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Purification of α-Amylase from *Bacillus* sp. GHA1 and Its Partial Characterization

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Bacillus sp. GHA1 was isolated from water samples and screened for the production of α-amylase. Maximum production of amylase by this strain occurs at 42 ºC, pH 6.5 and 72 h after cultivation in production medium. The enzyme was purified through successive applications of ammonium sulfate precipitation, ion exchange and hydrophobic interaction chromatography, resulting in a single band with an apparent molecular weight of 66 kDa, as judged by SDS-PAGE. Calcium analysis of the purified enzyme revealed that it contained three metal ions per molecule. The new extracellular α-amylase is active in a wide range of pH with its maximum activity at pH values 5.5-8.0. The optimum temperature for enzyme activity is 57 ºC and the presence of calcium has relatively low influence on its activity and thermostability. The *Bacillus* sp. GHA1 α-amylase with these properties may be suitable for use in detergent and food industries.

Keywords: α-Amylase, *Bacillus* sp. GHA1, pH profile, Broad range of pH, Industrial applications

INTRODUCTION

α-1,4-Glucan-4-glucanohydrolases (EC 3.2.1.1), are calcium-containing enzymes, which catalyze the cleavage of α-D-(1-4) glycosidic bonds in starch and related carbohydrates, producing α-anomeric products. α-Amylases can be found in microorganisms, plants and higher organisms where they play a major role in carbohydrate metabolism [1,2]. These enzymes are among the most important enzymes with a wide variety of industrial applications and account for about 30% of the world's enzyme production [2,3]. Projects for finding new microorganisms for enzyme production are being launched all around the world [4]. Furthermore, interest in starch-degrading amylases has increased in recent years and a number of papers have appeared in literature dealing with the production and purification of amylases from microorganisms [5,6]. A number of α-amylases with different molecular weights, optimum pH, and optimum temperatures have been identified and reported [5-7]. α-Amylases with desirable properties such as thermostability, metal ion independence, pH spectra, and so on can be applicable to the related industries [4,5,7,8]. For example, in bread making, the production of glucose and fructose syrup and fuel ethanol from starch materials, and in textile treatment, α-amylases from *Bacillus stearothermophilus* (BSTA), *B. licheniformis* (BLA) and *B. amyloliquefaciens* (BAA) have ordinarily been used [8]. Moreover, the demand for α-amylases for use in detergents has increased. However, most industrial amylases, such as BAA, BSTA and BLA, with optimum pH 5-7.5 [9,10] are neutrophilic enzymes [11] and, thus, not suitable for use in detergent industries [12]. Several papers have been concerned with reporting alkaline amylases [13-15]. These enzymes with pH optima in alkaline range can be used in laundry and

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automatic dishwashing detergents.

In this study, we report the purification and partial characterization of a novel enzyme exhibiting α-amylase activity isolated from *Bacillus* sp. GHA1. This enzyme is fully active at pH 8 which matches with the working pH range for detergent industries. Furthermore, other characteristics of this new amylase, such as its relatively low dependence on calcium ions for its activity and stability, may offer a good prospect for its application in starch as well as detergent industries.

EXPERIMENTAL

Chemicals

Yeast extract, soya meal peptone, meat extract, nutrient broth, nutrient agar, soluble starch, sodium chloride, calcium chloride, ethylenediaminetetraacetic acid (EDTA) and sodium *n*-dodecyl sulfate (SDS) were purchased from Merck (Darmstadt, Germany). Tris, phenylmethylsulfonyl fluoride (PMSF) and dinitrosalicylic acid (DNS) were obtained from Sigma (St Louis, MO, USA). *ρ*-Nitrophenyl α-D-maltoheptaoside-4-*O*-ethylidene (blocked EPS) and α-glucosidase were purchased from Boehringer Mannheim (Mannheim, Germany). DEAE-Sepharose and phenyl-Sepharose were obtained from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical grade and the solutions were prepared using doubly distilled water.

