

Purification and characterization of an extracellular thermostable alkaline α -amylase from the moderately halophilic bacterium, *Bacillus persicus*

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Abstract

Background: Today a large number of bacterial amylases are available commercially in industry. The goal of the present study was purification and biochemical characterization of an extracellular thermostable alkaline α -amylase from the novel moderately halophilic, *Bacillus persicus* from the Aran-Bidgol, Iran.

Methods: Purification of enzyme, was carried out by acetone precipitation, ultrafiltration and Q-Sepharose cation exchange chromatography.

Results: The purified native enzyme showed a molecular mass of 53 kDa composed of a monomer by SDS-PAGE. The optimum pH, temperature and NaCl concentration were 10, 45 °C and 0.85 M respectively. It retained 50% of activity at 1.25 M NaCl and about 73% of activity at highly alkaline pH of 10.5, therefore it was a moderately halophilic and also can activate by divalent metal ions especially Ca²⁺ and Mg²⁺. The apparent values of Km and Vmax were obtained 1.053 mg/ml and 356 μ M/min respectively.

Conclusion: In the present study we report the purification and characterization of a moderately halophilic α -amylase from a newly isolated *Bacillus persicus*. The purified enzyme shows interesting properties useful for industrial and biotechnological applications. The molecular cloning and structural studies of this α -amylase are in progress in our laboratory.

Keywords: α -Amylase, *Bacillus persicus*, Q-Sepharose, Haloalkaline, Thermostable

Introduction

There has been a meaningful increase in the use of the enzymes as industrial catalysts (Mojsov, 2012; Jegannathan & Nielsen, 2013; Sundarram & Murthy, 2014). Therefore, obtaining and preparation of pure enzyme that is active and stable under multiple extreme conditions

(alkaline pH, high salt concentrations and wide temperature) is scientifically and industrially significant (Gomes & Steiner, 2004; Gupta & Roy, 2004; Souza, 2010). α -Amylases (1,4- α -D-glucan glucanohydrolase [E.C. 3.2.1.1]) constitute a class of industrial and biotechnological enzymes, can be obtained from plants, animals and microbial sources. It is well evident that microbial α -amylases constitute one

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of the most important groups of industrial enzymes (Souza, 2010; Doss & Anand, 2012; Vengadaramana, 2013), as they account for at least a quarter of the total global enzyme production and covering many industrial processes such as sugar, paper, distilling industries and pharmaceuticals (Oyeleke et al., 2010). Today a large number of bacterial amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry (Souza, 2010). Amylases have been isolated and characterized from a number of animal, plant and fungal as well as bacterial sources, with different molecular weights, optimum pH, salt concentration and temperatures. α -Amylases with optimum pH values higher than 8.0 have potential applications in a wide number of industrial processes such as fermentation, paper, detergent, and pharmaceutical industries as well as the removal of starch sizing from textiles (Souza, 2010; Sajedi et al., 2005; Yang et al., 2011; Behal et al., 2006). Thus, the potential of alkaline amylases for industrial applications have stimulated the search for microbial strains expressing activities with desired properties. The genus "*Bacillus*" is an important source of industrial alkaline amylases and multiple *bacillus*-derived alkaline amylases have been purified and characterized (Oyeleke et al., 2010; Aygan et al., 2008; Barros et al. 2013).

Alkaliphilic *Bacillus* strains often produce enzymes active at alkaline pH, including alkaline α -amylases and proteases (Reddy et al., 2004). Several *Bacillus* strains such as *Bacillus sp. AB6* (Aygan et al., 2008), *Bacillus sp. PN5* (Saxena et al., 2007), *Bacillus subtilis A10* (Aygan et al., 2014), *Bacillus halodurans 38C-2-1* (Murakami et al., 2007), *Bacillus sp. isolate ANT-6* (Burhan et al., 2003), Alkaliphilic *Bacillus Isolate KSM-K38* (Hagihara et al., 2001) are known to produce industrially important alkaline amylases. The goal of the present study was isolation, purification and biochemical characterization of α -amylase from the novel moderately halophilic, *Bacillus persicus* from the Aran-Bidgol, Iran. Aran-Bidgol Lake, located in the center of Iran, is a hypersaline lake and the largest playa in Iran. α -Amylase from *Bacillus persicus* was characterized under various conditions of pH, temperature, salt concentrations, detergents and metal ions. The amylase has been used as a diagnostic tool in medicine and pathology, a higher than normal level of amylase may predict one of several medical conditions, including acute inflammation of the pancreas and perforated peptic ulcer.

Materials and methods

Bacillus persicus obtained from the Iranian Biological Resource Center (IBRC-M 10115). All other chemicals used were of analytical grade.

