Characterization of an α-amylase with broad temperature activity from an acid-neutralizing *Bacillus cereus* strain

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Abstract
Bacillus sp. GUF8, isolated from acidic soil samples of a tea farm was identified as *Bacillus cereus*, based on 16S rDNA sequencing and standard bacterial identification methods. Following optimization of enzyme production, the resulting α-amylase was purified by acetone precipitation and ion exchange chromatography. Consequently, thermostability and kinetic parameters of the purified enzyme were determined. The temperature profile of the enzyme indicated a very broad temperature range (from 10 to 70°C) with 50°C representing the optimum temperature for enzyme activity, which is different from those of the known *Bacillus* α-amylases. This enzyme was optimally active at pH 6.0 and retained 75 and 50% of its maximal activity at pH 8.0 and 9.0, respectively. It was also strongly inhibited by Zn^{2+} and partially inhibited by Ni^{2+} and ethylenediaminetetraacetic acid (EDTA). The α-amylase enzyme was found to hydrolyze starch forming various maltooligosaccharides, such as maltose (G2) and maltopentaose (G5) as major products.
Keywords: *α-Amylase; Bacillus cereus; Acid-neutralizing; Purification; Temperature profile*

INTRODUCTION

Amylolytic activity is an almost universal trait amongst heterotrophs. Several micro-organisms produce α-amylases having different characteristics and action patterns, which are widely used in industry. They have different substrate specificities, as well as huge variations in thermostability, temperature and pH optima (Gupta *et al*., 2003; Nielsen and Borchert, 2000). The suitability of any α-amylase to a particular process will depend on its specificity and characteristics (Van der Maarel *et al*., 2002). The *Bacillus* α-amylases have found widespread use in industrial processes, and much attention has been devoted to optimization of their production conditions (Sivaramakrishan *et al*., 2006; Nielsen and Borchert, 2000).

Bacteria inhabiting acidic environments either produce acidophilic enzymes, or raise the pH of their microenvironment and then secret acid-tolerant enzymes active at near-neutral pHs (Bachmeier *et al*., 2002; Marcus *et al*., 2001; Mann Hyung Lee *et al*., 1993). Soil from tea farms is intrinsically acidic, thus providing a potential habitat for acidophilic or acid-tolerant bacteria.

β-amylases from various *Bacillus cereus* strains have been studied and their characteristics extensively reported (Oyama *et al*., 2003; Nanmori *et al*., 1993; Nanmori *et al*., 1987). However, there are very few reports on the isolation of α-amylase produced by this species (Anto *et al*., 2006). As a result information on the purification and extensive biochemical characterization of the enzyme is lacking.

In previous studies, some unique *Bacillus* α-amylases isolated from native strains in Iran were characterized (Sajedi *et al*., 2005; Sajedi *et al*., 2004). In the present study, the purification, and biochemical characterization of an α-amylase produced by a new acid-neutralizing *Bacillus cereus* species isolated from soil of a tea farm in Iran is reported.

MATERIALS AND METHODS

Chemicals and reagents: Standard sugars [glucose (G1), maltose (G2), maltotriose (G3), maltotetraose

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(G4), maltopentaose (G5), maltohexaose (G6) and maltoheptaose (G7)], 3,5-dinitrosalicylic acid (DNS), phenylmethylsulfonyl fluoride (PMSF), sodium dodecyl sulfate (SDS), EDTA, and Tris were purchased from Sigma (St. Louis, MO. USA). DEAE-Sepharose was provided by Pharmacia (Uppsala, Sweden). Polymerase Chain Reaction (PCR) reagents were obtained from Boehringer Mannheim co. (Mannheim, Germany). Agarose was obtained from GibcoBRL (Maryland, USA). Microbiological media and all other chemicals (reagent grade) were from Merck (Darmstadt, Germany).

