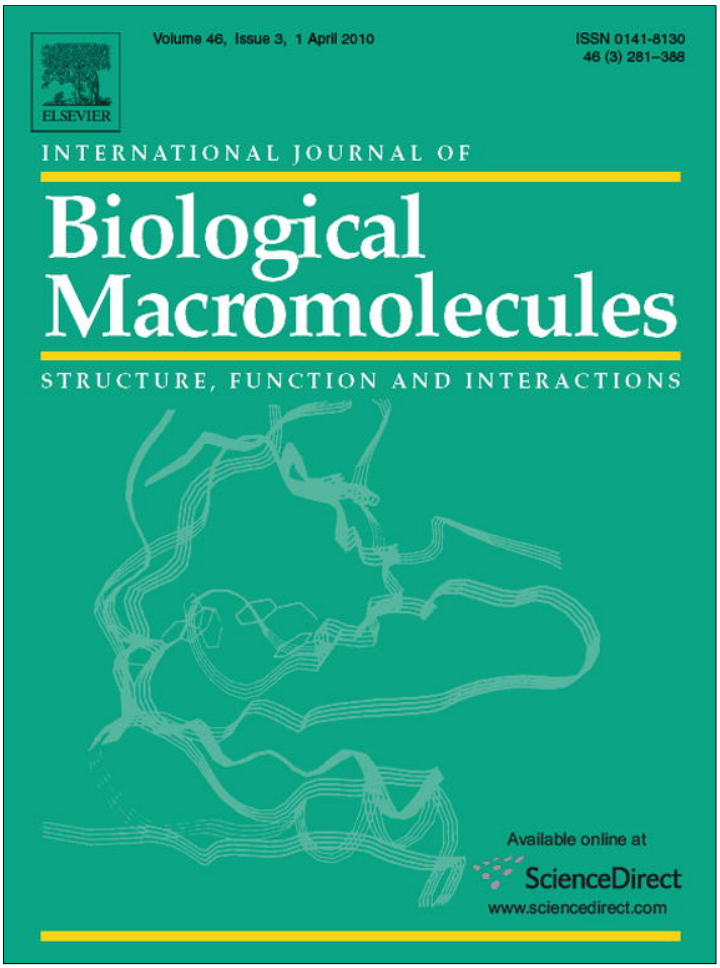

International Journal of Biological Macromolecules 46 (2010) 289–297

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

# A novel thermostable, acidophilic α-amylase from a new thermophilic "*Bacillus* sp. Ferdowsicous" isolated from Ferdows hot mineral spring in Iran: Purification and biochemical characterization

Ahmad Asoodeh[a,b,*], JamshidKhan Chamani[c], Milad Lagzian[a]

[a] *Department of Chemistry, Faculty of Sciences, Ferdowsi University of Mashhad, Azadi sq., Mashhad, Iran*
[b] *Cellular and Molecular Research Group, Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran*
[c] *Department of Biology, Faculty of Sciences, Islamic Azad University, Mashhad-Branch, Rahnamaei Ave., Mashhad, Iran*

ARTICLE INFO

*Article history:*
Received 26 September 2009
Received in revised form 15 January 2010
Accepted 18 January 2010
Available online 28 January 2010

*Keywords:*
Thermostable
α-Amylase
Purification
Acidophile
*Bacillus* sp. Ferdowsicous

ABSTRACT

This paper describes the purification and characterization of a novel acidophile α-amylase from newly isolated *Bacillus* sp. Ferdowsicous. The enzyme displayed a molecular weight of 53 kDa and it was stable over a range of pH from 3.5 to 7 with an optimum around 4.5. The optimum temperature for activity was found to be around 70 °C and the enzyme remained active to more than 75% up to 75 °C for 45 min. The enzyme activity was decreased by $Zn^{2+}$ and EDTA but inhibited by $Hg^{2+}$, whereas the activity was increased by approximately 15% by $Ba^{2+}$ and $Fe^{2+}$. $Na^{+}$, $Mg^{2+}$, $K^{+}$, $Ca^{2+}$, PMSF, Triton X-100 and β-mercaptoethanol had any considerable effect on its activity. The enzyme activity on the amylose as substrate was 1.98 times greater than amylopectin. Partial N-terminal sequencing demonstrated no significant similarity with other known α-amylases, indicating that the presented enzyme was new. Considering its promising properties, this enzyme can find potential applications in the food industry as well as in laundry detergents.



## 1. Introduction

Purified and characterized enzymes from microorganisms find their ways to the biotechnological industries in the globe; nowadays these enzymes reach to over than 3000 [1,2]. Bacteria belonging to the *Bacillus* genus and the genus closely related to *Bacillus* secrete proteases [3], cellulases [4], lipases [5], and amylases [6] in their culture. Amylases proportions from all produced industrial enzymes over the world were about 30%. Amylases, biosynthesized by the bacteria, show unique characteristics, exemplified by thermophilic [7], thermotolerant [8,9], alkaline [10–12], and acidophilic properties [13], and are used for food production, as well as in the textile and pharmaceutical industries, etc.

One of the use from thermo and alkaline stable alpha amylases is an additive in detergents that is used in washing processes, since these processes are generally performed on the alkaline pH in both hot and cold water. Alkaline and thermotolerant α-amylases have been purified from *Bacillus* species [11,12], *Bacillus licheniformis* [14], and *Bacillus halodurans* [9,10]. Some of them have been reported to be maltooligosaccharide-producing enzymes [11,12,15].

The thermostable, acidophile α-amylases were commonly used in the food processing industry. In the starch industry, the natural pH of starch slurry is usually around 4.5 [16] but, the optimum pH for most industrial alpha amylases are around 6.5 and are less stable at low pH. As result, the liquefaction step in the starch processing industry is currently focused on limited range of pH (5.5–6.5), and for this reason improving the thermal stability of the enzymes at low pH for removing pH adjustment step is necessary [17,18]. In recent years, a worldwide interest has been focused on the raw starch-digesting amylases capable of acting at low pH and high temperatures, which would be of value for efficiently simplifying the process of starch conversion. It has so far been reported that fungi and yeast, such as *Aspergillus* sp. [19], *Rhizopus* sp. [20], and *Cryptococcus* sp. [21], are good producers of raw starch-digesting amylases, capable of acting at low pH and high temperatures, while there are small number of reports on raw starch degradation by α-amylases purified from *Bacillus* sp. [17,22,23].

The present study, reports on the purification, characterization and partial N-terminal amino acid sequencing of an acidophilic thermostable α-amylase produced by "*Bacillus* sp. Ferdowsicous" which was newly isolated from the Ferdows hot mineral spring. The

* Corresponding author at: Tel.: +98 9155215590; fax: +98 5118795457.
*E-mail addresses:* Asoode_a@yahoo.com, Asoodeh@um.ac.ir (A. Asoodeh).

0141-8130/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijbiomac.2010.01.013

properties of the enzyme, including its pH profiles, thermostability and partial amino acid sequences, revealed that it can be considered a novel α-amylase.

## 2. Materials and methods

### 2.1. Material

DNS (3,5-dinitrosalicylic acid), maltooligosaccharide, TLC plate, starch, amylose and amylopectin, were purchased from Sigma (Sigma–Aldrich, USA). Q-Sepharose was purchased from Pharmacia (Pfizer and Pharmacia, Sweden). All culture mediums and their supplements were commercially purchased from Merck (Merck & Co., Inc.). Whole PCR and DNA extraction reagents were purchased from Fermentas (Fermentas life science, USA). All other chemicals used were of analytical grade.