Production and Purification of the α -amylase

For α -amylase isolation, the basal medium (100 ml) was inoculated with 1% of a stationary-phase culture and incubated at 45°C on a shaking platform at 150 rpm for 48 h. For the production of α -amylase a basal medium containing (g/l): yeast extract 6, peptone 12, maltose 4, NaCl 95, MgSO₄ 9.5 MgCl₂ 7, CaCl₂ 0.3, NaHCO₃ 2 and 1% soluble starch were used. After removal of cells by centrifugation (10000 g, 15 min) at 4 °C, the supernatant (crude enzyme) was used for α -amylase assay and purification. The α -amylase present in the culture supernatant were precipitated using acetone precipitation and dialyzing against 100 mM glycine–NaOH, pH 9.5 containing 50 mM NaCl for 20 h. The dialyzed crude enzyme was ultrafiltered using an Amicon Ultra centrifugal filter with a 30 kDa cutoff (Millipore, USA). The α -amylase activity was detected in the retentate fraction. Filtered crude was subjected onto Q-Sepharose column at a flow rate of 1ml/min, which was previously equilibrated with 25 mM Tris buffer. The α -amylase was eluted with a linear gradient of NaCl (0–500 mM). The fractions of 1 ml each were collected and monitored for α -amylase activity. Fractions showing the highest α -amylase activity were pooled, and desalted by dialyzing overnight against 100 mM glycine–NaOH, pH 9.5. All purification steps were performed at 4 °C. The purity of the

enzyme was investigated by SDS-PAGE and zymography. Also to determine molecular mass of the purified α -amylase, the protein was loaded onto SDS-PAGE and the gel stained with Coomassie brilliant blue R250.

Native PAGE

Native PAGE was carried out according to the method described by Singh (Singh et al., 2005), in a 8% (w/v) polyacrylamide gel with Tris/glycine buffer, pH 8.3. All the steps were performed at 4 °C. Then the gel was stained with Coomassie brilliant blue R-250 (0.2%).

SDS–PAGE

SDS-PAGE was carried out to test for purity and to determine the molecular weight of the enzyme as described by Laemmli (Laemmli, 1970), in a discontinuous system made of a 4% stacking gel and 12.5% resolving gel. Samples were heated at 90°C for 5 min in sample buffer prior to electrophoresis. A ready to use molecular marker (Fermentase) was used as a standard reference. Then the gel was stained with Coomassie brilliant blue R-250 (0.2%). All stages of purification were checked by electrophoresis.

Activity staining

Zymography was performed in polyacrylamide gel containing SDS. Samples were mixed with sample buffer without heat denaturation, and

electrophoresed. After electrophoresis, the gel was incubated in 2.5% (v/v) Triton X-100 for 45 min at room temperature to remove SDS. Triton-X-100 was removed by washing the gel with 50 mM Tris-HCl, pH 8.5. Prior to staining, the gels were slightly rinsed in distilled water. The rinsed gel was transferred to fresh buffer containing 1% soluble starch. The gel was incubated for 1 h, at 45°C, for the diffusion of the starch to the gel and that the enzymatic reaction occurs. Upon applying the Lugols solution at room temperature, protein bands with amylolytic activity became visible as clear zones against a dark blue background. All stages of purification were checked by zymography.

Protein estimation and α -amylase activity assay

Protein concentration was determined by the method of Bradford using bovine serum albumin as standard references (Bradford, 1976). The concentration of protein at different purification steps was determined by this method. The α -amylase activity was determined by measuring the formation of reducing sugars released during starch hydrolysis, using starch (1% v/v) as a substrate in 100 mM glycine-NaOH, pH 10.0, containing 50 mM NaCl and 10 mM CaCl_2 , the reaction mixture was then incubated at 45°C for 30 min. The activity was determined

using Dinitrosalicylic acid (DNS) method according to Miller (Miller, 1959). By reacting 0.5 ml of enzyme to 0.5 ml of soluble starch and incubating the enzyme mixture at 37°C for 15 minutes. The reaction was stopped by addition of 1ml of DNS reagent and boiled for 15 minutes. The Absorbance was read at 540 nm and results obtained were compared with standard of maltose. One unit of α -amylase activity is defined as the amount of enzyme that produces reducing sugar equivalent to 1 $\mu\text{mol/min}$ maltose under the assay conditions.

Biochemical properties of the purified α -amylase

Effect of sodium chloride on the enzyme activity

To investigate the effect of NaCl on amylolytic activity, the rate of starch degradation with the purified enzyme was investigated at NaCl concentrations ranging from 0 to 2.5 M in the enzyme reaction mixture.

Effect of pH on the enzyme activity and stability

Influence of pH on amylolytic activity was studied with varying pH range of 5–12. The buffer system used was potassium phosphate (pH 5.0-6.5), Na-phosphate buffer (pH 6.5-8), Glycine-NaOH buffer (pH 8.5-10.5) and Borax-NaOH buffer (pH 10.5-12). For pH

stability the purified α -amylase was pre-incubated during 180 min at 45°C, in buffers of various pH in the range of 6.0-11.5, followed by activity estimation at standard conditions.

Effect of temperature on the enzyme activity and stability

Amylolytic activity was measured at various temperatures ranging from 25 to 70°C (with 5°C intervals) in Glycine-NaOH buffer, pH 10.0, for 30 min. To determine the thermal stability, the purified α -amylase was pre-incubated for 3 h at 40 to 65 °C (with 5°C intervals) in Glycine-NaOH buffer, pH 10.0 and residual activity was measured at 45°C. Purified α -amylase that had not been incubated served as positive control.

Effect of metal ions on the α -amylase activity

Influence of different metal ions on the α -amylase activity was investigated in the presence of Co^{2+} , KCl, CaCl_2 and MgCl_2 . The purified α -amylase was incubated at 45°C for 30 min with each metal ion at final concentrations of 2 and 5 mM. The mixture was then incubated with 1mL of 1% (w/v) of starch as a substrate in Glycine-NaOH buffer, pH 10.0 at 45°C for 30 min, and residual activity was measured. The activity rate was determined as a percentage of the remaining activity (with metal ions) of the control activity (no metal ions).

