Recover (%)
Crude	150	5625	265.5	21.18	1	100
$(NH_4)_2SO_4$ (50%)	7	3750	57.21	65.54	3	66.6
DEAE-Sepharose	11	421.63	2.425	173.8	8.2	7.5

While purifying the α-amylase from *Bacillus* sp. WN11 by using DEAE-Sepharose, 2.7mg of total protein was obtained where the protein content of the initial sample was 6.7mg and the total activity recovered was 1015.0U while the initial activity was 1374.0U with the specific activity of 375.9.2Umg^{-1}, which was 16.5 fold higher than the initial sample and have obtained 49.5% of yield [15]. When α-amylase from *Bacillus subtilis* was purified using DEAE-Sephadex A-50, total protein recovered was 33.6mg with a total activity of 24024 U and the specific activity of 715Umg^{-1} which was 7.8 fold higher than that initial sample with 26.6% yield [18]. When the pooled sample of purified α-amylase was subjected to gel electrophoretic separation, and stained with coomassie brilliant blue the sample gave single band (Fig. 2). This single band indicated that one type of α-amylase was produced by this bacteria and further purification steps are not needed.

Figure 2: SDS-PAGE pattern of purified α-amylase from *Bacillus licheniformis* ATCC 6346. Standard proteins from Fermentas, certificate of analysis: protein Ladder. Lane A: marker proteins; Lane B: purified α-amylase sample.



The apparent molecular weight of the purified α-amylase was estimated by the method of Weber and Osborn [14]. The distance traveled by molecular markers and purified α-amylase were measured. The linear relationship existed between the logarithm of molecular weight of molecular markers and distance migrated by molecular markers (Table III). From this method the molecular weight of the purified α-amylase estimated to be 55540Da (Fig. 3). The molecular weight of this α-amylase (55540Da) closely resembled the molecular weight (56000 Da) of the α-amylase from *Bacillus subtilis* determined by SDS-PAGE and the value determined by gel permeation chromatography is 54000 Da [18] and higher than that reported for *B.licheniformis* 584 α-amylase (22500 Da; [19]).

Table III: The distance migrated by the molecular markers and purified α-amylase from starting point. The distances were measured from SDS-PAGE gel stained with coomassie brilliant blue

Molecular weight (kDa)	Molecular weight (Log)	Distance migrated from starting point (cm)
30	1.47	2.70
40	1.60	2.00
50	1.69	1.60
60	1.77	1.35
70	1.84	1.20
85	1.92	1.00
100	2.0	0.85
120	2.07	0.70
150	2.17	0.60
200	2.3	0.50
Purified α-amylase		1.45

Determining the optimum time for α-amylase activity measurement

Crude α-amylase preparation showed a linear relationship between the time and production up to 10minutes but purified enzyme showed up to 8 minutes (Fig. 4). Hence, it was decided to fix the reaction time for 5min.