Media and growth conditions

Isolation and identification of microorganisms: Soil samples were collected from a tea farm located in Fooman, Guilan province of Iran (pH 4.5). Twenty gram samples of soil were mixed with 100 ml of sterile water in Erlenmeyer flasks (Sajedi *et al*., 2005). Serial dilutions (10^{-1}-10^{-9}) of the resulting suspensions were prepared and 1 ml of each dilution was added and distributed onto isolation medium containing: agar-agar 3.5% (w/v), yeast extract 0.1% (w/v) and, starch 1% (w/v) (pH 4.0). Plates were then incubated at 35˚C for 24-48 h and different types of colonies were then chosen and purified. The isolated strains were checked for starch hydrolysis on nutrient agar supplemented with 1% (w/v) soluble starch. Hydrolysis was observed as clear zones around the margins of growth after flooding the plates with Lugol's iodine solution. The promising putative *Bacillus* strain was further examined for morphological, physiological and biochemical characteristics with reference to Bergey's Manual of Systematic Bacteriology (Garrity *et al*., 2005; Sneath, 1986).

Preculture medium for α-amylase production: The preculture medium consisted of: nutrient broth 8 (g/l); meat extract 10 (g/l); soy meal peptone 10 (g/l); potato starch 10 (g/l); NaCl 0.5 (g/l) and supplemented with 1% (w/v) trace elements, pH 6.8. Incubation was carried out at 35ºC, in an orbital incubator (Labcon, South African), with stirring at 180 rpm for 18-20 h.

Production medium for α-amylase production: The Production medium consisted of: potato starch 10 (g/l); soy meal peptone 4 (g/l); meat extract 3 (g/l); $CaCl_2.H_2O$ 0.5 (g/l); $MgSO_4.7H_2O$ 0.3 (g/l); and K_2HPO_4 1 (g/l), supplemented with 1% (v/v) trace elements, and incubated at the same condition as preculture.

Trace elements: Trace elements contained (w/v): Na salt EDTA 5%; $ZnSO_4.7H_2O$ 1.1%; $MnCl_2.4H_2O$ 0.25%; $CaCl_2.6H_2O$ 0.05%; $(NH_4)_6Mo_7O_{24}$ 0.05%; $FeSO_4.7H_2O$ 5%; $CuSO_4.5H_2O$ 0.2%, KH_2PO_4 0.3%; $(NH_4)_2SO_4$ 0.3%; $MgSO_4.7H_2O$ 0.05%; $CaCl_2.2H_2O$ 0.025%; $K_2S_4O_7$ 0.32%.

Polymerase Chain Reaction amplification and 16S rDNA sequencing: Genomic DNA was extracted and purified according to Sambrook and Russell (2001) and its purity was assessed by the A260/A280. Forward (5′-AGTTTGATCCTGGCTCAG-3′) and reverse (5′-GGC/TTACCTTGTTACGACTT-3′) primers were used in the amplification of 16S rRNA genes (Desantis *et al*., 2003). For the PCR procedure, the DNA thermal cycler (Applied Biosystems, Foster City, CA USA) was programmed as follows: initial denaturation at 94ºC for 5 min; 30 cycles of denaturation at 94ºC for 45 s, annealing at 48ºC for 45 s, extension at 72ºC for 90 s, and a final extension at 72ºC for 5 min. The amplification product was sequenced on both strands directly by SEQLAB (SEQLAB GmbH, Gottingen, Germany) according to the super long run. Multiple sequence alignment of 16S rDNA sequences from13 *Bacillus* species was carried out. The 16S rDNA sequences were obtained from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) and multiple sequence alignment was performed with the Clustal W version 1.82 program (Thompson *et al*., 1994). A phylogenetic tree was then constructed by the neighbor-joining method (Saitou and Nei, 1987), using the Mega 4.0.2 software (Tamura *et al*., 2000). The nucleotide sequence data were submitted to the GenBank.

Determination of α-amylase activity and protein concentration: α-Amylase activity was determined at room temperature in 20 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.4. Reducing sugar concentration, obtained from the catalyzed reaction for 10 min was determined by the dinitrosalicylic acid method of Bernfeld (1955). One unit of α-amylase is defined as the amount of the enzyme that liberates 1.0 µmol of reducing sugar/min with maltose as a standard (Sajedi *et al*., 2005). Protein concentration was determined by the Bradford method (Bradford, 1976).

Production of α-amylase: Prior to cultivation of the isolated strains in production medium, a loopful of each strain was precultured in medium as mentioned in media and growth conditions section. Precultured cells

were then used to inoculate production medium at a concentration of 5% (v/v). The cells were then incubated under the same conditions as the preculture. To determine the incubation period for optimum enzyme production, the production of α-amylase was examined at various time intervals during cultivation. The effect of trace elements on the α-amylase production was also studied.