Microorganisms

Water samples were collected from Kermanshah and Ilam mineral hot springs in western Iran. 1 ml of each sample was added to 9 ml of the defined broth media. To prepare the isolation medium, 1 ml of the defined broth media was added and distributed on an isolation medium containing: potato starch 10 g, meat extract 5 g , agar 20 g and 1,000 ml of distilled water. Plates were incubated at 37 ºC for 24-48 h and different types of colonies were chosen and isolated. Nutrient agar supplemented with 10 g l^{-1} soluble starch was used for checking out the starch hydrolysis ability of the isolated strains. The hydrolysis ability was examined by flooding plates with Lugol's iodine solution and recording the appearance of the clear zone around the margins of growth. The largest halo-forming zone was considered as the most promising strain and was further examined for morphological, physiological and biochemical characteristics based on Bergey's manual of systematic bacteriology [16] and color atlas of *Bacillus* sp. [17].

Polymerase Chain Reaction (PCR) Amplification and 16S rDNA Sequencing

Genomic DNA was extracted and purified using DNA extraction kit (Roche). Universal 16S rDNA PCR forward primer (5'-AGTTTGATCCTGGCTCAG-3') and reverse primer (5'-GGC/TTACCTTGTTACGACTT-3') were used in the amplification of 16S rDNA genes. A DNA thermal cycler (Eppendorf) was used and programmed as follows: (1) an initial temperature of 94 ºC for 5 min, (2) a run of 30 cycles with each cycle consisting of 45 s at 94 ºC, 45 s at 48 ºC and 90 s at 72 ºC and (3) 5 min at 72 ºC to allow for the extension of any incomplete products [18]. The amplification products were also purified using DNA extraction kit (Roche) and DNA sequencing on both strands was directly performed by SEQ-LAB (Germany).

Production of α-Amylase

A loopful of each strain was cultivated on preculture medium containing: nutrient broth 8; meat extract 10; soyameal peptone 10; potato starch 10; and NaCl 0.5 (g l^{-1}). Incubation was carried out in an orbital incubator at 37 ºC, with the stirring rate of 160 rpm for 24 h. Subsequently, each strain was transferred to the production medium aseptically at 5% of the production medium containing: potato starch 10; soyameal peptone 4; meat extract 3; $CaCl_2.H_2O$ 0.5; $MgSO_4.7H_2O$ 0.3; and K_2HPO_4 1 (g l^{-1}), and incubated under the same conditions as for the preculture medium. The isolates were examined periodically during cultivation to determine the optimum time of enzyme production. The effect of various pH values (3.0-9.0) on the amylase production was also studied.

Determination of Enzyme Activity and Protein Concentration

α-Amylase activity was determined at room temperature using a Cary-100 Bio (Varian) UV-Vis spectrophotometer, in a 200 µl reaction mixture containing 100 µl of a 1.0% (w/v) potato starch solution in 20 mM Tris-HCl, pH 7.4 and 100 µl of enzyme solutions. The concentration of reduced sugars liberated from the catalyzed reaction for 3 min was measured by Bernfeld method [19]. One unit of α-amylase is defined as the amount of the enzyme which liberates 1.0 µmol of reduced

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sugar per minute using maltose solution as the standard. The activity of α-amylase was also assayed using its absolute specific substrate (ρ-nitrophenyl α-D-maltoheptaoside-4-*O*-ethylidene) for α-amylase and the excess of α-glucosidase as the coupling enzyme under the same conditions [20,21]. Protein concentration was determined according to the Bradford method [22].

Purification of α-Amylase

After 72 h of growth in production medium, the culture medium was centrifuged at 5,000 × *g* for 10 min at 4 ºC. The supernatant was collected and adjusted to 1 mM PMSF. Ammonium sulfate was added to the supernatant to 85% saturation at 4 ºC for 4 h. The mixture was centrifuged at 10,000 × *g* for 15 min at 4 ºC and the precipitates were dissolved in a minimum volume of 20 mM Tris buffer, pH 8. The concentrated enzyme was dialyzed against the same buffer overnight at 4 ºC. The dialyzed solution was applied to DEAE-Sepharose fast flow column (1 cm × 10 cm) at the flow rate of 0.6 ml min^{-1}, pre-equilibrated with 20 mM Tris buffer, pH 8. Proteins were eluted with a linear gradient of 0-0.3 M NaCl in the same buffer and flow rate. The active fractions were combined and applied onto phenyl-Sepharose column, pre-equilibrated with 20 mM Tris-HCl, pH 8 containing 0.5 M ammonium sulfate. Before applying the enzyme solution onto the column, it was also adjusted to 0.5 M ammonium sulfate. A linear decreasing gradient of 0.5-0 M ammonium sulfate was used for washing the column in the same buffer at the flow rate of 1 ml min^{-1}. The effluent was collected as 1 ml fractions. The active fractions were collected and concentrated by Vaco 5 (Zirbus, Germany) freeze dryer.