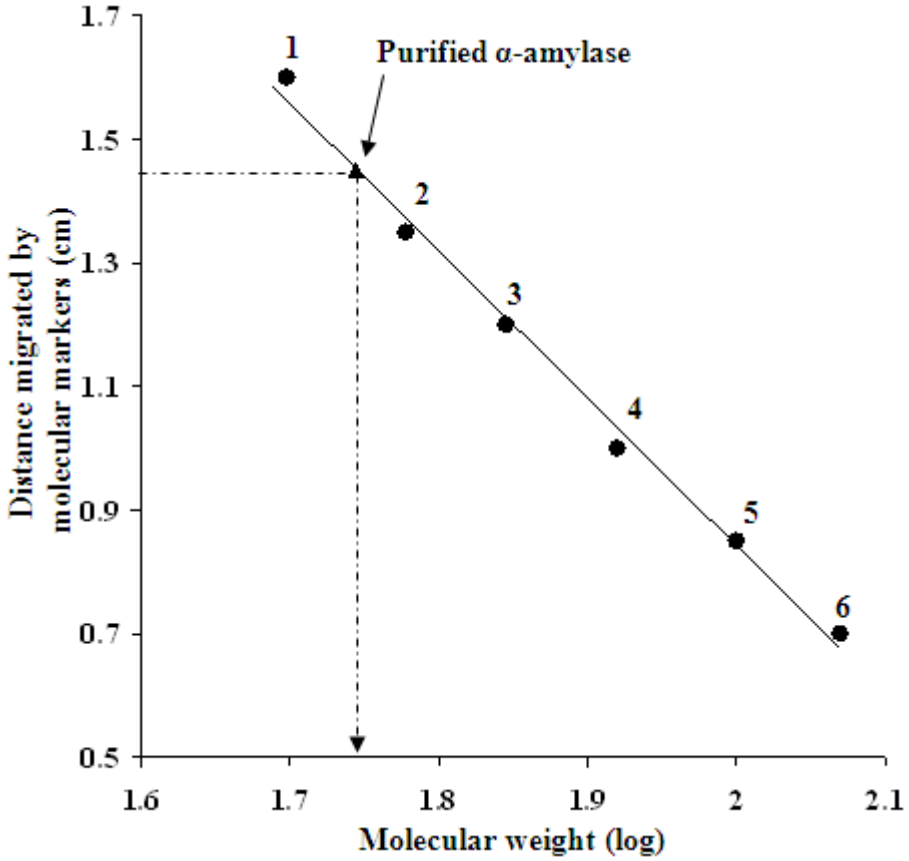


Figure 3: Determination of molecular weight of purified α-amylase by SDS-PAGE and purified by DEAE-Sepharose. The molecular markers used were from Fermentas, certificate of analysis: protein Ladder co Ltd, 1:-50; 2:-60; 3:-70; 4:-85; 5:-100 and 6:-120kDa.

Effect of temperature on the activity of crude and purified α-amylase

The initial relative activity of crude and purified α-amylase increased to 100% as the temperature increased up to 85^{o}C (Fig. 5). Maximum activity was obtained at 85^{o}C and pH 7.0 for the substrate starch (20gL^{-1}). Above 85^{o}C, α-amylase activity was decreased sharply due to thermal denaturation of the enzyme and lost the activity. Hence 85^{o}C was chosen as the optimum temperature for the assay of crude and purified α-amylases.

Bacillus licheniformis ATCC 6346 producing crude and purified α-amylases showed highest activity at 85^{o}C. The proteins, other than enzyme proteins present in the crude enzyme has not influenced the activity of enzyme at different temperatures. The purified α-amylase of *Bacillus licheniformis* CUMC 305 showed maximal activity at 90^{o}C and pH 9.0 [16]. The purified α-amylase obtained from *Bacillus subtilis* was optimally active at 80^{o}C and pH 5.6 [18]. Maximum activity of α-amylase from *Bacillus licheniformis* BLM 1777 was obtained at 85^{o}C and at pH 6.0 [20].

Effect of pH on the activity of crude and purified α-amylases

When the pH was increased, the maximum activities of crude and purified α-amylases were obtained at pH 7.0 (Fig. 6). Increase in the activities were observed of crude enzyme at pH 9.0 but the activities were less than that obtained at pH 7.0. Neutral pH was found to be optimal for amylase production by *B.thermooleovorans* NP54 as also reported in *B.coagulans* [21], *B.licheniformis* [16] and *B. Brevis* [22]. The dependence of enzyme activity on pH is a consequence of the amphoteric properties of proteins [23]. The majority of thermostable α-amylases from *Bacillus spp*, heretofore purified, have shown maximal activity in the acidic to neutral pH range [16]. The optimum pH of activity of purified α-amylase from *Bacillus* sp. TS-23 and *Thermus sp* were 9.0 and 5.5-6.5 respectively [24].