Purification of α-amylase: Eighteen hours after cultivation of cells in production medium, the culture medium was centrifuged at 3000 g for 10 min at 4ºC, and 1 mM PMSF was then added to the resulting supernatant. To concentrate the supernatant, prechilled acetone (-20ºC) was added slowly to the solution of crude culture supernatant up to 50% (v/v). The proteins were allowed to precipitate overnight at 4ºC. The precipitates were centrifuged at 3500 g for 10 min at 4ºC and the pellet was air dried at room temperature and dissolved in a minimum volume of 20 mM Tris buffer, pH 7.4. The resulting solution was applied to a DEAE-Sepharose column (10 × 100 mm) at a flow rate of 1 ml/min, previously equilibrated with 20 mM Tris, pH 7.4. Proteins were then eluted with a linear gradient of NaCl (0 to 1 M) in the same buffer and at the same flow rate. The active fractions were pooled and concentrated by ultrafiltration (Amicon, Beverly, MA, USA). During these experiments, the BioRad 2110 Fast Performance Liquid Chromatography System (BioRad, Hercules, CA, USA) was used.

Polyacrylamide gel electrophoresis and zymogram analysis: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (Laemmli, 1970), using a 10% (w/v) polyacrylamide gel, protein bands were detected by staining gels with Coomassie brilliant blue R250. For zymography, non-denaturing PAGE (10% (w/v)) was carried out as described by Davis (Davis, 1964) and electrophoresis was performed at 4ºC. Afterward, the gel was rinsed with deionized water and washed for 45 min at 40ºC in 0.2 M sodium phosphate buffer (pH 7.0). The washed gel was incubated in fresh buffer containing 1% (w/v) soluble starch at 40ºC for 45 min. After being washed with distilled water, the gel was subjected to staining with Lugol solution at ambient temperature until the appearance of clear zones against a dark blue background, in protein bands with α-amylase activity.

Effect of pH on enzyme activity and stability: The pH profile of α-amylase was determined at room temperature in a mixed buffer containing 25 mM phosphate-glycine-acetate adjusted to various pH values. The pH stability of the enzyme was established by incubating the enzyme in the mixed buffer (with different pH values) for 2 h at room temperature. Aliquots were withdrawn and amylolytic activity was determined at pH 7.4. (Sajedi *et al*., 2005; Bernfeld, 1955)

Effect of temperature on enzyme activity and stability: The activity of the enzyme at different temperatures was determined by incubating the reaction mixture at temperatures ranging from 10 to 80ºC in 20 mM Tris, pH 7.4. Thermal stability of the enzyme was examined by incubating the enzyme at 60ºC and 70ºC in 20 mM Tris, pH 7.4 for a series of time intervals, followed by cooling on ice, and determining residual activity under standard assay conditions. (Sajedi *et al*., 2005; Bernfeld, 1955)

Analysis of hydrolysis products of α-amylase: The hydrolysis products of the α-amylase from the isolated strain were analyzed by thin-layer chromatography (TLC) using Aluminum coated TLC silica gel plates (Kieselgel 60 F254; Merck, Darmstadt, Germany). After development of the products in a solvent system of water-acetonitrile 2:8 (v/v), the spots were visualized by spraying with methanol-sulfuric acid 1:1 (v/v) reagent and then baking it.

RESULTS

Isolation of an acid-tolerant amylolytic bacterium: Initial incubation of soil samples on starch agar plates at pH 4.0 resulted in the selection of the GUF8 strain, which exhibited the highest level of amylase activity, as determined by the Lugol's plate test. The presence of a lag period of approximately two days prior to active growth on media at pH 4.0 suggested that the bacterium may be affecting changes in its microenvironment. Incubation of the bacterium on the same medium, but at higher pH values (pHs 5.0, 6.0 and 7.0) indicated that the isolate grew faster at close to neutral pH values. Incorporation of phenol red into the starch medium, with an initial pH of 5.0, confirmed that the pH of the medium had risen to approximately pH 7.0 during the course of growth (Fig. 1).