Polyacrylamide Gel Electrophoresis and Estimation of Molecular Mass of α-Amylase

Molecular mass of the product was estimated by SDS-PAGE in a 12% polyacrylamide resolving gel according to the Laemmli method [23]. The protein bands were visualized by Coomassie Brilliant blue R-250. Ovotransferrine (78 kDa), serum albumin (66.2 kDa), ovoalbumin (45 kDa), actinidin (29 kDa), β-lactoglobulin (18 kDa) and lysozyme (14.4 kDa) were used as molecular mass standards.

Determination of Molar Absorption Coefficient of the Enzyme and Its Calcium Content

Molar absorption coefficient (ε_{280}) of the purified α-amylase was determined using the method proposed by Pace *et al.* [24]. The calcium bound to the α-amylase was determined by ICP-AES (Varian Libery 150 Ax Turbo) spectrophotometer at 317.93 nm after the dialysis of the purified enzyme against deionized water for 24 h. A Cary-100 Bio (Varian) UV-Vis spectrophotometer was used for recording the UV spectrum of α-amylase in 20 mM Tris-HCl, pH 7.4 at room temperature, as well as determining ε_{280} of the enzyme.

Activity and Stability of Purified α-Amylase

Activity of the purified enzyme was assayed at different pH values (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0) after adding 1 volume of enzyme to 4 volumes of mixed buffer (50 mM) containing phosphate, glycine, acetate and Tris adjusted to each pH.

To assess the pH stability, the enzyme solutions were incubated at different pH values (3.0-9.0) at room temperature for 30 min in 10 mM of mixed buffer, and used for measuring the residual activity under the standard assay conditions.

The activity of purified α-amylase was determined at different temperatures (20-75 ºC) in 20 mM Tris-HCl, pH 7.4. For determining the thermal stability, the purified enzyme was incubated at 60 ºC in the same buffer in the presence and absence of 10 mM $CaCl_2$ and 5 mM EDTA, for a series of time intervals, then cooled on ice bath, and finally the residual activity was determined under standard assay conditions. The purified enzyme was also dialyzed twice against 20 mM Tris-HCl, pH 7.4 containing 10 mM EDTA after which the aforementioned experiment was carried out in this buffer.

The effects of Ca^{2+} ions and EDTA on the activity of enzyme were also studied. Enzyme assays were performed in the presence of 5 and 10 mM of $CaCl_2$ and 5 mM of EDTA. The enzyme was incubated for 30 min at room temperature and then the residual activity was measured in the standard conditions of enzymatic assay.

RESULTS AND DISCUSSION

Identification of Bacteria

The bacterial strains obtained from hot springs were screened for α-amylase activity. After Lugol's staining, five strains which had considerable halo-forming zone on plates supplemented with starch were selected for further experiments. Finally a Gram positive *Bacillus*, GHA1 strain,

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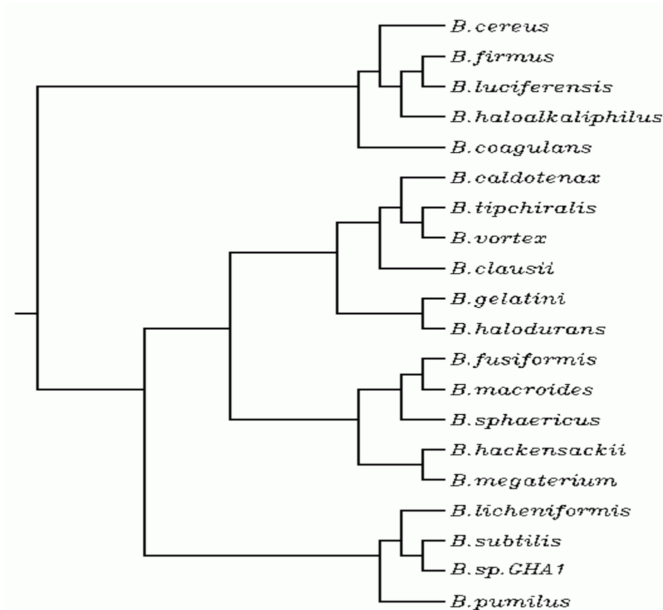

Fig. 1. Phylogenetic tree of GHA1 strain and related *Bacillus* inferred from sequence of 16S rDNA created by MEGALIGN.