### 2.2. Isolation of the microorganism

Water samples were collected from the Ferdows hot mineral spring in the east of Iran. Samples were then cultivated on nutrient agar plates and incubated at 42 °C for 48 h to identify possible microorganism existing in the water samples. A total of 2 isolates were screened for α-amylase activity on a starch agar plate containing 1% (w/v) peptone, 1% (w/v) starch and 28 g/l nutrient agar powder at 42 °C for 48 h.

### 2.3. Identification of microorganism and phylogenetic analysis

The isolate was identified as *Bacillus* sp. according to the methods described in "Bergey's Manual of Determinative Bacteriology" and on the basis of the 16S rDNA sequence analysis. The 16S rDNA gene of the isolate was amplified using the upstream primer 5′-AGTTTGATCCTGGCTCAG-3′ and the downstream primer P2: 5′-GGCTTACCTTGTTACGACTT-3′ which generate a DNA fragment of approximately 1.4 kb. Amplification of DNA was performed by means of the Techne FT Gene 2D thermocycler under the following conditions: denaturation at 93 °C for 5 min followed by 35 cycles at 93 °C for 45 s, 50 °C for 45 s, 72 °C for 1.30 min and a final extension at 72 °C for 5 min. The DNA nucleotide sequence was determined by "Institute Pasture-Iran branch". Sequence similarity searches were carried out with the BLAST program. The base identification and quality score at each position in a sequence were made using the Phred program. Additionally, sequences were analyzed via RDPII using SEQUENCE_MATCH (Version 2.7) to identify the most closely related database sequences. Multiple alignments of nucleotides were performed with ClustalX. Evolutionary distances were calculated by way of the Kimura-2-parameter algorithm and the phylogenetic trees were determined using the neighbor-joining method. Bootstrap analyses with 100 replications were performed on the phylogenetic tree to estimate the reproducibility of the tree topology [24].

The 16S rDNA sequence of *Bacillus* sp. Ferdowsicous has been deposited in the NCBI GenBank with accession number GQ365212.

### 2.4. Partial optimization of culture conditions

In order to select the best medium for amylase production, 4 different mediums were selected [25,26]. These mediums were: 1—nutrient broth supplemented with 1% starch; 2—nutrient broth with 1% of fructose, starch and peptone; 3—starch 1%, peptone 1% and 1% of maltose; 4—yeast extract 2%, peptone, starch and maltose 1%. Moreover, the effect of three temperatures, i.e., 37, 42 and 50 °C, on the bacterial growth was determined.

### 2.5. Culture conditions for enzyme production

Positive isolates for amylase activity testing were first grown in the nutrient broth medium and then transferred to an amylase production medium based on the nutrient broth and supplemented with 1% starch, 1% peptone, 1% fructose at 42 °C in rotary shaker with stirring at 150 rpm for 28 h.

### 2.6. Purification of the enzyme

All purification steps were carried out at 4 °C, and a 50 mM phosphate buffer (pH 6.8) was used throughout the purification unless otherwise stated. All preparations were stored at 4 °C.

#### 2.6.1. Ammonium sulfate precipitation

Ammonium sulfate was added to the crude culture supernatant to 20% saturation. After centrifugation, the supernatant was assayed for presence of the enzyme, and this assay indicated that the enzyme did not precipitate in 20% saturation of ammonium salt. As a result, the concentration of salt was increased from 20% to 50% and subsequently to 80%. In the 80% saturation, the supernatant did not contain any measurable amount of the enzyme, indicating that it had precipitated. The precipitates were dissolved in a minimum volume of 50 mM phosphate buffer (pH 6.8), and dialyzed overnight against the same buffer.

#### 2.6.2. Ion exchange chromatography

The dialyzed enzyme preparation was applied to a Q-Sepharose Fast Flow column (3 cm × 10 cm) previously equilibrated with 50 mM Tris buffer (pH 8.5). After washing through all the unbound proteins, the column was eluted using the same buffer containing 0.1–0.8 M NaCl at a flow rate of 1 ml/min. The fractions containing protein (absorbance 280 nm) were pooled and tested for enzyme activity, and the active fractions for enzyme assays were concentrated, and then dialyzed.

#### 2.6.3. Amylase activity assay

The α-amylase activity was measured with the DNS according to the method described by Bernfeld [27] using 1% starch dissolved in a 50 mM phosphate buffer pH 6.8 and 42 °C. One unit of amylase activity was defined as the amount of enzyme that released 1 μM of reducing end groups per minute at 42 °C. D-Maltose was used as standard of reducing end sugar.

#### 2.6.4. Determination of protein concentration

The protein concentration was determined by the method of Bradford [28] using bovine serum albumin as standard, in the final step and during the purification procedure.

#### 2.6.5. Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in order to determine the purity and molecular weight of the α-amylase. A 6.5% (w/v) stacking gel and a 15% (w/v) separating gel were employed [29]. Bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde 3-phoshpate dehydrogenase (35 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (21 kDa) and lysozyme (14 kDa) were used as molecular mass markers.

#### 2.6.6. Zymography of enzyme

The nondenaturing polyacrylamide gel electrophoresis was carried out as described by Lim et al. with certain modifications [30]. The separating gel contained 10% acrylamide, 0.5% bisacrylamide, and 1% starch to detect the activity of electrophoresed α-amylase. The sample was mixed with β-mercaptoethanol free loading buffer in a ratio of 3:1 (v/v) and was, without heating, electrophoresed

at 100 V at room temperature until the tracking dye migrated to the bottom of the gel. Active staining of the amylase activity in a nondenaturation gel was performed after washing the gel in Triton X-100 for 30 min and twice in 10-mM phosphate buffer (pH 6.8) for 15 min. The acrylamide gel was stained with Lugol's solution, which left a white halo zone pattern where the starch has been degraded.

#### 2.6.7. N-terminal amino acid sequencing

The purified band of enzyme on SDS-PAGE was firstly transferred to a PVDF membrane and then the band corresponding to the α-amylase was excised and the first 20 N-terminal amino acid sequences were determined. The determined sequence has been submitted to UniPortKB and assigned the accession number P86331.

### 2.7. Biochemical properties of the enzyme

#### 2.7.1. Effect of pH on the enzyme activity and stability

The effect of pH on the α-amylase activity was performed in the pH range of 3.0–9.5 at 42 °C using three buffers: 50 mM sodium acetate buffer (pH 3–5.0) 50 mM sodium phosphate buffer (pH 5.5–7.5) and 50 mM Tris–HCl buffer (pH 8.0–9.5). For measuring the pH stability, the enzyme was incubated at 42 °C for 1 h in buffers of varying pH and the residual activity was determined under the enzyme assay conditions. The activity of the enzyme in the beginning of the test was considered as 100% activity. The BLA (*Bacillus Licheniformis* α-amylase) was used as basis for comparison in activity and stability tests.

#### 2.7.2. Effect of temperature on the enzyme activity and assaying of residual activity

The effect of temperature on the α-amylase activity was studied from 30 to 85 °C at the optimum enzyme's pH. The residual activity of the purified enzyme was examined by incubating the enzyme preparation for different times at various temperatures from 30 to 80 °C after which the remaining activity was determined under the enzyme assay conditions. The activity of the enzyme at the beginning of the test was considered as 100% activity. The activity of the enzyme was also tested by incubating the purified enzyme for 150 min at temperatures ranging from 30 to 85 °C in the presence and absence of 5 mM $CaCl_2$. Aliquots were withdrawn at 30-min intervals and the remaining activity was measured under the enzyme assay conditions. The non-heated amylase enzyme was considered as the control sample (100%).