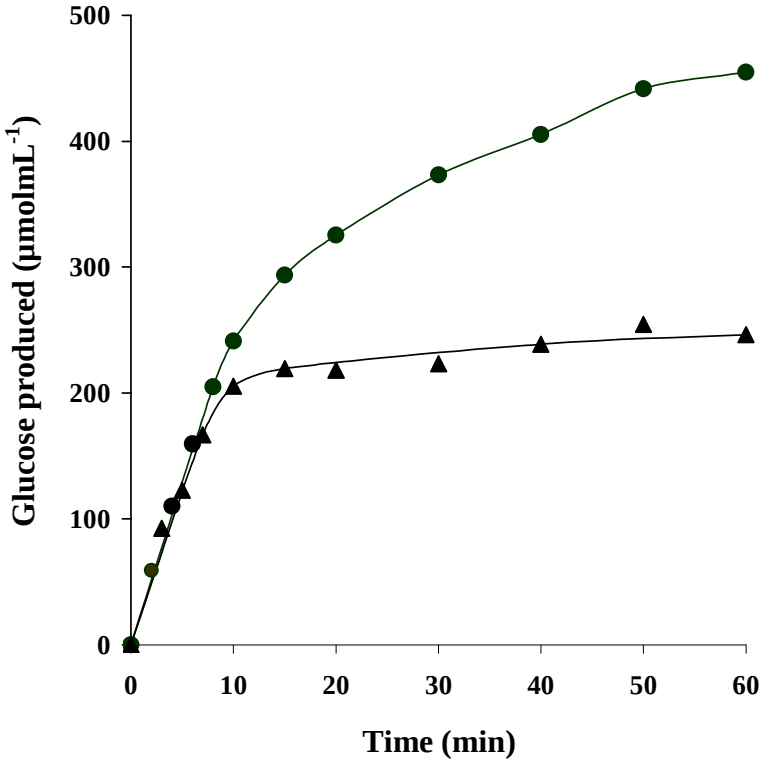


Figure 4: Production of glucose (●), crude and (▲), purified α-amylase preparation on starch (20gL^{-1})-0.01M phosphate buffer (pH 7.0) at 85^{o}C.

Stability of crude and purified α-amylases at 85^{o}C

When the crude α-amylase was pre-incubated at 85^{o}C and pH 7.0 for 30min it retained 31.2% of its initial activity and when it was pre-incubated at 85^{o}C for 60min, lost all its activity (Fig. 7). The purified α-amylase retained 10.69 and 9.57% of its initial activity at 30 and 60min respectively at 85^{o}C and pH 7.0 (0.01M Tris buffer). Half-life of crude and purified α-amylases were 13.9 and 4.7min respectively. Therefore in this case purification has reduced the α-amylase stability at 85^{o}C. Presence of some proteins other than enzyme protein could support the stability of crude α-amylase. The extra thermostability of the thermophilic α-amylase was found to be mainly due to additional salt bridges involving a few specific lysine residues (Lys-385 and Lys-88 and/or Lys-253). These stabilizing electrostatic interactions reduce the extent of unfolding of the enzyme molecule at high temperatures, consequently making it less prone to forming incorrect (scrambled) structures and thus decreasing the overall rate of irreversible thermoinactivation [25]. α-Amylase from *Bacillus* sp. WN11 retained 50% of its initial activity

at 4h when the enzyme was incubated at 80°C [15]. Half-life of *Bacillus thermooleovorans* NP54 producing α-amylase was 3h at 100°C and pH 8.0 [26].

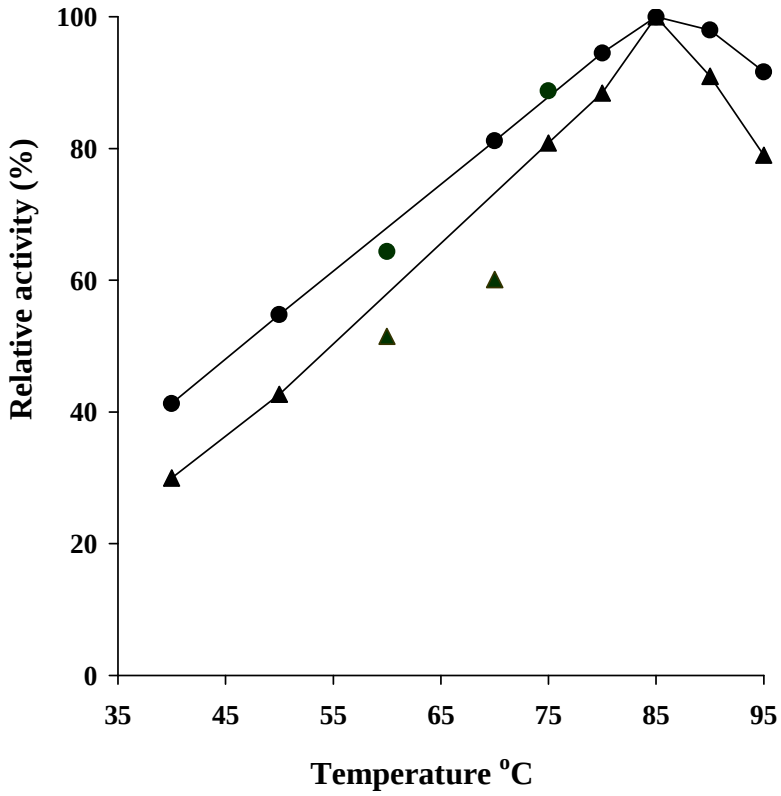


Figure 5: Effect of temperature on the activity of (●), crude and (▲), purified α-amylases with starch (20gL^{-1}) at pH 7.0. α-Amylases activity were measured at different temperatures of 40, 50, 60, 70, 75, 80, 85, 90 and 95°C, using 20gL^{-1} starch as substrate by incubating for 5minutes at pH 7.0 (0.01M phosphate buffer).