Effect of oxidizing agents on enzyme activity

The enzyme was incubated with 2 and 5 mM of different oxidizing agents, sodium perborate (NaBO_3), sodium dodecylsulphate (SDS), hydrogen peroxide (H_2O_2) and sodium hypochlorite (NaClO), for 2 h. After incubation, the residual activity was determined by the standard enzyme assay.

Determination of kinetic parameters

Determination of the kinetic parameters for the hydrolysis of α -amylase was calculated according to the method of Lineweaver-Burk plot (Lineweaver & Burk, 1934). The effect of increasing substrate concentration (from 0 to 2 g/100 ml) on the activity rate was measured at pH 10 and 45°C. Michaelis-Menten constant K_m and Values of maximum rate V_{\max} were determined from the Lineweaver–Burke plot. In addition, turnover number or k_{cat} was obtained by dividing the V_{\max} by the enzyme molar concentration.

Results

The enzyme purification and molecular weight

The α -amylase produced by *Bacillus persicus*, was purified in three steps involving acetone precipitation, ultrafiltration and Q-Sepharose column. The results obtained by this purification procedure are summarized in Table 1. Acetone precipitation showed total activity of 53.98 U with specific activity of

20.08 U/mg protein and ultrafiltration of the amylase leading to specific activity of 52.87 U/mg protein and yield of 63%. In lastly, approximately 42.14-fold purification to a specific activity as high as 494.25 U/mg of protein was obtained for the α -amylase when assayed at pH 10 (50 mM glycine-NaOH buffer) at 45°C and the purified enzyme was moved as a single band in native and SDS gels, indicating its homogeneity. The apparent molecular mass of the purified enzyme was

estimated to be 53 kDa by SDS-PAGE (Fig.1).

Table 1 Effect of metal ions and oxidizing agents on purified amylase from *B. persicus*

Ions	Residual activity (%)	
	Final concentrations	
	2mM	5mM
K ⁺	102	99
Mg ⁺	112	119
Ca ⁺	105	112
Co ²⁺	66	31
Oxidizing agents	2mM	5mM
Sodium perborate	94	85
Sodium hypochlorite	54	28
SDS	47	34
H ₂ O ₂	106	89

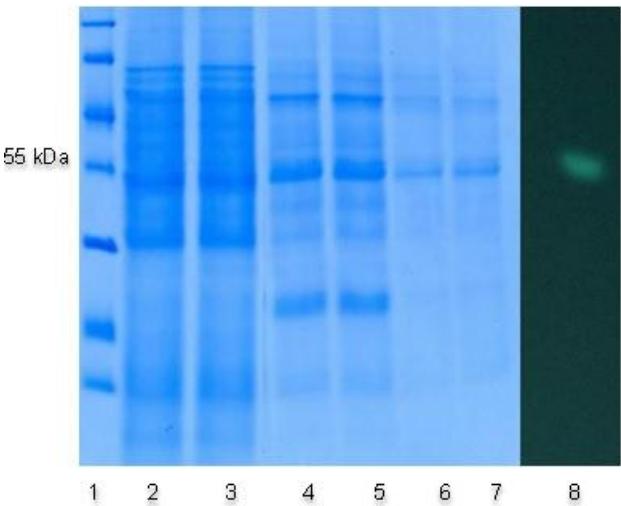


Fig. 1 Determination of purity and molecular weight of purified α -amylase by SDS-PAGE and zymography. Lane 1 molecular mass marker proteins, Lane 2,3 crude enzyme, Lane 4,5 acetone precipitation, Lane 6,7 purified α -amylase with Q-Sepharose column. Lane 8 zymogram of purified α -amylase.

Effect of NaCl and pH on the enzyme activity and stability

The purified enzyme retained 37 and 31 % of its initial activity in 0 and 2.5 M NaCl, respectively. The maximum activity was obtained with 0.85 M NaCl. Also, the purified enzyme showed that more than 50% of the

original activity was retained at NaCl concentration of 1.25 M (Fig.2) The effect of pH on the activity of purified enzyme was evaluated with soluble starch as the substrate at various pH values of 5–12. The purified α -amylase showed amylolytic activity from pH 6.5 to 11.5, where an optimum pH plateau was

observed in the range of pH from 8.5 to 10, also more than 40% of the maximum activity was detectable between pH 7.5 and 11. At pH values of 7 and 11.5, the amylolytic activity was reduced to 18% and 21%, compared with a control, respectively. As shown in Fig., pH

stability indicated that purified enzyme was very stable in a wide pH range (7.5–11) and retained about 54% of its original activity at pH 10 after 3 h of incubation at 45°C. This revealed that it is an alkalophilic α -amylase (Fig.3).