#### 2.7.3. Effect of metal ions, enzyme inhibitors and denaturing agents

The influence of various metal ions at three concentrations (i.e., 1, 5, 10 mM) on the enzyme activity was investigated using $CaCl_2$, $ZnCl_2$, $FeCl_2$, $HgCl_2$, $BaCl_2$, $MgCl_2$, NaCl and KCl. Also the effect of 1% Triton X-100 detergent on the enzyme activity was studied. For these purposes, the enzyme was incubated in the presence of the above-mentioned agents for 15 min at optimum temperature and pH conditions and then assayed for the remaining activity. The activity of the enzyme without any additive was taken as 100%. The influence of enzyme inhibitors on the α-amylase activity was studied using 1, 5 and 10 mM of phenylmethylsulfonyl fluoride (PMSF), β-mercaptoethanol and ethylenediaminetetraacetic acid (EDTA). The purified α-amylase was pre-incubated with inhibitors at 70 °C for 15 min after which the residual activity was calculated using 1% potato starch as a substrate in standard assay conditions. The activity of the enzyme assayed in the absence of inhibitor was taken as 100%.

#### 2.7.4. Thin-layer chromatography of enzyme products

The reaction products for raw potato starch produced by the enzyme at different intervals were identified by thin-layer chromatography (TLC) according to the method of Zhang et al. [31]. A precoated silica gel plate (Merck 60 HPTLC plate, Darmstadt, Germany) was employed. The reaction products on the TLC plate were developed with a solvent system consisting of n-butanol/formic acid/water, 4:8:1 (v/v). The developed plates were stained using a solution of 1 ml of 37.5% HCl, 2 ml of aniline, 10 ml of 85% $H_3PO_3$, 100 ml of ethyl acetate and 2 g of diphenylamine as described by Zhang et al. [31].

#### 2.7.5. Substrate specificity

A substrate solution (0.9 ml) containing 1.0% (w/v) each of amylopectin, amylose, α/β-cyclodextrin, maltooligosaccharide and glycogen in 50 mM sodium–acetate buffer (pH 4.5) was mixed with 0.1 ml of the enzyme solution, after which the enzyme assay was performed under the standard conditions. The value for hydrolyzing starch 1.0% (w/v) was considered as 100% activity.

## 3. Results and discussion

### 3.1. Biochemical identification

The isolate from the spring water was confirmed to be a member of the genus *Bacillus* by means of colony morphology, Gram staining appearing as Gram-positive rod-shaped cells and the ability to form spores.

### 3.2. Molecular identification

16S rDNA gene product with approximately 1418 bp was sequenced for the isolated microorganism. This sequence, containing at least 1100 bp constituted by nucleotides with Phred scores ≥20, was used for the database query. The phylogenetic position of the rDNA sequences was determined by the construction of a phylogenetic tree (Fig. 1).

### 3.3. Effect of temperature on bacterial growth

The strain Ferdowsicous showed good growth in the temperature range of 37, 42 and 50 °C and probably also at higher temperatures (although this was not verified) (Fig. 2). The optimum growth was observed at 42 °C but recorded data for temperature of 37 and 50 °C did not show any significant differences as compared to 42 °C. Values for bacterial growth ($OD_{600\,nm}$) were recorded during 46 h. The logarithmic phase was started after 15 h of incubation which indicated that this strain had a slow growth rate.

### 3.4. Effect of various mediums on the bacterial growth and enzyme production

As shown in Fig. 3, the production of α-amylase and growth of microorganism for *Bacillus* sp. Ferdowsicous varied with the type of medium and was also dependent on the incubation time—a fact that has been reported by numerous authors for microbial α-amylase synthesis [33–35]. The present work demonstrated that nutrient broth with 1% of fructose, starch and peptone supported maximum α-amylase productions post-28 h of incubation, evidencing that these mediums influenced the α-amylase synthesis machinery in the bacteria. Potato starch was found to be a superior substrate for the induction of α-amylase secretion in medium by *Bacillus* sp. Ferdowsicous. A minimum α-amylase production (~40% relative activity) was observed when starch 1%, peptone 1% and 1% of maltose were used as medium (Fig. 3C). The other mediums were not

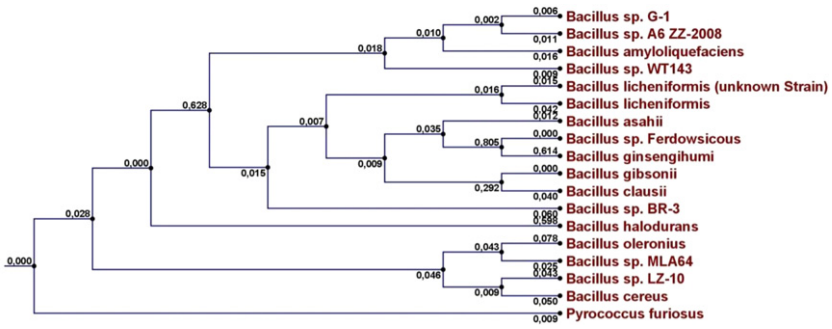


**Fig. 1.** Phylogenetic tree of 16S rDNA gene sequences. The isolated bacterium is represented by *Bacillus* sp. Ferdowsicous. DNA sequences of the 17 reference strains obtained from GenBank were incorporated into the tree by using the neighbor-joining method. Name of the each sequence typed in the end of the corresponding branch. Reliability of the tree was assessed by bootstrap analysis with 100 replications. The substitutions per nucleotide position, typed above of the each branch. The sequence of *Pyrococcus furiosus* was used as the *Outgroup*.

significantly superior to each other, but had little effect in comparison to the second medium.

### 3.5. Purification of the enzyme

The purification of the enzyme was performed as described in Section 2. The ion exchange chromatography of Q-Sepharose is presented in Fig. 4, and the results of the purification are summarized in Table 1. The purified enzyme exhibited 61% of the total initial activity, which corresponded to a 23-fold increase in specific activity when compared to the crude culture filtrate.

As shown in Fig. 5, the purified enzyme demonstrated an apparent single protein band on SDS-PAGE (Fig. 5A). The molecular weight of the single protein band was estimated to be 53 kDa from its mobility relative to those of standard proteins on SDS-PAGE. In Fig. 5B, the zymography of amylase was shown in 10% native-PAGE. The mass of α-amylases from various microbial sources vary from 22.5 to 184 kDa [32] but the most of them are in the range of 50–60 kDa (see Table 2).

The N-terminal amino acid sequence of the 20 amino acids of the purified α-amylase was found to be AHQLPMGTLCNFYEWYRRDD. As shown in Fig. 6, the N-terminal amino acid sequence alignment analysis of the purified amylase showed a 50% identity with other well-known amylases from various *Bacillus* sp. such as: amylases from *B. licheniformis*, *B. stearothermophilus* and *B. amyloliquefaciens*. These results suggested that the purified amylase could be a new enzyme in the thermostable amylase family.

### 3.6. Effect of pH on the enzyme activity and stability

The amylase activity was measured at various values of pH in buffers of identical ionic concentrations. The results were presented in Fig. 7A and the maximum activity was observed at pH 4.5. The pH stability was tested by 60 min of pre-incubation of the purified enzyme in appropriate buffers (cf. Section 2). Our enzyme in comparison with BLA as control was more acidophile. As shown in Fig. 7A, the BLA had a broad range of pH activity from 4 to 9. This finding was confirmed with results of previously reported by Hmidet et al. [9]. It can be seen from the results in Fig. 7B that the enzyme was stable from pH 4.0–7.5. The enzyme activity kept above 75% after treatment at pH values ranging from 3.5 to 6.0. For values beyond 7.5 or less than 3.5, the enzyme activity decreased drastically. These results suggest that the pH optimal of our enzyme was more acidophile than recently reported α-amylase from *B. licheniformis* NH1 [9], *B. stearothermophilus* US100 [36], *Bacillus* sp. YX-1 [23], *Geobacillus* sp. LH8 [32], *B. subtilis* JS-2004 [37]. Altogether, the α-amylase isolated from *Bacillus* sp. Ferdowsicous is one of novel amylolytic acidophile enzyme that was reported.