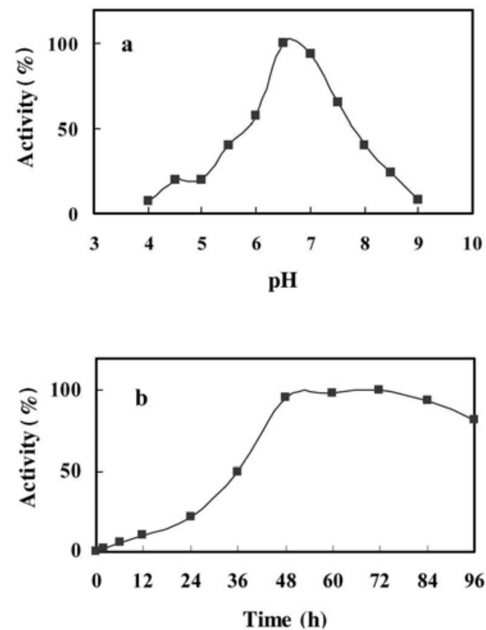

Fig. 2. Effect of pH (a) and time (b) on amylase production by *Bacillus* sp. GHA1.

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was selected as the best potent producer of α-amylase. It was nitrate, catalase and oxidase positive and its penicillin sensitivity was also positive. This strain was gram positive and able to grow well at 42 ºC and also at 50 ºC and 55 ºC and acid compounds were produced from glucose and mannitol. Furthermore, this strain had considerable motility. These characteristics suggest that the GHA1 strain is a *Bacillus* species. Ribosomal RNAs are essential elements in protein synthesis and are conserved in all living organisms. 16S rDNA sequences from 19 types of *Bacillus* species were obtained from the national center for biotechnology information (NCBI, http://www.ncbi.nlm.nih.gov/) and multiple sequence alignment was performed using ClustalW version 1.82 [18]. The 16S rDNA sequence of isolate GHA1 strain has been deposited in GenBank under Accession No. EU109536. The phylogenetic tree was constructed by neighbor-joining method. Multiple alignment and phylogenetic tree showed that the isolate GHA1 had the highest similarity to *Bacillus subtilis* (Fig. 1).

α-Amylase Production and Purification

The maximum production of α-amylase was obtained after 72 h of inoculation in the production medium at pH 6.5-7 (Figs. 2a,b). Precipitation by ammonium sulfate 85% resulted in the increment of the specific activity of the amylase from 1.9 to 4.1 U mg^{-1} (Table 1). Anion-exchange chromatography on DEAE-Sepharose was used for the partial purification of the enzyme after ammonium sulfate precipitation step. The elution pattern (Fig. 3a) showed a major peak with amylase activity which was pooled, adjusted to 0.5 M ammonium sulfate, and then applied onto a phenyl-Sepharose column. The fractions of 51-60 (corresponding to 51-60 ml of the effluent) which were eluted immediately at the end of the ammonium sulfate gradient showed α-amylase activity (Fig. 3b). The combined and concentrated active fractions showed homogeneity and appeared as a single band on SDS-PAGE (Fig. 4). The molecular weight of the enzyme was estimated as 66 kDa. A brief account of the purification procedure is given in Table 1. This procedure yielded pure amylase with the specific activity of 250.0 U mg^{-1}, purification factor of 131.6 and yield of 28%.