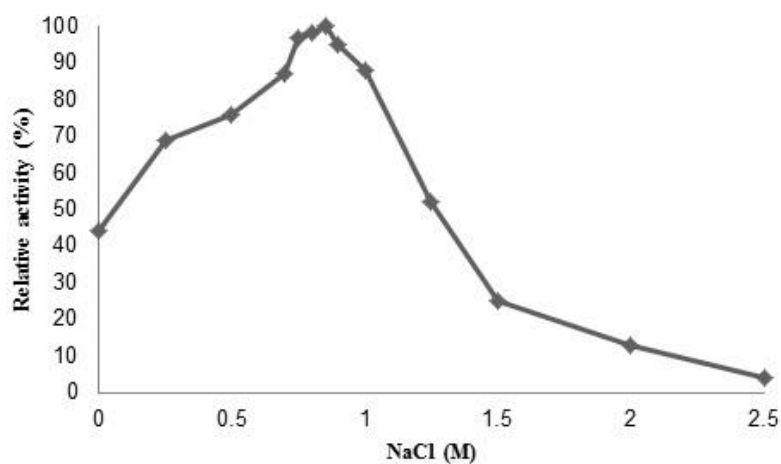


Fig. 2 Effect of NaCl on purified α -amylase activity from *Bacillus persicus*. The assay was carried out at 45 °C, in the presence of varying salt concentrations 0–3.0 M.

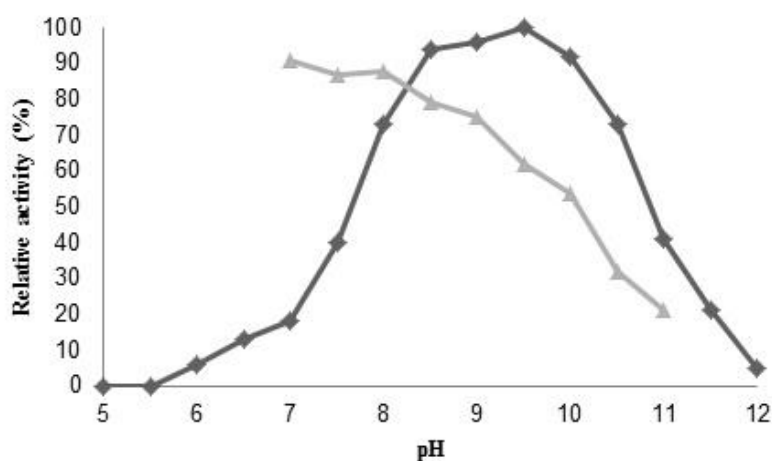


Fig. 3 Effect of pH on purified α -amylase activity from *Bacillus persicus*. Relative activity is expressed as the percentage of the maximum activity (100%) under standard assay conditions: (■) pH profile, (▼) pH stability

Effects of temperature on the enzyme activity

The amylolytic activity was measured at a temperature range from 25 to 70°C. The results revealed that the enzyme exhibited optimum temperature for highest activity at 45°C, although at higher temperatures (up to 60°C) activity still

remained. Therefore, 45°C was used for subsequent analysis. Heat stability assays of the purified enzyme showed that more than 50% of the initial activity was retained after 3 h of incubation at 45°C. The temperature profile of amylolytic activity is presented on Fig. 4.

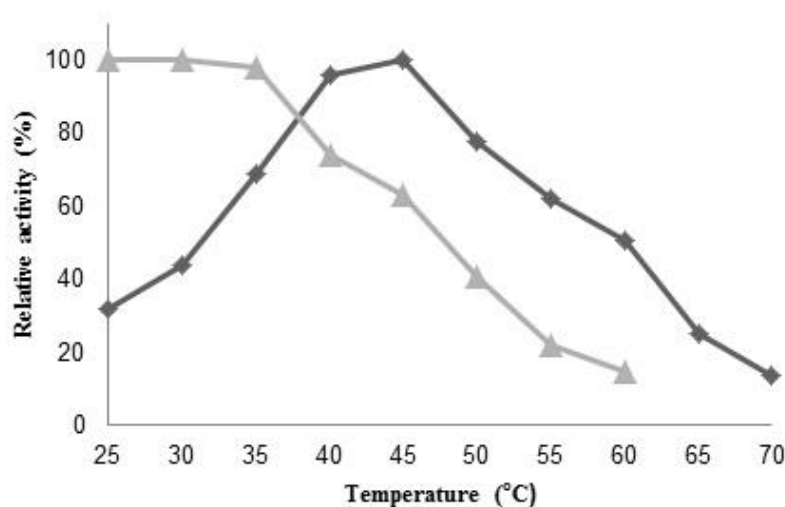


Fig. 4 Effect of temperature on purified α -amylase activity from *Bacillus persicus*. Relative activity is expressed as the percentage of the maximum activity (100%) under standard assay conditions (■) thermal profile, (▼) thermal stability.

Effect of metal ions on the enzyme activity

The effects of various metal ions on the purified enzyme are presented in Table 1. The enzyme activity was enhanced in the presence of CaCl_2 and MgCl_2 . The addition of 5 mM Ca^{2+} and Mg^{2+} ions to the reaction mixture increased the amylolytic activity to about 12% and 19% compared to a control, respectively. The activity of the α -amylase was not significantly affected by 5 mM of K^+ , while studies on the effect of metal ions on α -

amylase activity revealed that Co^{2+} had an inhibitory effect on the amylolytic activity at concentrations of ≥ 2 mM (Table 1).