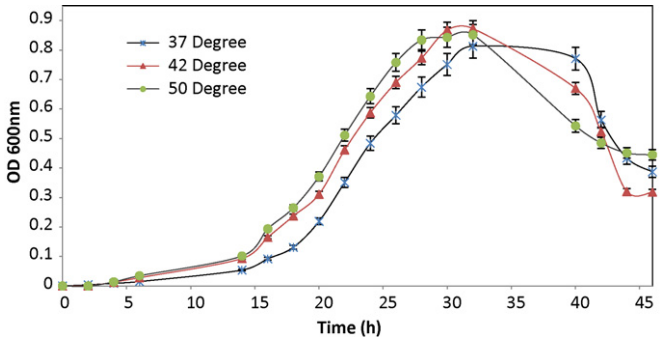


**Fig. 2.** Effect of temperature on the bacterial growth. Optimum temperature for growth of bacteria was 42 °C. Each data point represents the mean of three independent assays (the standard errors were less than 5% of the means).

### 3.7. Effect of temperature on the enzyme activity and residual activity

The effect of the temperature on the amylase activity was tested at various temperatures. The amylase was found to be active from 30 to 75 °C, with an optimum around 70 °C (Fig. 8A) . The relative activities at 55 and 65 °C were approximately 79% and 90% of the activity at 70 °C, respectively. The temperature activity profile of the enzyme showed that the amylase was very stable at temperatures between 30 and 70 °C after 150 min of incubation, but that it became completely inactivated at temperatures higher than 85 °C for this period (Fig. 8A). As shown in Fig. 8B, from 30 to 50 °C, the enzyme remained completely active after 45 min of incubation, the α-amylase from *Bacillus* sp. Ferdowsicous had a half-live of 48 min at 80 °C (based on the slope inactivation at this temperature) and maintained 100% of its initial activity after 1 h incubation at 70 °C. The half-live of our enzyme was more than α-amylases from *B. licheniformis* NH1 [9], *Lactobacillus manihotivorans* [39], *Bacillus* sp. YX-1 [23], *Bacillus* sp. KR-8104 [17] and *Geobacillus* sp. LH8 [32].

The activity of the purified amylase was also examined by incubating the enzyme at 30–85 °C in the presence of 5 mM $CaCl_2$. As shown in Fig. 8C, no considerable change occurred in activity of the enzyme in the presence or absent of $CaCl_2$ indicating that the activity of this enzyme was independent of the presence of $Ca^{2+}$.

### 3.8. Effect of various metal ions and inhibitors

An investigation was performed on the effect of metal ions and inhibitors on the purified enzyme pre-incubated at 42 °C for 15 min. Subsequently, the residual activity was measured at optimum pH and temperature (cf. Section 2). Among the tested

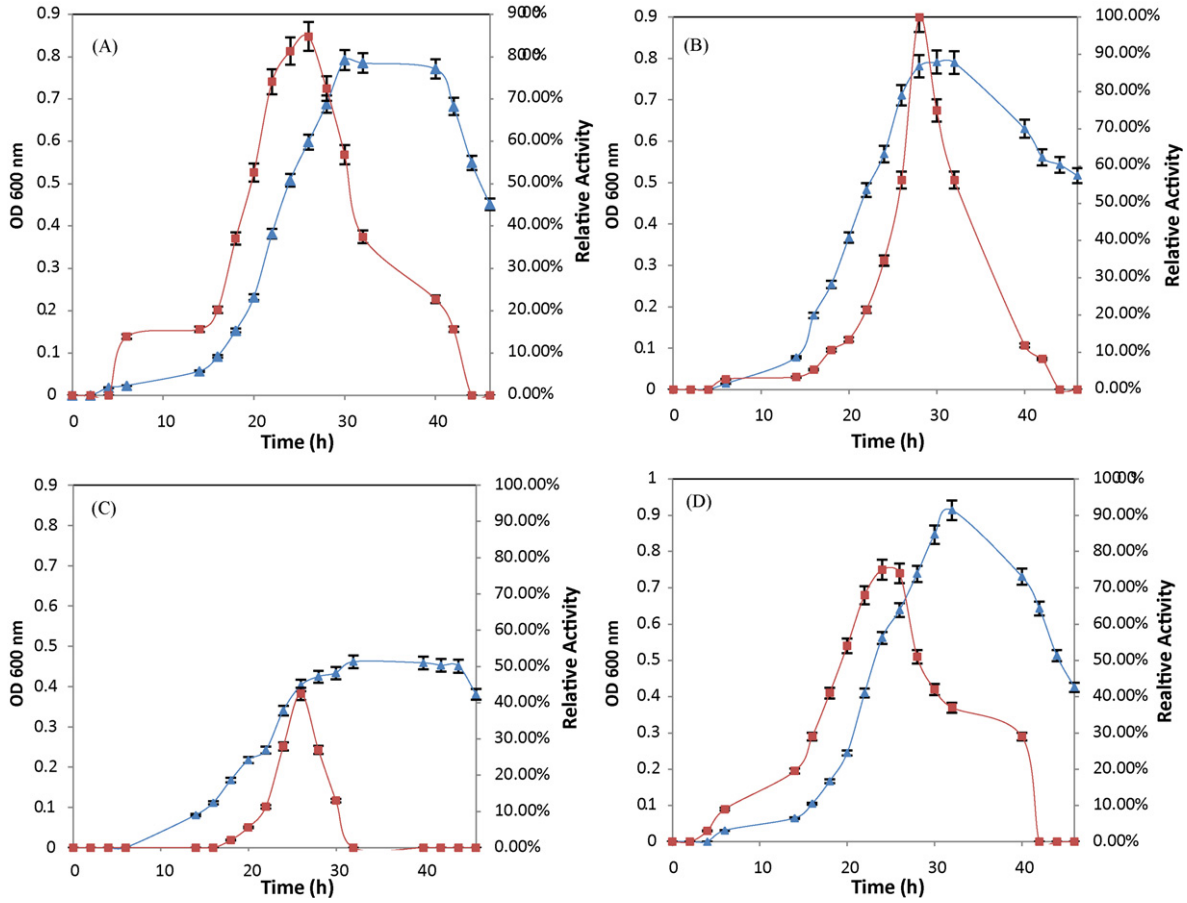


**Fig. 3.** Influence of different mediums on the enzyme activity (■)and bacterial growth (▲). (A) Nutrient broth + 1% starch; (B) nutrient broth + 1% starch, peptone, and fructose; (C) 1% starch + 1% peptone and 1% maltose; (D) 2% yeast extract + 0.1% maltose, 1% starch and 1% peptone. Each data point represents the mean of three independent assays (the standard errors were less than 5% of the means).