Calcium Content and ε_{280} of α-Amylase

All amylases are metalloenzymes and the number of bound

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Table 1. Purification Table of α-Amylase from *Bacillus* sp. GHA1

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Culture extract	270	142.5	1.9	100	1.0
Ammonium sulfate precipitation	148	36.4	4.1	55	2.2
DEAE-Sepharose chromatography	103	3.2	32.2	38	17.0
Phenyl-Sepharose chromatography	75	0.3	250.0	28	131.6

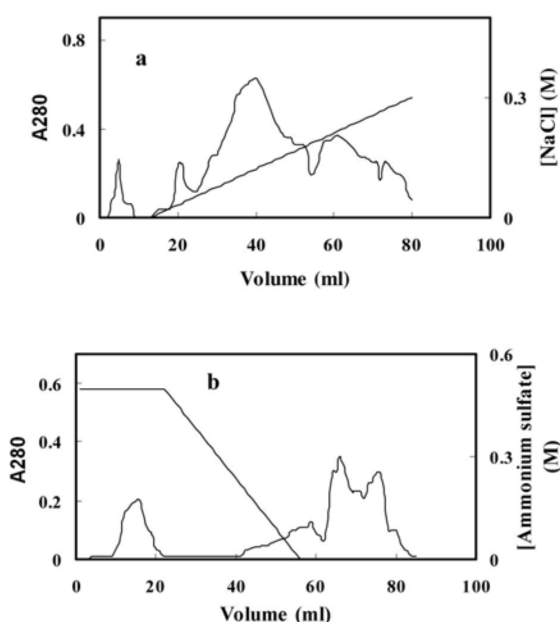

Fig. 3. Elution profiles of *Bacillus* sp.GHA1 α-amylase on DEAE-Sepharose (a) and phenyl-Sepharose (b) columns as described in experimental section. The active peaks that exhibits amylase activity showed by arrows in the profiles. The extended lines in the figure shows the NaCl (a) and ammonium sulfate (b) gradients.

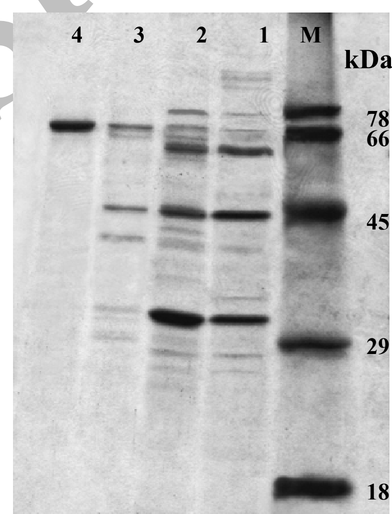

Fig. 4. SDS-PAGE depicts different steps of α-amylase purification. Lane 1 (17 μg), supernatant of bacterial culture; lane 2 (15 μg), crude enzyme after precipitation with ammonium sulfate; lane 3 (2.7 μg), after ion exchange chromatography using DEAE-Sepharose column; lane 4 (0.5 μg), the purified enzyme after hydrophobic interaction chromatography using phenyl-Sepharose column and lane M, molecular size markers.

calcium is between one and ten per enzyme molecule [25]. Calcium is essential for the manifestation of activity and the maintenance of structural stability of α-amylases reported to date [26]. The analysis of calcium content of the purified enzyme revealed the presence of three bound calcium ions per molecule of the protein. The UV spectrum of α-amylase is depicted in Fig. 5. The intense peak around 204 nm corresponds to the peptide bond absorption, whereas the minor peak at 280 nm accounts for the absorption of tryptophan and tyrosine side chains of the protein. The ε_{280} of the purified α-

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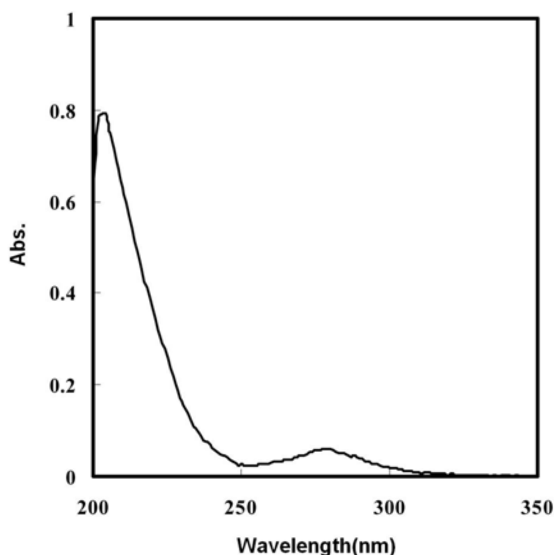

Fig. 5. UV spectrum of *Bacillus* sp.GHA1 α-amylase in 20 mM Tris-HCl, pH 7.4. The enzyme concentration was 1.4×10^{-6} M.