Effect of oxidizing agents on the enzyme activity

The effect of different oxidizing agents (at a final concentration of 2 and 5mM in the reaction mixture) on the activity of the purified enzyme was investigated. The purified enzyme exhibited more than 85% activity when

incubated with 5 mM of sodium perborate for 120 min. In contrast, the presence of sodium dodecyl sulfate and sodium hypochlorite reduced the enzyme activity to 34% and 28% respectively in comparison to control (no agents). On the other hand, the surprising, α -amylase activity was increased in the presence of 2 mM of H_2O_2 showing 106% of its original activity (Table 1).

Determination of K_m and V_{max}

According to different V_0 for various starch concentrations, kinetic constants K_m and V_{max} for purified enzyme were calculated from a Lineweaver-Burk plot. The accurate values of K_m and V_{max} were obtained 1.053 mg/ml and 356 μ M/min respectively.

Discussion

Haloalkaliphiles are an interesting class of extremophiles that live in very extreme environments (high salt and alkaline pH) and therefore, their extracellular enzymes might be active and stable under these conditions. Regarding bacteria, *Bacillus* strains and the related genera produce a large variety of extracellular enzymes, of which amylases are of particular significance to the industry e.g., *Bacillus sp. PS-7* (Sodhi et al., 2005), *Bacillus amyloliquefaciens P-001* (Deb et al., 2013), *B. licheniformis* (Zare Mirakabadi et al., 2012), *B. cereus* (Hodes et al., 1987) and *B. subtilis* (El-

Banna et al., 2007). Bacterial α -amylases are mostly extracellular, easily produced in larger amounts, thermostable, and active in a wider pH range. The advantages of using thermostable and alkalophilic α -amylase in industrial processes include the increased diffusion rate, cost of external cooling and decreased risk of contamination. Despite the fact that many different α -amylases have been purified and characterized so far, and some of them have been used in biotechnological and industrial applications, the present known α -amylase are not sufficient to meet most of the industry demands. Typically, a moderately thermostable, alkaline α -amylase needs to be active and stable in alkaline environments (pH 9–11), at 30–60 °C and in environments with high salt concentrations (Saxena et al., 2007; Burhan et al., 2003; Najafi et al., 2005). In this study, a novel moderately thermostable, alkaline α -amylase was purified from the culture supernatant of *B. persicus*. The α -amylase produced by *B. persicus* was purified by a three step purification procedure involving acetone precipitation, ultrafiltration and Q-Sepharose column. The apparent molecular weight of the purified enzyme, determined by SDS-PAGE and native gel, was 53 kDa. Molecular weight of α -amylase from *Bacillus* species ranges between 50 and 60 kDa though some exception exists in case of α -amylase (molecular weight 31 kDa) isolated

from *Bacillus licheniformis* (Raul et al., 2014). In support of the present study, similar molecular masses have been reported earlier for other *Bacillus* amylases. Forty eight kDa (Marco et al., 1996) and 59-68 kDa from *B. stearothermophilus* strains (Ali et al., 2001; Chakraborty et al., 2000), 63 kDa from *Bacillus subtilis* BS5 (Femi-Ola & Olowe, 2011), 52 kDa from *Bacillus alcalophilus* (Archana Mehta, 2013), 55 kDa from *Bacillus licheniformis* AI20 (Abdel-Fattah et al., 2012). In contrast, various molecular weights of the α -amylases from different *Bacillus* sp. and other group of organisms have been provided by many researchers, such as 101 kDa from *Bacillus clausii* (Duedahl-Olesen et al., 2000), 126 kDa from thermophilic and alkaliphilic *Bacillus* sp. DM-15 (Ozcan et al., 2010) and 159 kDa from alkaliphilic *Bacillus* sp. IMD 370 (Mc Tigue et al., 1995) and 97 kDa from *S. megasporum* (Dey & Agarwal, 1999) are some of them. Among these amylases, the highest and lowest molecular weight, 210 kDa and 48 kDa, belong to *Chloroflexus aurantiacus*, and *B. stearothermophilus*, respectively. Some studies have found that the alkaline amylase of different species of *Bacillus* was not thermostable (Burhan et al., 2003), However, the present results revealed that the optimum temperature and pH of the purified amylase were 45 °C and 10, respectively. The effect of pH on the enzyme

activity was analyzed by carrying out assays at different pH values. The highest activity was found at alkaline pH and the enzyme was active and stable at alkaline pH and also showed maximal activity with 0.85 M NaCl. The result was bell-shaped curve showing an optimal activity at pH 10, also the pH stability studies revealed that the purified α -amylase has acceptable stability (> 70%) between pH 8 and 10.5 and retained about 54 % of its initial activity at pH 10 after 3 h of incubation at 45°C. These results revealed the haloalkaline property of purified enzyme. The amylolytic activity was active in a temperature range of 25-70 °C and the maximum activity was detected at 45°C, also the enzyme showed 79 and 42% of original activity at 35 and 55°C, respectively. Heat stability assays of the purified enzyme showed that more than 50% of the initial activity was retained after 2 h of incubation at 55°C, which suggested the moderate thermostable nature of this enzyme. The same optimum temperature was obtained with the amylase from *Bacillus subtilis* strain AS-S01a (Roy et al., 2012), *Bacillus subtilis* AX20 (Najafi et al., 2005). Most amylases are known to be metal ion dependent enzymes (Gupta et al., 2003). The thermophilic and alkaliphilic *Bacillus* sp. TS-23 amylase activity remained up to 115% while thermostable activity from *B. stearothermophilus* was retained up to 122% with 1 mM CaCl₂