Identification of the amylolytic strain and 16S rDNA analysis: Initial tests showed the isolate to be a

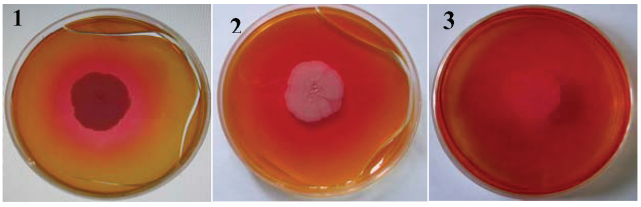

Figure 1. Incubation of the GUF8 isolate on nutrient agar with an initial pH 5.0, in the presence of 0.002% (w/v) phenol red. Plate 1: 16 h post-inoculation (pi); plate 2: 24 h pi and plate 3: 36 h pi. Phenol red in the agar changes color from yellow to orange and; then to red as the pH of the medium increases.

Gram-positive, facultatively anaerobic sporulating *Bacillus*. The isolate was 5 µm in length and 0.9 µm in width. A series of other tests according to Bergey's manual (1984) were performed on the GUF8 strain. Accordingly, the isolate was positive for catalase, oxidase, protease, and gelatinase activities. On the other hand, GUF8 was unable to utilize sodium citrate, lactose, and sucrose. At the same time the isolate was negative for indole and H_2S production. The methyl red (MR) test was negative and the Vogues-Proskauer (VP) test positive. It was able to grow between 30-40˚C but no growth was exhibited at 45˚C. The combination of morphological, physiological, and biochemical data resulted in the preliminary identification of the isolate as *Bacillus cereus,* although the tests also indicated that the isolate is closely related to *Bacillus*. *circulans*. Subsequently, PCR amplification and sequencing of its 16S rDNA gene was carried out. The PCR amplification product was approximately 1500 bp (data not shown). 16S rDNA sequence was edited to a total length of 1435 bp after direct sequencing. The results from the 16S rDNA sequencing and the neighbor joining method (Fig. 2) showed *Bacillus* sp. GUF8 to be as closely related to *B*. *cereus* as it was to *B*. *circulans*. However, the MR/VP test, which distinguishes between the putative *B*. *cereus* and *B*. *circulans*, confirmed that the isolate was a *B*. *cereus* strain (*B*. *circulans* being predominantly VP negative).

Nucleotide sequence accession number: The nucleotide sequence data have been submitted to the GenBank under the accession number EU164579 for 16S rDNA.

α-Amylase production during growth: Measurements of α-amylase activity in culture supernatants showed that maximum enzyme production occurred after 18 h of incubation at 35˚C and pH 6.5 (Fig. 3A). Addition of trace elements more than doubled maximum α-amylase production (Fig. 3B). Native PAGE followed by activity staining of the cul-