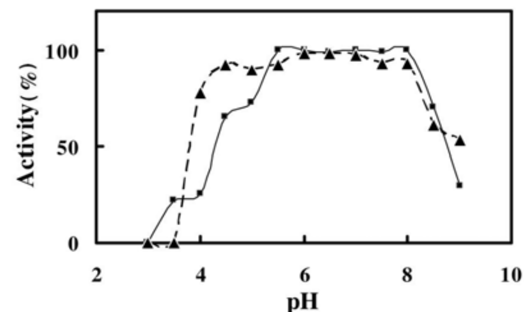

Fig 6. Effect of pH on the activity and stability of α-amylase from *Bacillus* sp.GHA1. The pH activity and stability are shown by solid and dotted lines, respectively. Enzyme activity was measured over pH range of 3-9. Assays were performed at room temperature in the mixed buffer (50 mM) containing phosphate, glycine, acetate and Tris which adjusted to each pH. For the pH stability, the enzyme was preincubated at the indicated pH in 10 mM mixed buffer for 30 min at room temperature and then residual activity was measured at standard conditions in the mixed buffer (50 mM). The values are shown as the percentage of the original activity.

amylase was determined as 4.19×10^4 M^{-1} cm^{-1}.

Effect of pH on α-Amylase Activity and Stability

The pH profile of the α-amylase is presented in Fig. 6. The results showed that the amylase exhibited a very broad pH activity profile with the optimum pH 5.5 to 8 and more than 70% of the maximum activity was detectable at pH 4.5 and 8.5. To examine the pH stability of the amylase, the enzyme samples incubated in various pH values for 30 min, were assayed to determine the remaining activity (Fig. 6). The results show that the amylase was stable in the pH range of 4.5 to 8 losing only 12% of its activity at pH 4. Bacterial α-amylases have very different pH profiles some of which have sharp pH profiles with rapid decline of the activity occurring at the higher and lower pH ranges [27,28]. However, in several α-amylases, activity exists in a very broad pH range. For the sake of a comparison, the optimum pH, pH stability, and the profile of some *Bacillus* sp. α-amylases are listed in Table 2.

Influence of Temperature on α-Amylase Activity and Stability

The effect of temperature on the α-amylase activity is shown in Fig. 7a. The enzyme showed the optimum activity at 57 ºC losing its activity sharply at 70 ºC (Fig. 7a). The irreversible thermoinactivation of the enzyme was carried out at 60 ºC by incubating the enzyme samples in the appropriate buffer at 60 ºC. The residual activities were measured in 0, 5, 10, 15, 20, 25 and 30 min after incubation. After 30 min, the activity of enzyme decreased to 48.6% of its original one (Fig. 7b).

The optimum temperature for an amylase activity is usually related to the growth temperature of the microorganism. However, Vieille *et al*. showed that extracellular enzymes were optimally active at temperatures above and beyond the host organism's optimum growth temperature [33]. The *Bacillus* sp. GHA1 has optimum growth temperature around 42 ºC while the purified amylase from this bacterium is optimally active at 57 ºC, which is comparable with that described for *B. amyloliquefaciens* α-amylase [25].

Effects of Calcium Ions and EDTA on the α-Amylase Activity and Stability

To asses the effects of Ca^{2+} and EDTA on the activity of

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Table 2. *Bacillus* α-Amylases Having Different Optimal pH and Stability

Bacillus sp.	pH range	Residual activity (%)[a]	pH optimum	Ref.
GHA1	5.5-8.0	88 (pH 4 for 30 min)	5.5-8.0	This work
WN11	5.0-8.0	80 stable	5.5	[29]
JS-2004	6.5-9.0	94 (pH 8.0 for 24 h)	8.0	[30]
Moderately thermophilic	5.0-7.5	–	6.5	[31]
KR-8104	3.5-7.0	80 (pH 3.5 for 1 h)	4.0-6.0	[5]
KSM-K38	5.5-10.5	80 (pH 11 for 30 min)	8.0-9.5	[26]
ANT-6	10.5-13.0	55 (pH 10 for 15 h)	10.5	[32]

[a]Residual activity (%) refers to the percentage of remaining activity of the enzyme after incubation at the defined pH in the appropriate buffer for a given period of time under the standard assay conditions.