(Burhan, 2008). Similarly, purified amylase in this study, showed an enzymatic activity around 112% and 119% in the presence of 5 mM Ca^{2+} and Mg^{2+} , compared to a control, respectively. In addition, the activity was not significantly affected by 5 mM of K^{+} , while Co^{2+} had an inhibitory effect on the amylolytic activity at concentrations of more than 2 mM. The results showed that the amylase did not have an obligate requirement for divalent metal ions to be active and its activity was not significantly stimulated in the presence of metal ions. In a similar study the inhibition of *Bacillus sp* strain SMIA- 2 amylase by Co^{2+} ions reported by Carvalho (Carvalho et al., 2008). Tripathi et al. (2011) reported that the inactivation of the enzyme by Co^{2+} may be due to their binding to the catalytic residues in the active site of the enzyme. Also, the influence of different oxidizing agents on the activity of the purified amylase was tested. In the presence of general oxidizing agents, sodium perborate, hydrogen peroxide, sodium dodecyl sulfate and sodium hypochlorite, the enzyme retained 85, 106, 34, and 28 % activity upon 1 h of incubation, respectively. Carvalho et al. have reported the amylase from *Bacillus sp.* SMIA-2 retained more than 70% activity after incubated for 1 h at 50°C with sodium dodecyl sulfate (Carvalho et al., 2008). Also, Saxena et al. have showed the amylase from *Bacillus sp.* PN5 retained more than 80% activity when

incubated with sodium perborate and sodium dodecyl sulfate and more than 70% activity when incubated with hydrogen peroxide for an hour (Saxena et al., 2007). In addition, Hmidet et al. have reported the amylase from *Bacillus licheniformis* NH1 retained more than 57% activity when incubated with 1% (w/v) sodium perborate for an hour (Hmidet et al., 2008). In most published studies, the amylase activity was completely inhibited by hydrogen peroxide (Carvalho et al., 2008; Saxena et al., 2007), however, the surprising α -amylase activity was increased in the presence of 2 mM of H_2O_2 showing 106% of its original activity. The K_m and V_{max} values for the purified enzyme, which are indicators of affinity of the enzyme towards its substrate (starch), were determined as 1.053 mg/ml and 356 $\mu\text{M}/\text{min}$, respectively. We compared our results with the K_m and V_{max} values reported for other amylase isolated from *Bacillus* species, our purified enzyme indicated a higher substrate affinity compared with the earlier reports. The K_m and V_{max} values in the literature for the comparable α -amylase are as follows: *Bacillus subtilis* AX20 (K_m 1.86 mg/ml, V_{max} 62.3 U/ml) (Najafi et al., 2005), *Bacillus subtilis* KIBGE HAS (K_m of 2.68 mg/ml and V_{max} of 1773 U/ml) (Bano et al., 2009) and *Bacillus licheniformis* SKB4 (K_m of 6.2 mg/ml and V_{max} of 1.04 $\mu\text{mol}/\text{mg min}$) (Samanta et al., 2014). According to the features mentioned, the purified enzyme has

potential application in a wide range of industrial and biotechnological processes such as ethanol production to break starches in grains into fermentable sugars. Alkaline α -amylases have potential applications for hydrolyzing starch under high pH conditions in the starch and textile industries and as ingredients in detergents for automatic dishwashers and laundries (Gurung et al., 2013; Pandey et al., 2000; Yang et al., 2011). The widely used thermostable enzymes in the starch industry are the amylases (Jensen et al., 2002).

Conclusions

To conclude, the present study describes the purification and characterization of an extracellular thermostable alkaline α -amylase from newly isolated *Bacillus persicus*. Purification of enzyme, was carried out by acetone precipitation, ultrafiltration and Q-Sepharose chromatography. In lastly, approximately 42.14-fold purification to a specific activity as high as 494.25 U/mg of protein was obtained for the α -amylase when assayed at pH 10 (50 mM glycine-NaOH buffer) at 45°C. SDS-PAGE and zymographic analyses indicated monomeric nature of the purified α -amylase with a molecular weight of 53 kDa. The molecular cloning and structural studies of this α -amylase are in progress in our laboratory. This is the first report of purification and characterization of an

extracellular thermostable alkaline α -amylase from *Bacillus persicus*.

Acknowledgments

We gratefully acknowledge the University of Guilan for their financial support.

References

- [1] Abdel-Fattah YR, Soliman NA, El-Toukhy NM, El-Gendi H, Ahmed RS. 2013. Production, purification, and characterization of thermostable α -amylase produced by *Bacillus licheniformis* Isolate AI20. Journal of Chemistry, 2013:1-11
- [2] Ali MB, Mhiri S, Mezghani M, Bejar S. 2001. Purification and sequence analysis of the atypical maltohexaose-forming α -amylase of the *B. stearothermophilus* US100. Enzyme and Microbial Technology, 28: 537-42.
- [3] Archana Mehta RK. 2013. Isolation, Optimization and Characterization of α -Amylase from *Bacillus alcalophilus*. International Journal of Science and Research, 2: 171-4.
- [4] Aygan A, Arikan B, Korkmaz H, Dincer S, Colak  m. 2008. Highly thermostable and alkaline α -amylase from a halotolerant-alkaliphilic *Bacillus* sp. AB68. Brazilian Journal of Microbiology, 39: 547-53.
- [5] Aygan A, Sariturk S, Kostekci S, Tanis H. 2014. Production and characterization of