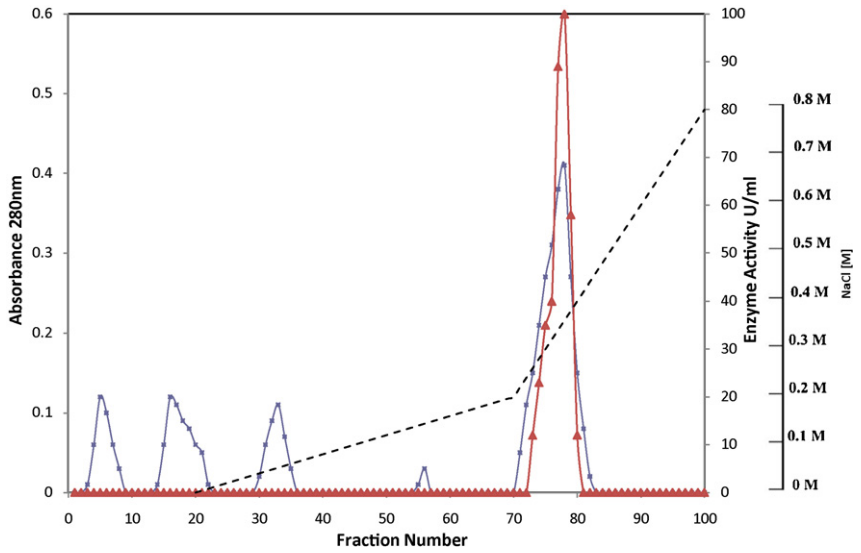


**Fig. 4.** Q-Sepharose column chromatography. (▲) Amylase activity; (■) protein concentration; (- - -) NaCl gradient. Column: 3 cm × 10 cm; flow rate: 60 ml/h.

**Table 1**
Summary of the purification of α-amylase from *Bacillus* sp. Ferdowsicous.

| | Crude extract | Supernatant 20% AS[a] | Supernatant 50% AS | Supernatant 80% AS | Percipitate-80% AS-dialyzed | Q-Sepharose |
|---|---|---|---|---|---|---|
| Total protein (mg) | 5.5 | 0.54 | 0.42 | 0.01 | 0.29 | 0.15 |
| Total activity (U/ml) | 65 | 59 | 51.5 | 0 | 45 | 40 |
| Specific activity | 12 | 109 | 123 | 0 | 155 | 267 |
| Purification fold | 1 | 9 | 10 | - | 13 | 23 |

[a] As: ammonium sulfate.

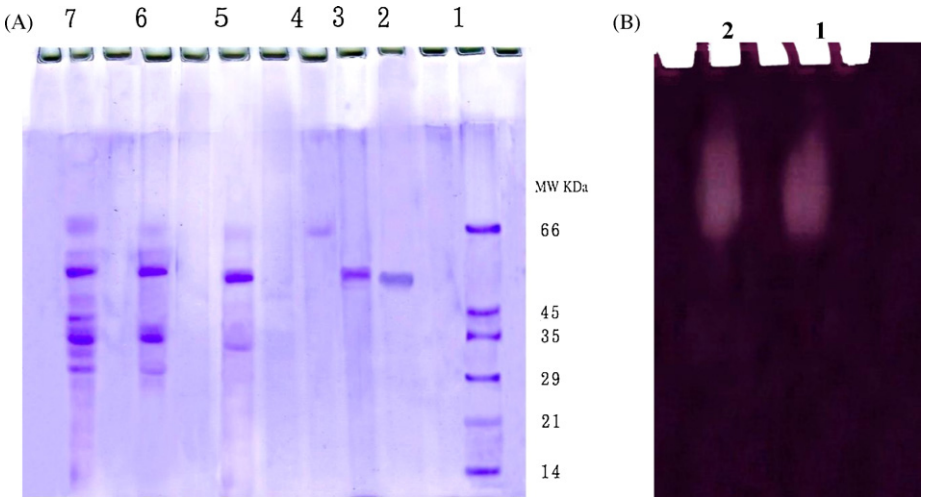


**Fig. 5.** Polyacrylamide gel electrophoresis of the purified amylase from *Bacillus* sp. Ferdowsicous. (A) SDS-PAGE. Lane 1: low molecular mass standard protein; Lane 2: purified enzyme from Q-Sepharose; Lane 3: precipitate of 80% ammonium sulfate; Lane 4: 80% ammonium sulfate supernatant; Lane 5: 50% ammonium sulfate supernatant; Lane 6: 20% ammonium sulfate supernatant; Lane 7: crude enzyme extract. (B) Native-PAGE of the purified enzyme. Lanes 1 and 2: purified enzyme.

**Table 2**
The comparison of various biochemical properties of α-amylase from *Bacillus* sp. Ferdowsicus with other *Bacillus* species.

| Bacteria species | MW (kDa) | Optimal pH, temperature Ca $^{2+}$-dependency | Half-life | Ref |
|---|---|---|---|---|
| *Bacillus* sp. Ferdowsicous | 53 | pH 4.5, 70 °C $Ca^{2+}$-independent | 48 min at 80 °C | This study |
| *Bacillus licheniformis* NH1 | 58 | pH 4–9, 90 °C $Ca^{2+}$-independent | 8 min at 85 °C | Hmidet et al. [9] |
| *Bacillus stearothermophilus* US100 | | pH 5.6, 80 °C $Ca^{2+}$-dependent | | Khemakhem et al. [36] |
| *Lactobacillus manihotivorans* | 135 | pH 5.5, 55 °C | 10 min at 60 °C | Aguilar et al. [39] |
| *Bacillus* sp. strain WN11 | 76, 53 | pH 5.5,75–80 °C $Ca^{2+}$-independent | 4 h at 80 °C. | Gashaw and Amare [38] |
| *Bacillus stearothermophilus* US100 | 59 | pH 5.6, 80–82 °C | >90 min at 90 °C | Ben Ali et al. [40] |
| *Bacillus* sp. YX-1 | 56 | pH 5.0, 40–50 °C | >1 h at 60 °C | Liu and Xu [23] |
| *Bacillus* sp. KR-8104 | 59.0 | pH 4.0–6.0, 75–80 °C, $Ca^{2+}$-independent | <10 min at 70 °C | Sajedi et al. [17] |
| *Bacillus subtilis* JS-2004 | | pH 8.0 and 70 °C, $Ca^{2+}$-dependent | 1 h at 90 °C | Asgher et al. [37] |
| *Geobacillus* sp. LH8 | 52 | pH 5–7, 80 °C, $Ca^{2+}$-dependent | 30 min at 80 °C in present of $Ca^{2+}$ | Mollania et al. [32] |

effectors, $HgCl_2$ strongly inhibited the enzyme activity, and $ZnCl_2$ inhibited the activity up to 60%. When the purified amylase was incubated in minimal concentrations of EDTA, $CaCl_2$, NaCl, KCl, PMSF and β-mercaptoethanol, the enzyme activity was retained at 85%, 92%, 100%, 100%, 100% and 100% of the original activity, respectively. EDTA demonstrated a non-significant inhibition of the enzyme activity and a slight inhibition was evidence of the purified enzyme being a metallo-enzyme. On the other hand, a slightly enhanced activity was observed in the presence of $FeCl_2$, $MgCl_2$ and $BaCl_2$—around 115% as compared to the original activity (Fig. 9). The non-enhanced activity in the presence of $Ca^{2+}$ also confirmed the previously obtained result that the enzyme was independent of the presence of calcium ions. Calcium-independent α-amylases were previously reported for *Bacillus thermooleovorans* NP54 [8], *B. licheniformis* NH1 [9], *Bacillus* sp. WN11 [38], *Bacillus* sp. KR-8104 [17]. Therefore, the α-amylase secreted by *Bacillus* sp. Ferdowsicous is a novel $Ca^{2+}$-indepnedent amylase. The most of reported α-amylases were calcium-dependent and a few of them is $Ca^{2+}$-dependent (see Table 2).