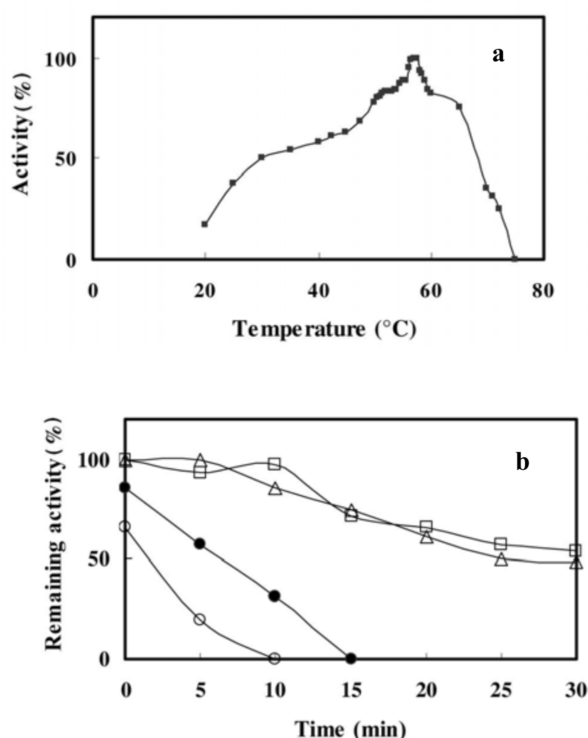

Fig. 7. (a) Effect of temperature on the activity of *Bacillus* sp. GHA1 α-amylase. (b) Thermal stability of α-amylase at 60 ºC in the absence of Ca^{2+} and EDTA (Δ), in the presence of 10 mM Ca^{2+} (□), in the presence of 5 mM EDTA (●) and in the presence of 10 mM EDTA after dialyzing against 10 mM Tris-HCl, pH 7.4 containing 10 mM EDTA (○).

Bacillus sp. GHA1 α-amylase, the purified enzyme was incubated with Ca^{2+} and EDTA at concentrations of 5 mM and 10 mM, respectively. The addition of Ca^{2+} stimulated the activity by 3% at 5 mM and 7% at 10 mM whereas EDTA at 5 mM and 10 mM inhibited the enzyme activity by 14% and 34%, respectively.

In order to study the effect of calcium and EDTA on thermostability of the purified enzyme, the experiments were carried out in four conditions: in the absence of Ca^{2+} and EDTA, in the presence of 10 mM Ca^{2+}, in the presence of 5 mM EDTA, and after dialysis against 10 mM EDTA. The thermal stability of the enzyme increased (~6%) by the addition of Ca^{2+} after 30 min at 60 ºC (Fig. 7b). These observations suggest that the activity and thermal stability of the enzyme is not influenced considerably upon addition of Ca^{2+}. However, EDTA at 5 mM and 10 mM, as shown in Fig. 7b, fully inhibited the enzyme after 15 and 10 min, respectively.

CONCLUSIONS

In this study, we reported the purification and partial characterization of a new *Bacillus* sp. α-amylase. The purification process is a simple, low-costing and straightforward one since it only involves ammonium sulfate precipitation of the culture extract followed by DEAE- and phenyl-Sepharose chromatography. *Bacillus* sp. GHA1 α-amylase is a metalloenzyme (three bound metal ions) which is inhibited by EDTA and its activity and thermostability show

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little dependency on the added Ca^{2+} ions. The use of enzyme (with low dependence on calcium ions) in starch hydrolysis eliminates the use of calcium in starch liquefaction as well as the need for subsequent removal of Ca^{2+} by ion exchange chromatography [7]. This α-amylase is active and stable in a relatively broad range of pH which eliminates the requirement for pH adjustment in its industrial applications. This again reduces the cost of ion exchange media and chemicals required for downstream processing [34], and thus increasing the cost-efficiency of starch processing. Since the GHA1 α-amylase has a broad working pH range (5.5-8) and a relatively moderate thermostability, it may be a suitable candidate to use in detergent industries. On the other hand, the activity and thermostability of this enzyme show little dependency on calcium ions whose presence causes some difficulties in many industrial processes [35]. Further characterization and enzymatic analysis of this enzyme is a task which warrants investigation. *Bacillus* sp. GHA1 α-amylase with the aforementioned considerable characteristics may find some biotechnological applications in starch and detergent industries.

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