- alkaliphilic α -amylase from *Bacillus subtilis* A10 isolated from soils of Kahramanmaras, Turkey. African Journal of Microbiology Research, 8: 2168-73.
- [6] Bano S, Qader SAU, Aman A, Syed MN, Azhar A. 2011. Purification and characterization of novel α -amylase from *Bacillus subtilis* KIBGE HAS. AAPS Pharm Sci Tech, 12: 255-61.
- [7] Barros FFbC, Simiqueli APR, de Andrade CJ, Pastore GuM. 2013. Production of enzymes from agroindustrial wastes by biosurfactant-producing strains of *Bacillus subtilis*. Biotechnology Research International, 2013: 1-9.
- [8] Behal A, Singh J, Sharma MK, Puri P, Batra N. 2006. Characterization of alkaline α -amylase from *Bacillus* sp. AB 04. Int J Agric Biol, 8: 80-3.
- [9] Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72: 248-54.
- [10] Burhan A. 2008. Highly thermostable, thermophilic, alkaline, SDS and chelator resistant amylase from a thermophilic *Bacillus* sp. isolate A3-15. Bioresource Technology, 99: 3071-6.
- [11] Burhan A, Nisa U, Gokhan C, Omer C, Ashabil A, et al. 2003. Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic *Bacillus* sp. isolate ANT-6. Process Biochemistry, 38: 1397-403.
- [12] Carvalho RVd, CÃ´rrea TLvR, Silva JICMd, Mansur LRCdO, Martins MLL. 2008. Properties of an amylase from thermophilic *Bacillus* SP. Brazilian Journal of Microbiology, 39: 102-10.
- [13] Chakraborty K, Bhattacharyya BK, Sen SK. 2000. Purification and characterization of a thermostable α -amylase from *Bacillus stearothermophilus*. Folia Microbiologica, 45: 207-10.
- [14] Deb P, Talukdar SA, Mohsina K, Sarker PK, Sayem SMA. 2013. Production and partial characterization of extracellular amylase enzyme from *Bacillus amyloliquefaciens* P-001. Springer Plus, 2: 1-12.
- [15] Dey S, Agarwal SO. 1999. Characterization of a thermostable α -amylase from a thermophilic *Streptomyces megasporus* strain SD12. Indian J Biochem Biophys, 36: 150-7.
- [16] Doss A, Anand SP. 2012. Purification and characterization of extracellular amylolytic enzyme from *Aspergillus* species. African Journal of Biotechnology, 11: 14941.
- [17] Duedahl-Olesen L, Kragh KM, Zimmermann W. 2000. Purification and characterisation of a malto-oligosaccharide-forming amylase active at high pH from *Bacillus clausii* BT-21. Carbohydrate Research, 329: 97-107.

- [18] El-Banna TE, Abd-Aziz AA, Abou-Dobara MI, Ibrahim RI. 2007. Production and immobilization of alpha-amylase from *Bacillus subtilis*. Pakistan Journal of Biological Sciences: PJBS, 10: 2039-47.
- [19] Femi-Ola TO, Olowe BM. 2011. Characterization of alpha amylase from *Bacillus subtilis* BS5 isolated from *Amitermes evuncifer* Silvestri. Research Journal of Microbiology, 6: 140.
- [20] Gomes J, Steiner W. 2004. The biocatalytic potential of extremophiles and extremozymes. Food Technology and Biotechnology, 42: 223-35.
- [21] Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B. 2003. Microbial α -amylases: a biotechnological prospective. Process Biochem, 38: 1599-616.
- [22] Gupta MN, Roy I. 2004. Enzymes in organic media. European Journal of Biochemistry, 271: 2575-83.
- [23] Gurung N, Ray S, Bose S, Rai V. 2013. A broader view: microbial enzymes and their relevance in industries, medicine, and beyond. BioMed Research International, 2013: 1-18.
- [24] Hagihara H, Igarashi K, Hayashi Y, Endo K, Ikawa-Kitayama K, et al. 2001. Novel α -Amylase That Is Highly Resistant to Chelating Reagents and Chemical Oxidants from the Alkaliphilic *Bacillus* Isolate KSM-K38. Applied and Environmental Microbiology, 67: 1744-50.
- [25] Hmidet N, Bayoudh A, Berrin JG, Kanoun S, Juge N, et al. 2008. Purification and biochemical characterization of a novel α -amylase from *Bacillus licheniformis* NH1: cloning, nucleotide sequence and expression of amyN gene in *Escherichia coli*. Process Biochemistry, 43: 499-510.
- [26] Hodes C, Strasser J, Friedberg F. 1987. Sequence of an active fragment of *B. polymyxa* beta amylase. Nucleic acids Research, 15: 3934.
- [27] Jegannathan KR, Nielsen PH. 2013. Environmental assessment of enzyme use in industrial production-a literature review. Journal of Cleaner Production, 42: 228-40.
- [28] Jensen B, Nebelung P, Olsen Jr, Reeslev M. 2002. Enzyme production in continuous cultivation by the thermophilic fungus, *Thermomyces lanuginosus*. Biotechnology Letters, 24: 41-5.
- [29] Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.
- [30] Lineweaver H, Burk D. 1934. The determination of enzyme dissociation constants. Journal of the American Chemical Society, 56: 658-66.
- [31] Marco JL, Bataus LA, Valencia FF, Ulhoa CJ, Astolfi-Filho S, et al. 1996. Purification and characterization of a truncated *Bacillus*