In Table 2 a comparison of biochemical properties of the purified α-amylase from this study with those reported in relevant literatures was shown. Interestingly, our enzyme was highly active and stable over a range of pH (3.5–7.5) with the optimum pH around of 4.5. The other hallmark of our enzyme is calcium independency which is valuable for industrial starch liquefaction occurring at acidic pH [17].

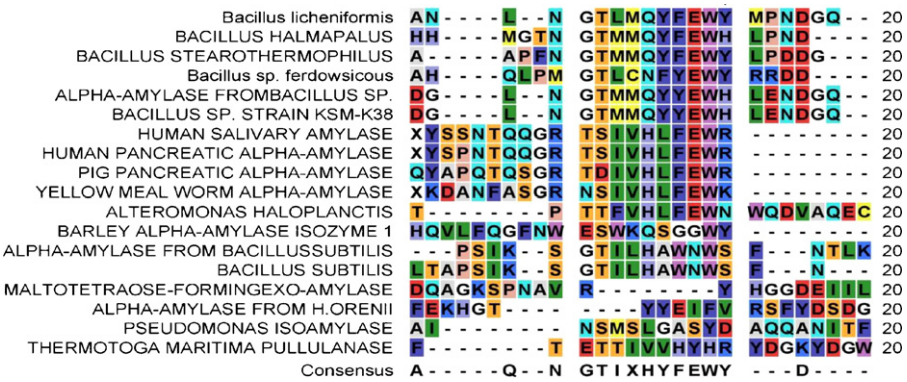


**Fig. 6.** Alignment of the N-terminal amino acid sequence of the purified amylase with the sequences of other amylase. Alignment was carried out with CLC Main Work Bench Ver.4 software.

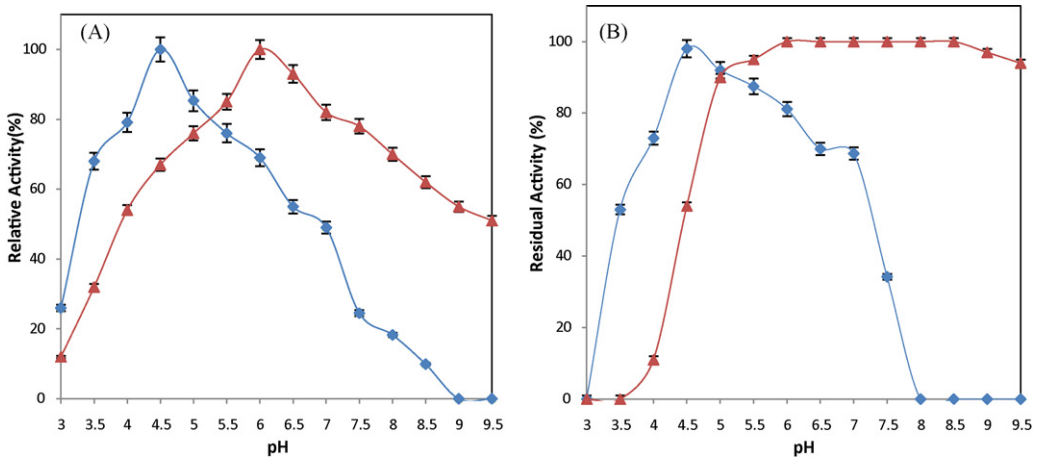


**Fig. 7.** Effect of pH on activity (A) and stability (B) of the purified amylase (♦). The enzyme activities were assayed as described in Section 2. For pH activity and stability BLA (▲, *Bacillus licheniformis* α-amylase) was tested as standard sample in conditions same as purified enzyme. Each data point represents the mean of three independent assays (the standard errors were less than 5% of the means).

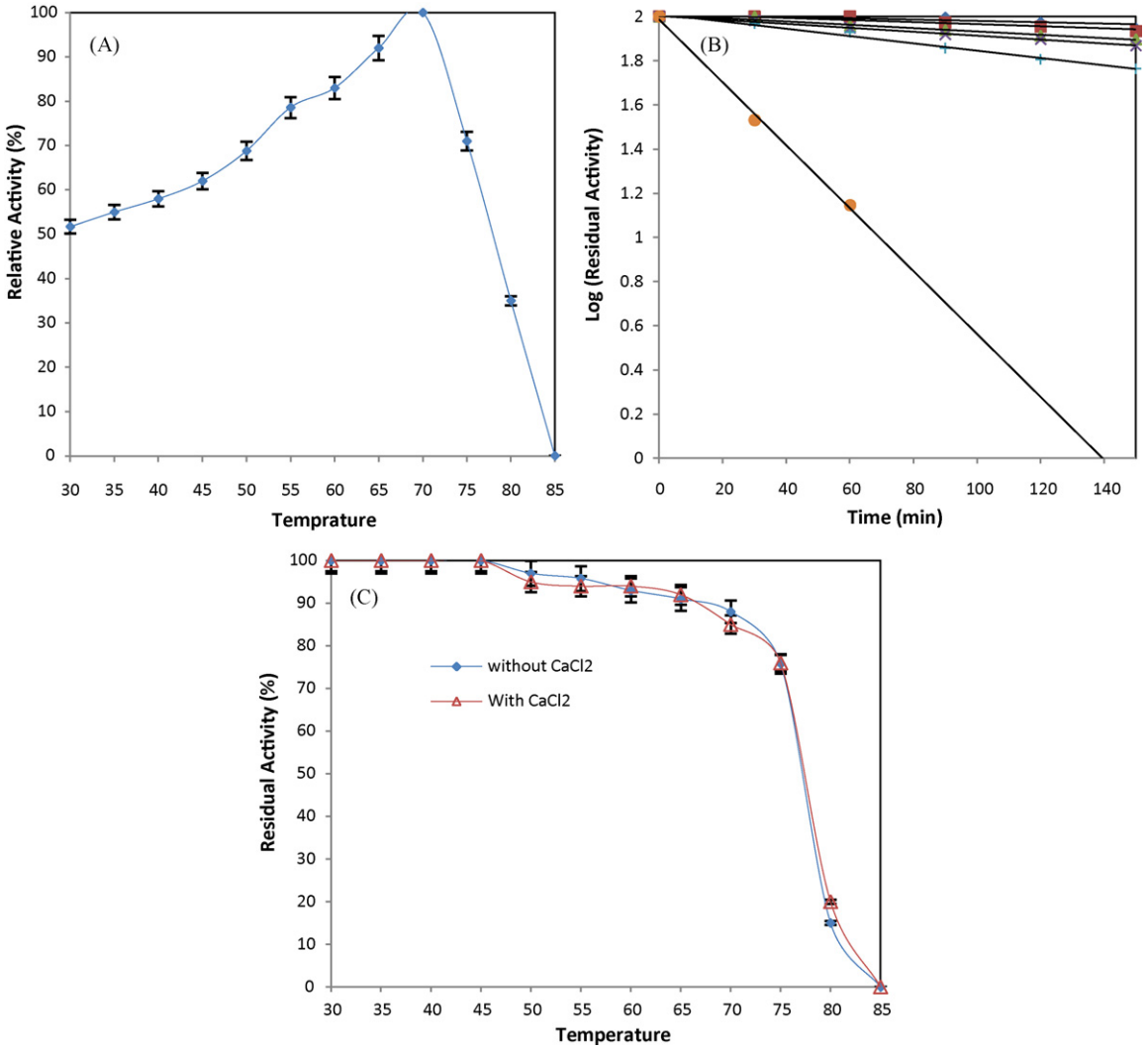


**Fig. 8.** Temperature activity profile, residual activity and the effect of $CaCl_2$ on activity of the purified amylase. In temperature activity profile (A), the activity of the enzyme at 70 °C was taken as 100%. In residual activity profile (B), the remaining of activity was determined by incubating the purified enzyme at temperatures from 30 °C (♦), 40 °C (■), 50 °C (▲), 60 °C (×), 70 °C (+) and 80 °C (●) for 150 min (sampling carried out at 30 min intervals). The residual enzyme activity was measured under the standard conditions assay. The original activity at the beginning of reaction was taken as 100% for each temperature. (C) Effect of $CaCl_2$ (Δ) on activity of the enzyme was determined by same method as above but sampling carried out at the end of incubation time. Each data point represents the mean of three independent assays (the standard errors were less than 5% of the means).