Comparison of substrate specificity of the crude and purified α-amylases

Different substrates were hydrolyzed by crude and purified α-amylases. When 20gL^{-1} of amylose, amylopectin, pectin, chitin, xylan and maltose were used as substrates to crude enzyme that showed 119.3, 77.7, 25.5, 27.4, 5.7 and 20.3% of relative activity (Table IV) when compared to soluble starch and no activity was observed when cellulose, pullulan and sucrose were used as substrates at 85°C and pH 7.0 (0.01M phosphate buffer). When 20gL^{-1} of amylose, amylopectin, pectin, chitin, xylan and maltose were used as substrate to purified enzyme, it showed 107.42, 60.02, 19.64, 21.17, 0, and 20.3% of relative activity (Table IV) when compared to soluble starch at 85°C and pH 7.0. α-Amylase does not hydrolyse cellulose because it dose not hydrolyse ß(1→4) linkages. But from chitin reducing sugars were obtained at slower rate at 85°C.

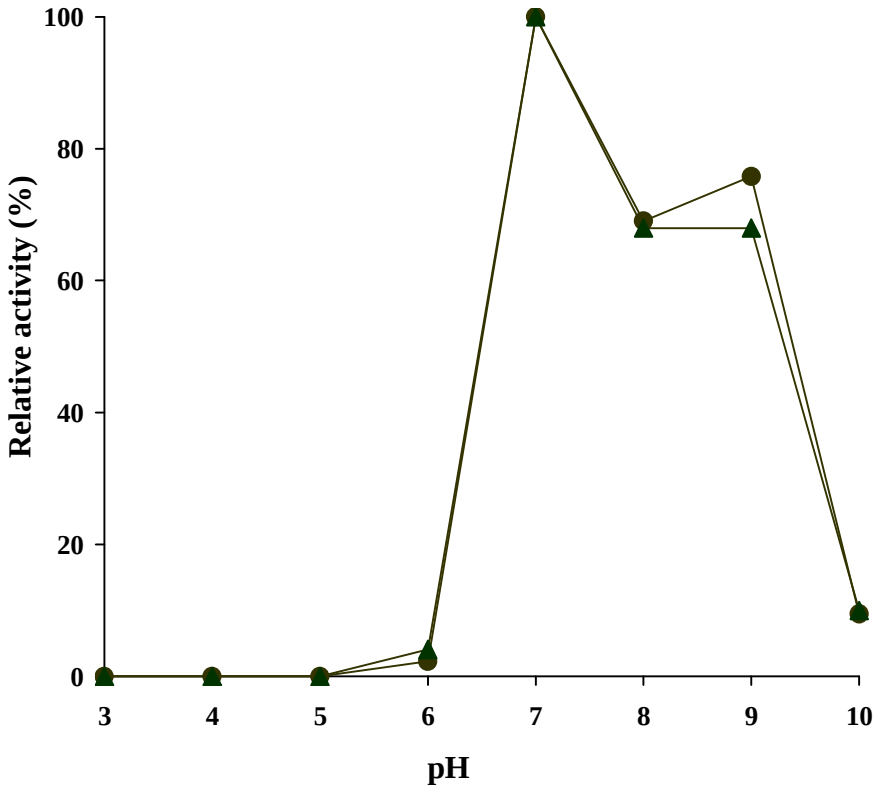


Figure 6: Effect of pH on the activity of (●), crude and (▲), purified α-amylases with starch (20gL^{-1}) at pH 85^{o}C. Activities were measured at different pH, using 20gL^{-1} starch as substrate by incubating for 5minutes at 85^{o}C.

Chitin contains ß(1→4) linkage here the products should have been obtained by the enzyme activity. The results indicated that the substrate samples contained some impurities. Both crude and purified enzymes hydrolysed maltose at slower rate. Study of the substrate specificity indicated that the enzyme was able to hydrolyse mainly starch, amylopection and amylose. Maltose was slowely hydrolyzed at 85^{o}C and at pH 7.0.

The relative rates of hydrolysis of amylase, soluble starch, amylopectin and dextrin by α-amylase from *Bacteroides amlophilus* were 100, 97, 92 and 60% respectively [27]. Krishnan *et al* [16] showed that the substrate specificity of purified α-amylase from *Bacillus licheniformis* CUMC305 with different 1% substrates, release of reducing sugar were very rapid from amylase (129%) but was slower from soluble starch (101.4%), amylopectin (58.3%) and glycogen (100%). α-Amylase from *Streptococcus bovis* JB1, exhibited neither pullulanase nor dextranase activity and hydrolysis of amylase, starch and amylopectin were 100, 100 and 70% respectively [28]. Starch, amylase and amylopctin were the substrates preferentially hydrolysed by α-amylase from *Aspergillus tamari* [29].

