

Isolation, Purification and Characterization of Thermostable α -amylase Enzyme from *Bacillus TULH*

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This research reported the isolation, purification and characterization of thermostable α -amylase enzyme from *Bacillus TULH*. Studies on the α -amylase production were carried out with a bacterial strain isolated from a hot water spring of gir national forest. The addition of yeast extract, $MgSO_4$, Vitamins and amino acids to the mineral medium shortened the lag period and improved the growth and α -amylase synthesis. The bacteria showed optimum growth at pH 6.8 and optimum temperature for the growth at 70°C. The optimal pH and temperature of the amylase activity were 6.8 and 68°C, respectively. The enzyme was found to be stable in the pH range of 5 to 8. Maximum α -amylase activity was determined in 2% starch. The enzyme was purified using 60% ammonium sulphate precipitation, dialysis and DEAE cellulose ion exchange chromatography which resulted in 11.1 fold purity with specific activity of 9.40 units/mg protein/ml. SDS-PAGE showed a single band equal to molecular weight of about 67 kDa which is equivalent to microbial amylases. The activity of the purified α -amylase increased with increasing enzyme concentration and incubation time.

Key words: *Bacillus TULH*, thermophile, α -amylase, DEAE cellulose, Dialysis.

One of the enzymes widely used in industrial sectors is α -amylase enzyme (α -1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) which catalyses the breakdown of α -D-(1,4)-glucosidic bond in glycogen and oligosaccharide (Collins *et al.*, 1993a; Bolton *et al.*, 1997; Dey *et al.*, 2002; Messaoud *et al.*, 2004; Hasim *et al.*, 2005). α -amylases has extensive commercial applications in starch liquefaction, brewing, sizing in textile industries, and paper and detergent manufacturing processes and constitute a class of industrial enzyme having approximately 30% of the world enzyme production (Vander Maarel *et al.*, 2002). Though they originate from different sources (plants, animals and microorganism) (Vander Maarel *et al.*, 2002; Aquino *et al.*, 2003) and show varying pattern of action depending on the source and origin (Hagenimana *et al.*, 1992), in industry, they are mainly produced from microbes (Crueger *et al.*, 1989; Kathiresan *et al.*, 2006). Enzymes from

microbial sources generally meet industrial demands due to their higher yield and thermo stability, which allow them to work at elevated temperatures (Adams *et al.*, 1998; Filter *et al.*, 2000; Burhan *et al.*, 2003). In this research, α -amylase was isolated and purified from bacterial isolate *B. tulh* in three step purification processes, namely coagulation with ammonium sulphate salt in a variety of saturated degree, dialysis and DEAE-cellulose ion exchange column chromatography. The enzyme was then characterized at its pH optima and temperature optima. The enzyme activity was determined based on Fuwa 1954 and Mandels 1976 methods, while protein content was determined by Lowry method (Lowry *et al.*, 1951).

MATERIALS AND METHODS

Sample collection and screening of α -amylase producing bacteria

Bacteria named *Bacillus TULH*. was isolated from hot water spring located at gir national park for the production of thermostable α -amylase.

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Organism grows best at 68°C on media containing (grams per liter): 10.0 g Peptone, 3.0 g Meat/beef extract, 5.0 g Sodium chloride, 3.0 g Agar, and 2% starch pH (7.4). Plates were flooded with a solution of 0.5% (w/v) I2 and 5.0% (w/v) KI (Thippeswamy *et al.*, 2006). The clear zone surrounding the colony was measured in (cm) from the edge of the colony to the limit of clearing and the diameter of colony was recorded. The colonies forming clear zones around them were picked up and streaked on nutrient agar plates to get pure culture and to confirm zone formation.

Purification procedures of *B. Tuh* α - amylase

All experiments were carried out in triplicates and all purification procedures carried out at 4°C as the work was related to thermostable enzymes as follows:

Preparation of the crude enzyme

The loopfull suspension of bacteria was inoculated in the fermentation media containing 3% starch. The strain was grown at 68°C for 48 h in liquid broth, centrifuged at 10000 rpm for 15 min. and the supernatant retained as the source of extracellular enzyme. The resulting culture supernatant was filtered through Whatman No. 1 filter paper and the filtrate used as crude enzyme solution. The obtained filtrate was then estimated for both protein content and amylolytic activity.