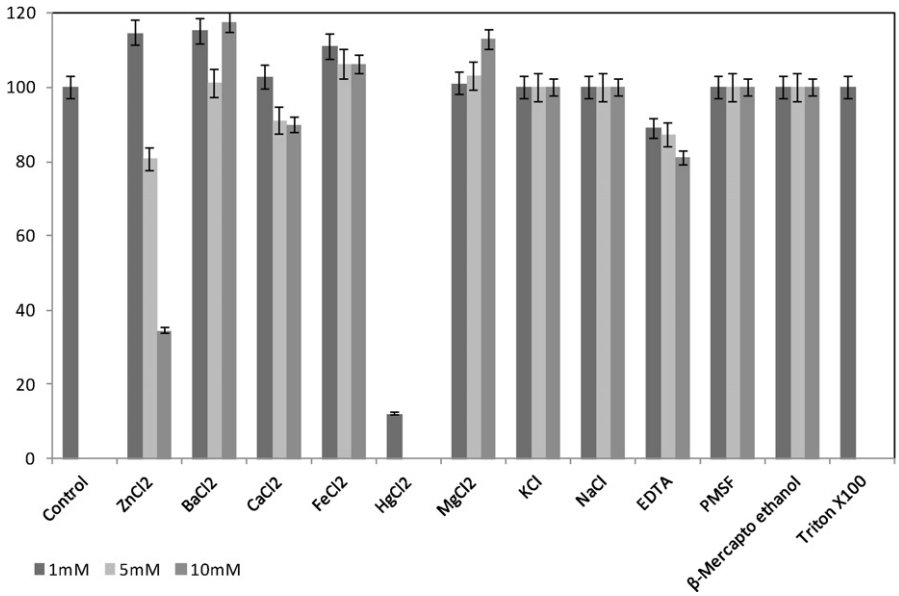


**Fig. 9.** Effect of various metal ions on the enzyme activity; for determined activity the enzyme incubated in the presence of various metal ions and then the remaining enzyme activity was determined after 5 min incubation in starch (1%) as a substrate at pH 4.5 and 70 °C. Each data point represents the mean of three independent assays (the standard errors were less than 5% of the means).

### 3.9. Product profile of the enzyme

The thin-layer chromatography (TLC) technique was used for analyzing of hydrolytic products of the enzyme action on soluble starch. After 30 min of reaction with the enzyme, G7 was observed as a predominant product (Fig. 10). When further hydrolysis was performed, glucose, G2, G3, G4, G5 and G6 appeared. Glucose and maltose were produced after 2 h of incubation, and after 24 h, the main products were G3, G2 and glucose. It could thus be concluded that the purified amylases were maltooligosaccharide-producing enzymes in the early time of incubation, albeit capable of producing glucose and maltose after long periods of hydrolysis. Hmidet et al. [9] showed that maltopentaose(G5), maltose(G2) and maltotriose(G3) are the end products of starch hydrolysis by the α-amylase of *B. licheniformis* NH1. Collectively our results show that α-amylase of *Bacillus* sp. Ferdowsicous is able to complete hydrolysis of starch for 24 h.

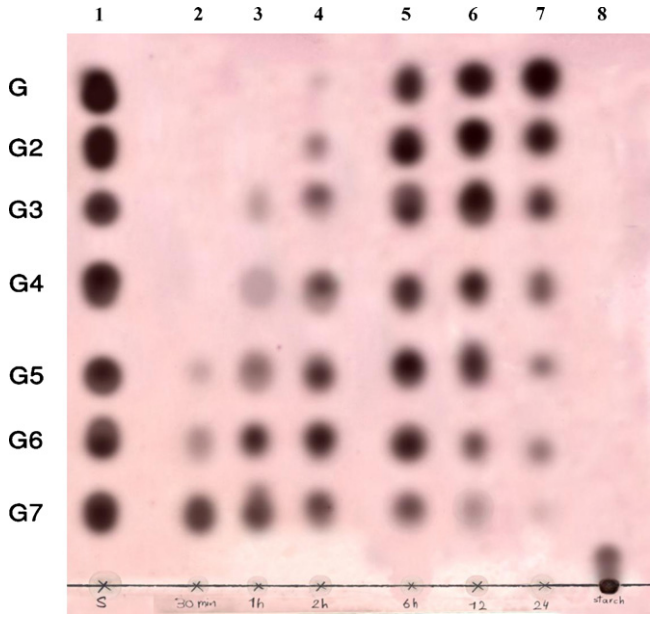


**Fig. 10.** TLC analyses of reaction products of the purified amylase. From left to right, standard oligosaccharides (Lane 1), reaction products for 30 min (Lane 2), 1 h (Lane 3), 2 h (Lane 4), 6 h (Lane 5), 12 h (Lane 6), 24 h (Lane 7) and starch sample that used as substrate (Lane 8). Lane 1 indicates the standard oligosaccharides (G, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexose; G7, maltoheptose).

### 3.10. Substrate specificity

Among the tested substrates, purified amylase showed a significant activity toward amylose (145% relative to that for soluble starch). The activity toward amylopectin, maltodextrins and glycogen was 73%, 85% and 14%, respectively, relative to that of soluble starch. No hydrolytic activity toward α/β cyclodextrin was observed (Fig. 11). Therefore, α-amylase from *Bacillus* sp. Ferdowsicous has a high affinity for hydrolysis of amylose than the other linear α-glycosides.

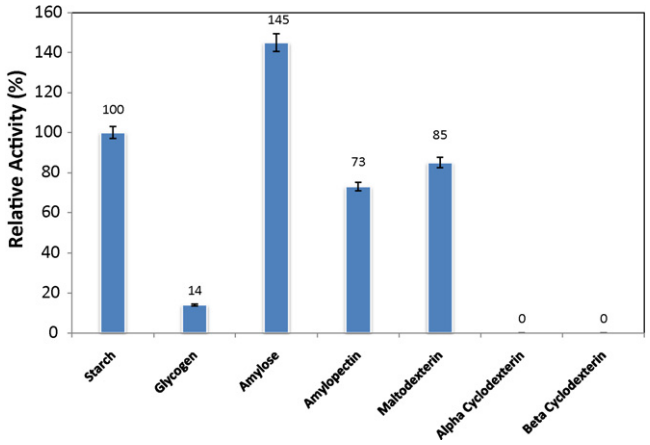


**Fig. 11.** Substrate specificity of the purified enzyme toward various substrates. Each data point represents the mean of three independent assays (the standard errors were less than 5% of the means).