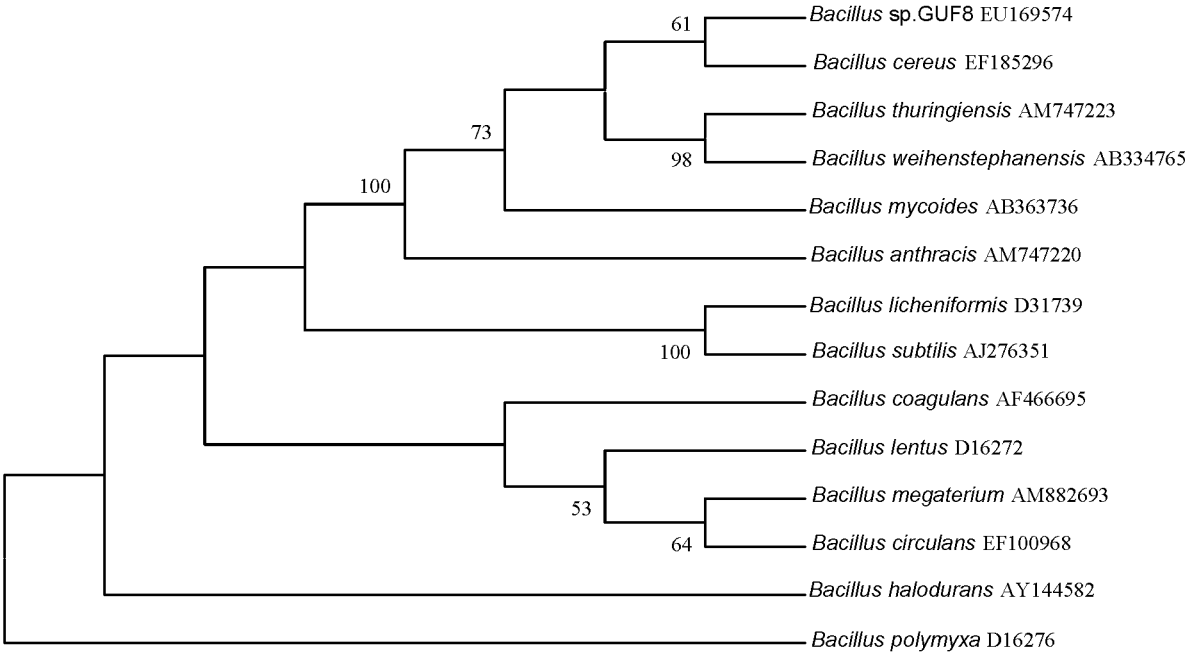

Figure 2. Phylogenetic tree of *Bacillus* sp. GUF8 based on the 16S rDNA sequence using Mega 4.0.2 software. (The numbers on the tree represent the percentage of bootstrap replicates supporting each branch).

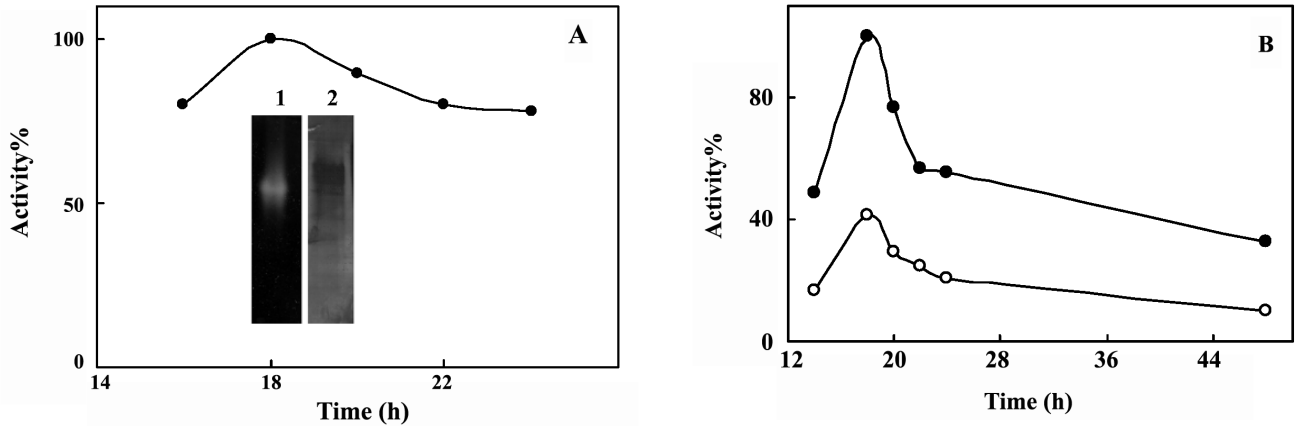

Figure 3. Measurement of α-amylase activity in starch broth from 16 h pi to 24 h pi. A: Maximum activity is seen at 18 h pi. The inset is the native PAGE of the culture supernatant from *Bacillus cereus* GUF8. Lane 1, activity staining; 2, silver staining. B: α-amylase activity in starch broth in the presence (●) and absence (○) of trace elements.

ture supernatant (Fig. 3A, inset) confirmed the secretion of an extracellular amylolytic enzyme by the GUF8 strain.

α-Amylase purification: Proteins were precipitated with saturation to 50% by acetone and the α-amylase from *B. cereus* GUF8 was subsequently purified with ion exchange chromatography using a DEAE-Sepharose column. The FPLC chromatogram showed several small peaks and a large protein peak. The large peak was symmetric and had α-amylase activity. The purified enzyme had a specific activity of 50 U/mg of protein. The molecular weight of enzyme was found to be approximately 56 kDa (Fig. 4).

Catalytic properties: The α-amylase of *B. cereus* GUF8 was shown to obey Michaelis-Menten kinetics when soluble starch was used as a substrate. As calculated from the Lineweaver-Burk plots, the K_m and V_{max} values for soluble starch at room temperature were 1.43 mg/ml and 0.27 µmol/min, respectively. The hydrolysis products of extensive hydrolysis of soluble starch were determined by TLC analysis. As shown in Fig. 5, the end products of the α-amylase were G7 to G2, but not glucose.