Ammonium sulfate precipitation

Ammonium sulfate was gradually added to the supernatant to different 20-70% saturations according to the method of Gomeri (Gomeri *et al.*, 1995). The precipitate of crude enzyme was dissolved in a minimum volume of 0.2 M phosphate buffer (pH 7.0); and dialyzed overnight in a dialysis bag against the same phosphate buffer at pH-7 at 4 °C. At each step of saturations, a pellet was collected and was kept in the refrigerator at 4 °C for further purification steps.

DEAE cellulose anion exchange chromatography

The concentrated enzyme preparation was loaded onto DEAE cellulose anion exchange column pre-equilibrated with 0.1 M KCL (pH 7.0), and sample can be eluted by using the gradients of KCl by eluting the sample each time with a given concentration of KCl like 0.1-0.5 M. Active fractions exhibiting amylolytic activity were collected, combined and assayed for both α -amylase activity and protein content.

Determination of the molecular weight of the purified enzyme

The molecular weight of the purified enzyme was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% (w/v) acrylamide gel that was performed as described by Laemmli, U.K. 1970 and modified later by Studier, F.W. 1973.

Amylase activity assay

Protein content of the enzyme extracts was estimated by the method of Lowry (Lowry *et al.*, 1951) using bovine serum albumin (BSA) as the standard. Amylase activity was determined by measuring the release of reducing sugar from soluble starch. The reaction mixture contained 0.5 ml of crude enzyme and 1 ml of sodium phosphate buffer (pH 7.0) containing 1% soluble starch and incubated at 68°C for 10 min, the amount of reducing sugar released in the mixture was determined by the addition of 2 ml of 3, 5 dinitrosalicylic acid method (Yang *et al.*, 2003) followed by boiling for 10 min and to develop color. The absorbance of the mixture was measured at 540 nm, and standard curve was developed with help of glucose. One unit of enzyme activity was defined as the amount of enzyme releasing reducing sugar equivalent to one μ mol glucose per minute under the assay condition. All analytical measurements were performed at least in triplicates.

Effect of pH-values on amylase activity

The effect of pH on the activity of amylase was measured by incubating 0.1 mL of the enzyme and 1.5 ml of buffers presenting pH from 5.8-8.0, containing 1% soluble starch for 30 min at 68°C. The buffers used were: Sodium acetate buffer, pH 4.6- 5.6; sodium phosphate buffer, pH 6.8-7.9. Then after the mixture were boiled for 10 min to measure reducing sugar content in order to know amylase activity.

Effect of temperature on amylase activity

The effect of temperature on the enzyme activity was determined by performing the previously described standard assay procedure for 10 min at pH 6.8 within a temperature range of 35°C-75°C.

RESULTS AND DISCUSSION

Bacteria and other microbes were the predominant in the extreme environment as they

were the first to evolve and provide excellent source of various industrial important raw materials such as enzymes and other polymers. Thermophiles are the main source of thermostable enzymes. The intrinsic thermal stability of the enzymes isolated from these sources makes it possible to study the molecular mechanisms governing structure and function in a system adapted for elevated temperatures. Although α -amylases can be derived from several sources, such as plants, animals and microorganisms, the enzymes from microbial sources are preferred in industrial sector and a large number of them are available commercially (Crueger *et al.*, 1989; Kathiresan *et al.*, 2006). Precipitation of α -amylase was performed by using different concentrations of ammonium sulfate, 60% concentration gave the highest enzyme activity of 0.316 (μ M/mg protein/ml) as illustrated in table 1. Enzyme purification using 60% ammonium sulfate for precipitation followed by purification using DEAE cellulose ion exchange chromatography resulted in 2 fold pure enzyme recovery, specific activity of 1.66 U/mg protein/ml as illustrated in table 1.