## 4. Conclusions

The present work involves the purification and characterization of a novel thermostable acidophilic α-amylase from *Bacillus* sp. Ferdowsicous which did not show a high homology to the usual α-amylase-producing strains based on the 16S rDNA sequence when comparing results of this strain to others related to *Bacillus* sp. The enzyme was purified to homogeneity by 80% ammonium sulfate precipitation, Q-Sepharose anion exchange chromatography. After the final purification step, the enzyme had obtained a 23-fold purification and a specific activity of 267 U/mg. The purified enzyme was single-band on SDS-PAGE and its molecular weight was estimated to 53 kDa. The optimum temperature for amylolytic activity was 70 °C. The partial amino acid sequences analyzing of the purified enzyme was shown that in comparison with well-known amylases, it may be a novel α-amylase. Besides being active and stable at low pH, the enzyme performs remarkable stability in a range of pH values. These features are very important in starch industry, which is lacking in the majority of α-amylases published to date [17,23]. From these results, the α-amylase from *Bacillus* sp. Ferdowsicous was proposed to be a novel α-amylase enzyme. From an application point of view, this enzyme could be a potential candidate to be used on its own for complete hydrolysis of high-concentration raw starch to glucose and maltose. Considering its high activity and stability in a wide range of pH (i.e., from 3.5 to 7, particularly at 4.5) and at high temperatures stability and $Ca^{2+}$ independent hydrolyzing mechanism, the *Bacillus* sp. Ferdowsicous α-amylase is a good choice for application in starch and food industries.

Future studies should be focused on determining the encoded gene sequence of this α-amylase in order to understand the mechanism of its activity at low pH, and also on the improving the stability of the enzyme through site-directed mutagenesis.

## Acknowledgements

This work was financially supported by the Ferdowsi University of Mashhad, Mashhad, Iran. The authors gratefully acknowledge the Cellular and Molecular Research Group, Institute of Biotechnology, Ferdowsi University of Mashhad, as well, the "Buali Research Institute of Mashhad" for beneficial help.

## References

[1] J. Eichler, Biotechnol. Adv. 19 (2001) 261–287.
[2] J.D. Rozell, Bioorg. Med. Chem. 7 (1999) 2253–2261.
[3] E. Setyorini, S. Takenaka, S. Murakami, K. Aoki, Biosci. Biotechnol. Biochem. 70 (2006) 433–440.
[4] K. Eckert, E. Schneider, Eur. J. Biochem. 270 (2003) 3593–3602.
[5] E. Lesuisse, K. Schanck, C. Colson, Eur. J. Biochem. 216 (1993) 155–160.
[6] H. Hagihara, K. Igarashi, Y. Hayashi, K. Endo, K. Ikawa-Kitayama, K. Ozaki, Appl. Environ. Microbiol. 67 (2001) 1744–1750.
[7] L.L. Lin, C.C. Chyau, W.H. Hsu, Biotechnol. Appl. Biochem. 28 (1998) 61–68.
[8] R. Malhotra, S.M. Noorwez, T. Satyanarayana, Lett. Appl. Microbiol. 31 (2000) 378–384.
[9] N. Hmidet, A. Bayoudh, J.G. Berrin, S. Kanoun, N. Juge, M. Nasri, Process Biochem. 43 (2008) 499–510.
[10] S.O. Hashim, O. Delgado, R. Hatti-Kaul, F. Mulaa, B. Mattiasson, Biotechnol. Lett. 26 (2004) 823–828.
[11] T.U. Kim, B.G. Gu, J.Y. Jeong, S.M. Byun, Y.C. Shin, Appl. Environ. Microbiol. 61 (1995) 3105–3112.
[12] T. Hayashi, T. Akiba, K. Horikoshi, Agric. Biol. Chem. 52 (1988) 443–448.
[13] B. Schwermann, K. Pfau, B. Liliensiek, M. Schleyer, T. Fischer, E.P. Bakker, Purification, Eur. J. Biochem. 226 (1994) 981–991.
[14] S. Medda, A.K. Chandra, J. Appl. Bacteriol. 48 (1980) 47–58.
[15] S.O. Hashim, O.D. Delgado, M.A. Martinez, R.H. Kaul, F.J. Mulaa, B. Mattiasson, Enzyme. Microb. Technol. 36 (2005) 139–146.
[16] S. Sivaramakrishnan, D. Gangadharan, K.M. Nampoothiri, C.R. Soccol, A. Pandey, Food Technol. Biotechnol. 44 (2006) 173–184.
[17] R.H. Sajedi, H. Naderi-Manesh, K. Khajeh, R. Ahmadvand, B.A. Ranjbar, A. Asoodeh, F. Moradian, Enzyme Microb. Technol. 36 (2005) 666–671.
[18] N. Goyal, J.K. Gupta, S.K. Soni, Enzyme Microb. Technol. 37 (2005) 723–734.
[19] T. Matsubara, Y. Ben Ammar, T. Anindyawati, S. Yamamoto, K. Ito, M. Iizuka, N. Minamiura, J. Biochem. Mol. Biol. 37 (2004) 422–428.
[20] H. Morita, Y. Fujio, Starch 52 (2000) 18–21.
[21] H. Iefuji, M. Chino, M. Kato, Y. Iimura, Biochem. J. 318 (1996) 989–996.
[22] L.M. Hamilton, C.T. Kelly, W.M. Fogarty, Process Biochem. 35 (1999) 27–31.
[23] X.D. Liu, Y. Xu, Biores. Technol. 99 (2008) 4315–4320.
[24] S.F. Altschul, T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, Nucleic Acids Res. 25 (1997) 3389–3402.
[25] H. Vahidi, B. Shafagi, Z. Mirzabeigi, Daru 13 (2005) 20–22.
[26] P.V. Aiyer, Afr. J. Biotechnol. 3 (2004) 519–522.
[27] P. Bernfeld, Methods Enzymol. 1 (1955) 149–158.
[28] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[29] U.K. Laemmli, Nature 227 (1970) 680–685.
[30] W.J. Lim, S.R. Park, C.L. An, J.Y. Lee, S.Y. Hong, E.C. Shin, E.J. Kima, J.O. Kim, H. Kim, H.D. Yun, Res. Microbiol. 154 (2003) 681–687.
[31] Z. Zhang, J. Xie, F. Zhang, R.J. Linhardt, Anal. Biochem. 1 (2007) 118–120.
[32] N. Mollania, K. Khajeh, S. Hosseinkhani, B. Dabirmanesh, Int. J. Biol. Macromol. 46 (2010) 27–36.
[33] Z. Baysal, F. Uyar, C. Aytekin, Process Biochem. 38 (2003) 1663–1668.
[34] S. Ramachandran, A.K. Patel, K.M. Nampoothiri, F. Francis, G. Szakacs, A. Pandey, Biores. Technol. 93 (2004) 169–174.
[35] J. Shukla, R. Kar, World J. Microbiol. Biotechnol. 22 (2006) 417–422.
[36] B. Khemakhem, M. Ben Ali, N. Aghajari, M. Juy, R. Haser, S. Bejar, Biochem. Biophys. Res. Commun. 385 (2009) 78–83.
[37] M. Asgher, M. Javaid Asad, S.U. Rahman, R.L. Legge, J. Food Eng. 79 (2007) 950–955.
[38] M. Gashaw, G. Amare, Enzyme Microb. Technol. 25 (1999) 433–438.
[39] G. Aguilar, J. Morlon-Guyot, B. Trejo-Aguilar, J.P. Guyot, Enzyme Microb. Technol. 27 (2000) 406–413.
[40] M. Ben Ali, S. Mhiri, M. Mezghani, S. Bejar, Enzyme Microb. Technol. 28 (2001) 537–542.