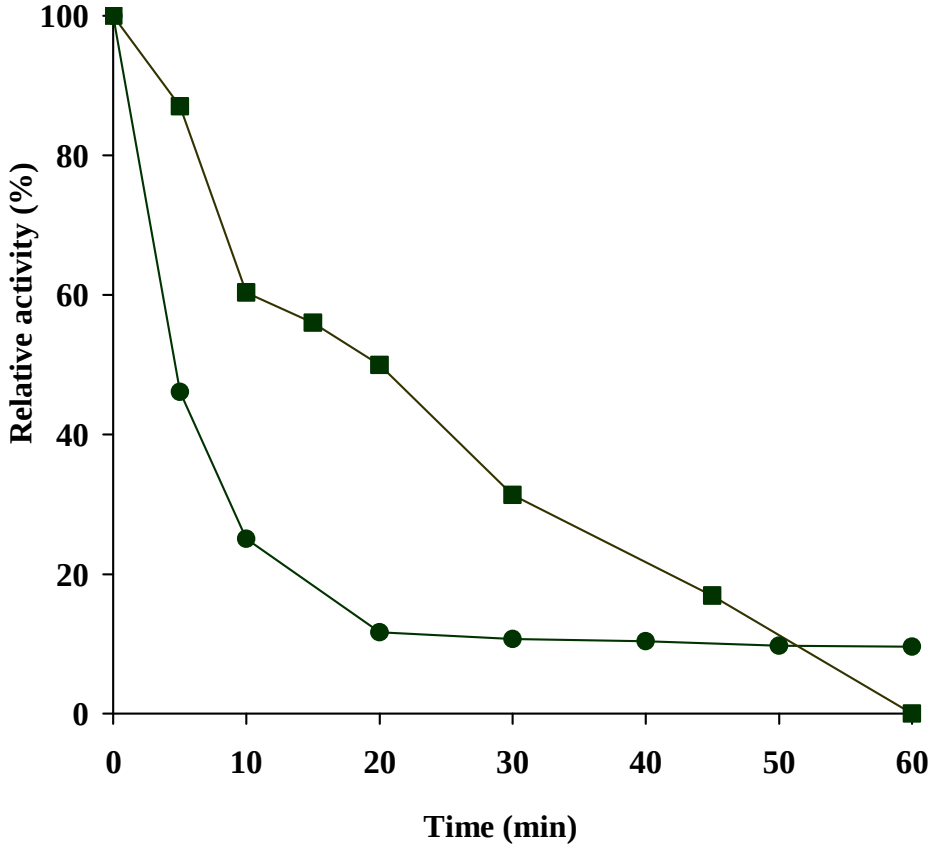


Figure 7: Stability of (●), crude and (▲), purified α-amylases. α-Amylase activity was measured at 85°C using 20gL^{-1} starch as substrate by incubating for 5minutes at pH 7.0 (0.01M phosphate buffer).

Table IV: Effect of different carbon sources on the activity of crude and purified α-amylase produced by *B.licheniformis*. α-Amylase activity was determined at 85°C and pH 7.0 (0.01M phosphate buffer) using 20gL^{-1} different substrate by incubating for at 5minutes.

Substrate (20gL^{-1})	Relative α-amylase activity (%)	
	Crude	Purified
Starch (control)	100	100
Amylose	119.3	107.42
Amylopectin	77.7	60.02
Pectin	25.5	19.64
Chitin	27.4	21.70
Xylan	5.7	0.00
Cellulose	0.0	0.00
Pullulan	0.0	0.00
Sucrose	0.0	0.00
Maltose	20.3	20.06

CONCLUSION

The specific activity of the crude enzyme was 21.18 Umg^{-1} protein and this was increased to 65.54 Umg^{-1}protein by $(NH_4)_2SO_4$ precipitation. Further purification by ion exchange chromatography had increased the specific activity to 173.8 Umg^{-1}protein. A thermostable α-amylase 55.540 kDa (MW) produced by mesophilic *Bacillus licheniformis* ATCC 6346, has been

purified to homogeneity. The optimum pH of crude and purified α-amylase from *Bacillus licheniformis* ATCC 6346 α-amylase was pH 7.0. The enzyme was more specific to hydrolyse α(1→4) linkages between glucose units. Future work includes effect of metal ions on the stability of purified α-amylase.

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