Temperature and pH profiles: The influences of temperature and pH on the activity of enzyme are shown in Figure 6. The temperature-activity profile of

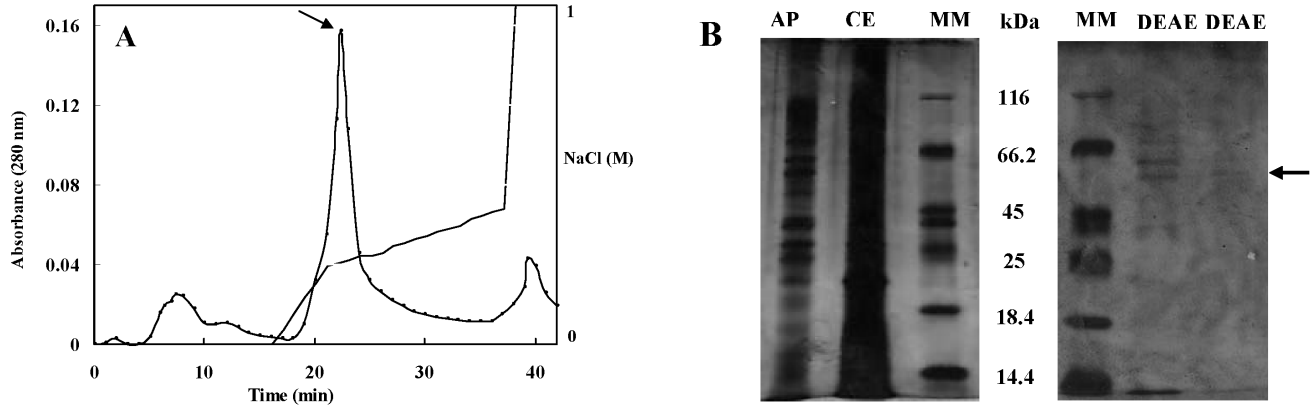

Figure 4. (A) Elution profile of GUF8 strain α-amylase on the DEAE-Sepharose column as described in "Materials and Methods". The active peaks are indicated with the arrow. (B) SDS-PAGE illustrates different steps of α-amylase purification. Lane CE, crude enzyme; Lane AP, acetone precipitation; Lanes DEAE, after ion exchange chromatography using the DEAE-Sepharose column; and Lane MM, Molecular size markers (MBI Fermentas, Vilnius, Lithuania).

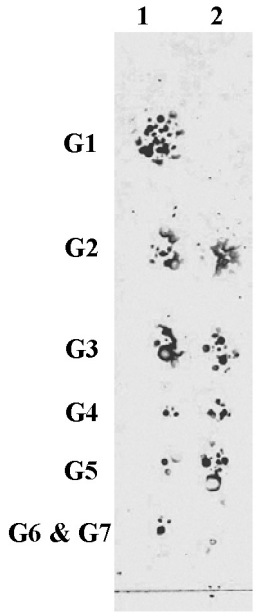

Figure 5. Thin-layer chromatogram for hydrolysis products of *B. cereus* GUF8 α-amylase. Lane 1: Standard maltooligosaccharides from glucose (G1) to maltoheptaose (G7); Lane 2: The products of soluble starch hydrolysis.

the enzyme shows a very broad temperature range at pH 7.4 (from 10 to 70ºC). The optimum temperature of the α-amylase was identified as 50ºC but low sensitivity to temperature was observed between 10-50ºC (Fig. 6A). There was a rapid loss of activity over 70ºC.

The optimum pH for α-amylase activity was 6 and at pHs 8 and 9, the enzyme retained 75 and 50% of its maximal activity, respectively (Fig. 6B).

Thermostability of the enzyme: The irreversible thermoinactivation of the enzyme was investigated in 20 mM Tris, pH 7.4, containing 10 mM $CaCl_2$ at 60 and 70ºC. *B. cereus* GUF8 α-amylase retains more than 80% of its activity after 60 min of incubation at 60ºC but it loses 50% of its original activity after 10 min of incubation at 70ºC, as shown in Fig. 6C. After 10 min, a complete inactivation of *B. cereus* GUF8 α-amylase was witnessed.