Moreover eluted product of ion exchanged chromatography showed specific activity of 9.40 U/mg of protein while it exhibited

11 fold purity then the crude one. Ibrahim *et al.*, in 1990 succeeded to precipitate and purify amylase secreted by *Streptomyces aureofaciens* 77 using a 50-70 % saturation ammonium sulfate and the amylase purification on Sephadex G-200 column chromatography resulted in an increase of purification up to 74 fold. Similarly, Sidkey *et al.*, 1997 purified α -amylase from *A. flavus*, S-7 by a process of ammonium sulfate precipitation at 80% saturation and sephadex G-200 column chromatography resulting in a purified enzyme with specific activity of 28.6 (units/mg protein/ml) and 6.7 purification folds. Moreover the obtained data showed that the fractions from 60% ammonium sulfate saturation correlated with high proteolytic and specific activities compared with the crude amylase and other concentrations. Similar concentration of 60% saturation ammonium sulfate was used by Carvalho *et al.*, 2004 to precipitate amylase from thermophilic *Bacillus* sp. strain SMIA2. Whereas, amylase from *Bacillus* strain GM8901 precipitated by 80% saturation of ammonium sulfate (Kim *et al.*, 1995). The results showed that the enzyme was purified 6.9 fold with a specific activity of 2487 U mg⁻¹ proteins after ammonium sulfate fractionation.

Table 1. A summary of the purification steps of *B. Tuhl* α -amylase

Sample	Sugar Estimated μ g/ml	Enzyme unit μ M/min./ml	Protein Estimated μ g/ml	Specific Activity	Fold Activity
Crude	500	0.073	86.79	.841	1
Precipitated	1625	0.316	1147	0.275	0.326
Dialyzed	4200	.818	492.52	1.66	1.97
Eluted	2557	0.498	53.98	9.40	11.1

Table 2. Comparison of various characteristics for amylase from *Bacillus* sp. strain *Tulh* with other *Bacillus* species

Source	Optimum pH	pH stability	Optimum temperature ($^{\circ}$ C)	MW (kDa)	References
<i>Bacillus Tulh</i>	6.8	6.8	68-70	67	This study
<i>Bacillus</i> YX-1	5.0	4.5-11.0	40-50	56	Liu and Yan <i>et al.</i> 2008
<i>Bacillus</i> GM8901	10.5	6.0-13.0	50	97	Kim <i>et al.</i> 1995
<i>Bacillus subtilis</i> JS-2004	8.0	5.5-10.0	70		Asgher <i>et al.</i> 2007
<i>Bacillus</i> ANT6	10.5	9.0-13.0	100 -		Burhan <i>et al.</i> 2007
<i>Bacillus</i> WN11 76;	5.5	5.6-9.0	75-80	53	Mamo and Gessesse 1999
<i>Bacillus</i> A3-15	8.5	6.0-12.0	60	86; 60	Burhan <i>et al.</i> 2007
<i>Bacillus licheniformis</i>	7.5	6.0-7.0	70		Iraj <i>et al.</i> 2008

Enzyme profile

Analysis of the purified enzyme by SDS-PAGE revealed a monomer band with a molecular mass of 67 kDa (Fig. 1). Vieille and Zeikus in 2001 reported that the molecular mass for amylase range

from 50-60 kDa. With regards to the findings of other workers, the enzyme obtained by Liu and Xu showed a molecular weight of 56 kDa by SDS-PAGE after purification using ammonium sulfate precipitation, ion exchange and gel filtration

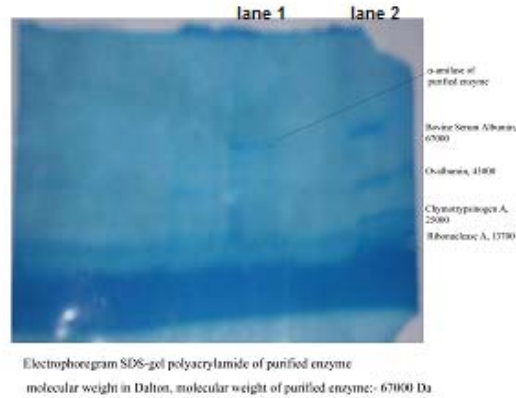


Fig. 1. SDS-PAGE of pure *Bacillus TULH* α -amylase. (1) Pure Enzyme after DEAE cellulose ion exchange chromatography. (2) Molecular size marker