- subtilis* α -amylase produced by *Escherichia coli*. Applied Microbiology and Biotechnology, 44: 746-52.
- [32] Mc Tigue MA, Kelly CT, Doyle EM, Fogarty WM. 1995. The alkaline amylase of the alkalophilic *Bacillus* sp. IMD 370. Enzyme and Microbial Technology, 17: 570-3.
- [33] Miller G L. 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Analytical Chemistry, 31: 426-8.
- [34] Mojsov K. 2012. Microbial alpha-amylases and their industrial applications: a review. International Journal of Management, IT and Engineering (IJMIE), 2: 583-609.
- [35] Murakami S, Nishimoto H, Toyama Y, Shimamoto E, Takenaka S, et al. 2007. Purification and characterization of two alkaline, thermotolerant α -amylases from *Bacillus halodurans* 38C-2-1 and expression of the cloned gene in *Escherichia coli*. Bioscience, Biotechnology, and Biochemistry, 71: 2393-401.
- [36] Najafi MF, Deobagkar D, Deobagkar D. 2005. Purification and characterization of an extracellular α -amylase from *Bacillus subtilis* AX20. Protein Expression and Purification, 41: 349-54.
- [37] Oyeleke SB, Auta SH, Egwim EC. 2010. Production and characterization of amylase produced by *Bacillus megaterium* isolated from a local yam peel dumpsite in Minna, Niger State. Journal of Microbiology and Antimicrobials, 2: 88-92.
- [38] Ozcan BD, Baylan M, Ozcan N, Tekdal D. 2010. Characterization of Thermostable α -amylase from Thermophilic and Alkaliphilic *Bacillus* sp. Isolate DM-15. Research Journal of Biological Sciences, 5: 118-24.
- [39] Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D, et al. 2000. Advances in microbial amylases. Biotechnology and Applied Biochemistry, 31: 135-52.
- [40] Raul D, Biswas T, Mukhopadhyay S, Kumar Das S, Gupta S. 2014. Production and partial purification of alpha amylase from *Bacillus subtilis* (MTCC 121) using solid state fermentation. Biochemistry Research International, 2014: 1-5
- [41] Reddy NS, Nimmagadda A, Rao KRSS. 2004. An overview of the microbial α -amylase family. African Journal of Biotechnology, 2: 645-8.
- [42] Roy JK, Rai SK, Mukherjee AK. 2012. Characterization and application of a detergent-stable alkaline α -amylase from *Bacillus subtilis* strain AS-S01a. International Journal of Biological Macromolecules, 50: 219-29.
- [43] Sajedi RH, Naderi-Manesh H, Khajeh K, Ahmadvand R, Ranjbar B, et al. 2005. A Ca-independent α -mylase that is active and stable at low pH from the *Bacillus* sp.

- KR-8104. Enzyme and Microbial Technology, 36: 666-71.
- [44] Samanta S, Das A, Halder SK, Jana A, Kar S, et al. 2014. thermodynamic and kinetic characteristics of an α -amylase from *Bacillus licheniformis* SKB4. Acta Biologica Szegediensis, 58: 147-56.
- [45] Saxena RK, Dutt K, Agarwal L, Nayyar P. 2007. A highly thermostable and alkaline amylase from a *Bacillus* sp. PN5. Bioresource Technology, 98: 260-5.
- [46] Singh R, Chenier D, Beriault R, Mailloux R, Hamel RD, et al. 2005. Blue native polyacrylamide gel electrophoresis and the monitoring of malate-and oxaloacetate-producing enzymes. Journal of Biochemical and Biophysical Methods, 64: 189-99.
- [47] Sodhi HK, Sharma K, Gupta JK, Soni SK. 2005. Production of a thermostable α -amylase from *Bacillus* sp. PS-7 by solid state fermentation and its synergistic use in the hydrolysis of malt starch for alcohol production. Process Biochemistry, 40: 525-34.
- [48] Souza PMd. 2010. Application of microbial α -amylase in industry-A review. Brazilian Journal of Microbiology, 41: 850-61.
- [49] Sundarram A, Murthy TPK. 2014. α -amylase production and applications: A review. Journal of Applied & Environmental Microbiology, 2: 166-75.
- [50] Tripathi P, Tomar R, Jagannadham MV. 2011. Purification and biochemical characterisation of a novel protease streblin. Food Chemistry, 125: 1005-12.
- [51] Vengadaramana A. 2013. Industrial Important Microbial alpha-Amylase on Starch-Converting Process. Scholars Academic Journal of Pharmacy (SAJP), 10: 209-21.
- [52] Yang H, Liu L, Li J, Du G, Chen J. 2011. Heterologous expression, biochemical characterization, and overproduction of alkaline α -amylase from *Bacillus alcalophilus* in *Bacillus subtilis*. Microbial cell Factories, 10: 1-10.
- [53] Zare Mirakabadi A, Ghorbanpour M, Sadeghi A, Sarzaeem A. 2012. Two-step purification and partial characterization of an extra cellular α -amylase from *Bacillus licheniformis*. Archives of Razi, 67: 155-60.