Effect of metal ions and EDTA on α-amylase activity: The activity of α-amylase was measured at pH 7.4 in the presence of various metal ions and EDTA (5 and 10 mM). The purified enzyme was dialyzed extensive-

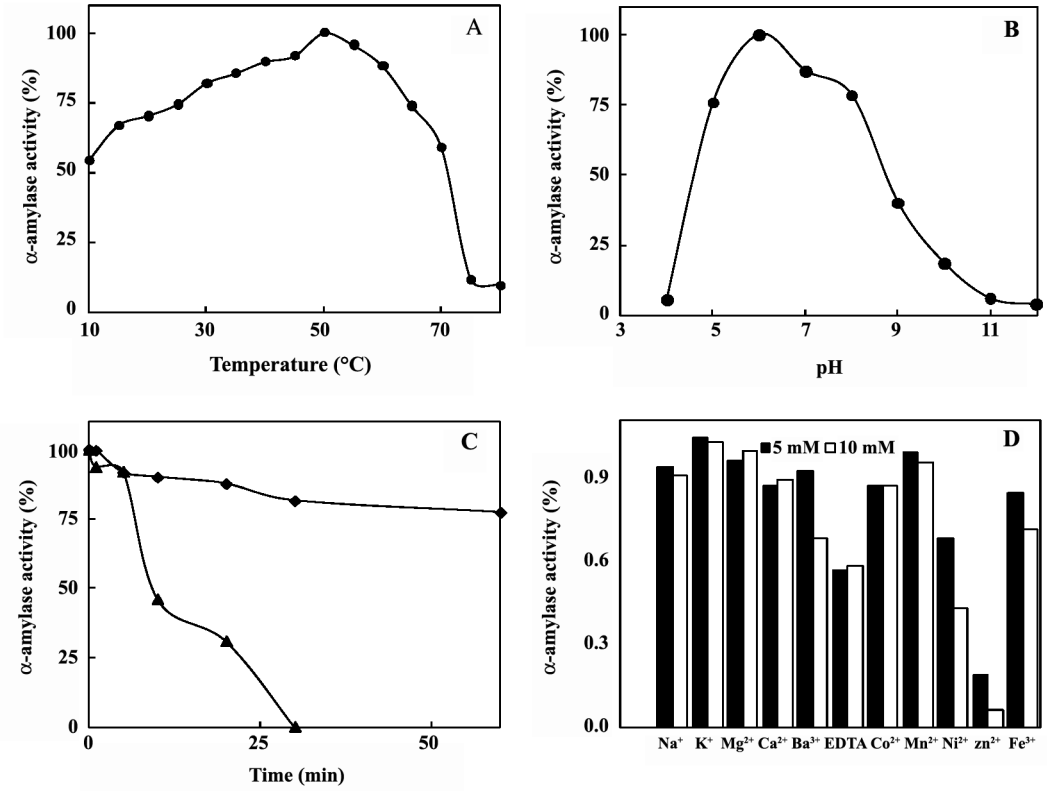

Figure 6. A: Effect of temperature on activity of *B. cereus* GUF8 α-amylase. B: Effect of pH on activity of *B. cereus* GUF8 α-amylase. C: Irreversible thermoinactivation of the GUF8 α-amylase at 60ºC (♦) and 70ºC (▲) in 10 mM Tris and 10 mM $CaCl_2$, pH 7.4. D: Effect of various metal ions and EDTA (5 and 10 mM) on α-amylase of *B. cereus* GUF8. All the metal ions were added as chloride salts.

ly at 4ºC against 20 mM Tris buffer (pH 7.4) prior to the experiment. As shown in Fig. 6D, the addition of EDTA and Ni^{2+} decreased enzyme activity by approximately 60%, whereas the addition of K^{+}, Na^{+}, Ca^{2+}, Ba^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+}, and Fe^{3+} ions had no significant effect on α-amylase activity. The enzyme was almost completely inhibited by the addition of Zn^{2+}.

DISCUSSION

α-Amylases from various *Bacillus* species are produced at a wide range of temperatures. *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus licheniformis an*d *Bacillus stearothermophilus* are among the most commonly used *Bacillus* species reported to produce α-amylase at temperatures of 37-60°C (Mendu *et al*., 2005b; Mishera *et al*., 2005a; Syu and Chen, 1997; Mielenz, 1983). *B*. *subtilis*, *B*. *licheniformis*, and *B*. *amyloliquefaciens*, like *Bacillus cereus* GUF8**,** require an initial pH of 7.0 for maximal amylase production (Haq *et al*., 2005a; Tanyildizi, *et al*., 2005b; Syu and Chen, 1997).