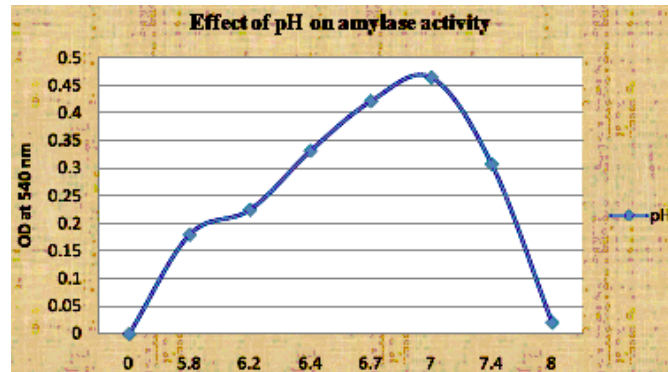


Fig. 2. Effect of pH on amylase activity for *Bacillus* strain *TULH*. The activity of the purified enzyme was measured in 100 mM acetate or phosphate buffer pH 5.8 to 8 for 10 min at 68°C

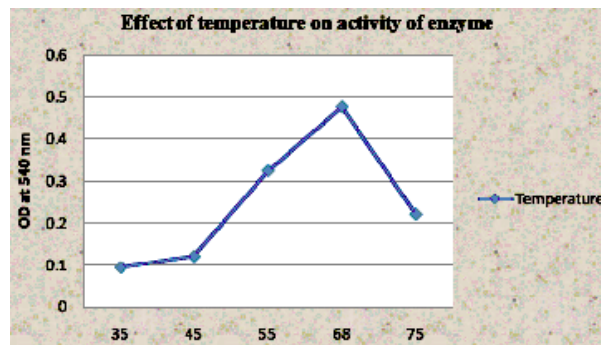


Fig. 3 Effect of temperature on amylase activity for *Bacillus* strain *TULH*. The activity of the purified enzyme was measured in 100 mM phosphate buffer pH 6.8 for 10 min at 35°C to 75°C

chromatography from a newly isolated *Bacillus* sp. YX-1. Different molecular masses for different amylases have been reported by gel filtration: 76-53 kDa for *Bacillus* WN11 (Mamo *et al.*, 1995); 56 kDa for *Bacillus* sp. YX-1 (Liu *et al.*, 2008); 86-60 kDa for *Bacillus* A3-15 (Burhan *et al.*, 2007) and 97 kDa for *Bacillus* GM8901 (Kim *et al.*, 1995) (Table 2).

In the present study maximum amylase activity was obtained with substrate concentration of 2%. Other researcher who found maximum α -amylase activity was attained at the least substrate (starch) concentration (Sidkey *et al.*, 1997) while other investigators as Moustafa, O.A. 2002 found that 1% and 4% starch solution gave the highest α -amylase activity in case of *T. lanuginosus* F4 and *S. moniliformis* B7, respectively. The production and stability of the enzyme is very sensitive to pH and temperature (Declerck *et al.*, 2003). Slight changes in temperature and pH have adverse effect on the growth of microorganisms as well as on the productivity of α -amylase (Anyangwa *et al.*, 1993). The Most of the *Bacillus* amylase have optimum pH values of from 5.0-8.5 (Asgher *et al.*, 2007; Liu and Yan *et al.*, 2008; Burhan *et al.*, 2007; Iraj *et al.*, 2008) However, our amylase has an optimal pH value of 6.8 and activity was found to be gradually declining as the pH goes to alkalinity, indicating that the enzyme is mesophilic. Also, temperature was found to play a significant role in the activity of the produced α -amylase. The supernatant amyolytic activities were assayed at different temperatures ranging from 35-75°C. Enzyme activity increased with temperature within the range of 35-70°C. A reduction in enzyme activity was observed at temperatures above 70°C. The optimum temperature of amylase activity was 68-70°C, which is comparable to that described for other *Bacillus* amylase (Zhang *et al.*, 1994; Takasaki *et al.*, 1994). Purified amylase was found to be moderately thermostable as compare to other amylase indicated in table 2.

CONCLUSION

The newly isolated *B. TULH* strain which produces α -amylase was active at pH 6.8 besides mesophilic in its characteristics; the most striking feature of the enzyme was its thermostability at

70°C. Also significant thermostability of the enzyme make it potential for industrial applications such as starch liquefaction, textile desizing and paper industries, which requires the process to be carried out in multiple steps at high temperature.

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