α-Amylase purified from *B*. *cereus* GUF8 appeared as a single polypeptide with a molecular weight of about 56 kDa similar to *B*. *licheniformis* and *B*. *amyloliquefaciens* α-amylases. Although it is difficult to compare the kinetic values of α-amylases reported by other groups in view of the different starch substrates and their assay conditions, the K_m value of *B*. *cereus* GUF8 α-amylase, 1.4 mg/ml, is within the range of majority of other α-amylases (0.35 to 4.7 mg/ml) (Aguilar *et al*., 2000). The hydrolysis products of soluble starch were determined by TLC analysis. The major end products of the enzyme were G2-G5 together with smaller amounts of higher maltooligosaccharides, however, it was not able to produce glucose. These indicate that the amylolytic enzyme from *B*. *cereus* GUF8 hydrolyzes starch by randomly cleaving internal α-1,4-glycosidic linkages and therefore the reaction proceeds in an endo-type fashion (Mezghani *et al*., 1999; Prieto JA, 1995).

The α-amylase showed higher activity from pH 5 to 9 with a maximum level at 5. α-Amylases produced by different *Bacillus* sources have a range of pH optima. Most of these enzymes show maximum activity in the pH ranges of 6.0-7.0 or 5.0-7. (Sivaramakrishan *et al*., 2006; Gupta *et al*., 2003; Malhotra *et al*., 2000). Some of these enzymes have low pH optima such as the α-amylase produced by *B*. *subtilis* X-23 (Ohdan *et al*., 1999), whilst others have a high pH optima such as the α-amylase produced by *B*. *firmus* KSM-1378 (Igarashi *et al*., 1998).

The temperature-activity profile of the enzyme shows a very broad temperature range at pH 7.4 (from 10 to 70ºC). The optimum temperature of the α-amylase is 50ºC, with a loss of activity over 70ºC. The data suggest a low sensitivity to temperature in this range of temperature, especially between 10-50ºC. This property might limit industrial applications of the enzyme at high temperatures, but favors its application in processes that require complete inactivation of the enzyme, such as the baking industry (Coronado *et al*., 2000). When the enzymatic properties of α-amylases from several *Bacillus* sources were compared, it was clear that the temperature profile of the purified α-amylase from *B*. *cereus* GUF8 was different from those of the known *Bacillus* α-amylases. The optimal temperatures of most bacterial α-amylases, including those from *Bacillus* sp. are in the range of 50-90ºC, but the activity is significantly decreased at temperatures lower than 50ºC (Sivaramakrishan *et al*., 2006; Gupta *et al*., 2003). In comparison to other *Bacillus* α-amylases, thermostability of this enzyme is slightly lower (Sajedi *et al*., 2005; Sajedi *et al*., 2004; Khajeh *et al*., 2001). The broad range of temperatures and the enzyme's high activity at both moderate and lower temperature values make this enzyme highly attractive for both basic research studies and industrial processes. A modern trend among consumers is to use colder temperatures for laundry or dishwashing. At lower temperatures, the removal of starch from cloth and porcelain becomes more problematic. Detergents with α-amylases working optimally at low or moderate temperatures can overcome this problem (Van der Maarel *et al*., 2002).

In conclusion, the present study indicates that the extracellular α-amylase secreted by the acid-neutralizing *B*. *cereus* GUF8 is more stable than the α-amylases of psychrophilic microorganisms, such as *Alteromonas haloplanctis* A23 (Feller *et al*., 1992), but less stable than those produced by thermophilic microorganisms. Since mesophilic and thermophilic enzymes are less active at lower temperatures and psychrophilic enzymes also have lower stability, their applications are limited. Therefore, an enzyme with moderate stability and a broad temperature profile, such as the α-amylase from *B*. *cereus* GUF8, could be considered as a suitable candidate for industrial applications. The present study for the first time provides detailed information on the biochemical characteristics of a *B*. *cereus* α-amylase. Further experiments including

cloning of the α-amylase encoding gene, sequence and molecular modeling analysis are currently under way on *B*. *cereus* GUF8 α-amylase.

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