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A Ca-independent α-amylase that is active and stable at low pH from the *Bacillus* sp. KR-8104

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Abstract

Bacillus sp. KR-8104 was selected from a set of 18 bacteria strains isolated from soil samples and screened for production of amylase. The maximum productivity obtained at pH 5–6 and 60–65 h after cultivation in production medium. New extracellular Ca-independent α-amylase was highly purified using ion exchange and hydrophobic interaction chromatography, which showed a single band with an apparent molecular weight of 59 kDa by SDS-PAGE. This enzyme is active in a wide pH range with its maximum activity at low pH values (4.0–6.0) and has the 90% of its maximum activity at pH 3.5. The α-amylase is optimally active at 75–80 °C. The presence or absence of Ca^{2+} and EDTA did not affect enzyme activity and thermal stability.

Keywords: *Bacillus* sp.; Ca-independent α-amylase; Alkaline protease; Broad range of pH; pH profile; Industrial applications

1. Introduction

α-Amylases are among the most important industrial enzymes [1,2] which can be obtained from several sources [3]. However, they are usually produced by bacteria belonging to the genus *Bacillus* for industrial applications such as *B. licheniformis*, *B. amyloliquefaciens*, *B. stearothemophilus*, and *B. subtilis* [4]. α-Amylases have potential application in wide number of industrial processes such as food, fermentation, textile, paper, detergent, and pharmaceutical industries [4–6]. The properties of each α-amylase such as thermostability, pH profile, pH stability, and Ca-independency must be matched to its application. For example, α-amylases used in starch industry must be active and stable at low pH but in detergent industry at high pH values [7,8]. The diversity in the applications and properties encourage us to take special attempts to search for new α-amylases.

Most of the bacterial α-amylases have pH optima at around 6.5 and generally stabilized by Ca^{2+} ions and destabilized and/or inhibited by chelating agents such as EDTA [9]. In the present study, we report purification, and partial characterization of a Ca-independent α-amylase produced by *Bacillus* sp. KR-8104 isolated from the soil. This enzyme has a broad range of pH activity with the maximum activity at pH 4.0–6.0 that makes it very attractive for further investigations and studies.

2. Materials and methods

2.1. Chemical

α-Amylase from *Bacillus amyloliquefaciens* (BAA), 3,5-dinitrosalicylic acid (DNS), and Tris were purchased from Sigma (St. Louis, MO, USA). DEAE-Sepharose and phenyl-Sepharose were provided by Pharmacia (Uppsala, Sweden). *ρ*-Nitrophenyl α-D-moltoheptaoside-4-6-*O*-ethylidene (blocked EPS) and α-glucosidase were obtained from Boehringer Mannheim (Mannheim, Germany) and all other chemicals were from Merck (Darmstadt, Germany) and were reagent grade.

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2.2. Isolation and identification of microorganisms

Soil samples of rhizospher and rhizoplane zone of potato were collected from Karaj and Hamadan in Iran. Twenty grams of an air-dried soil samples were added to 100 ml sterile water in an Erlenmeyer flask. The container was heated in a water bath for 10 min at 80 °C while the content was agitated. Subsequently, 1 ml of this suspension was added to 9 ml of sterile distilled water and a serial dilution (10^{-1} to 10^{-9}) was prepared. About 1 ml of each dilution was added and distributed on an isolation medium containing: potato starch 10 g, meat extract 5 g, and 1000 ml of distilled water. Plates were incubated at 37 °C for 24–48 h and different types of colonies were chosen and purified. Starch hydrolysis of the isolated strains was checked on nutrient agar supplemented with 10 g/l soluble starch. Hydrolysis was examined by flooding plates with lugol's iodine solution and recorded when clear zone appeared around the margin of growth. The promising strains were further examined for morphological, physiological and biochemical characteristics with reference to Bergey's manual of systematic bacteriology [10] and color atlas of *Bacillus* sp. [11].

2.3. Production of α-amylase

Prior to the cultivation of isolated strains on production medium, a loopful of each strain was cultivated on preculture medium containing (g/l): nutrient broth 8; meat extract 10; soyameal peptone 10; potato starch 10; and NaCl 0.5. Incubation was carried out at 37 °C, in an orbital incubator, with stirring at 160 rpm for 18 h. Subsequently, they were transferred to the production medium aseptically at 5% of the production medium containing (g/l): potato starch 10; soyameal peptone 4; meat extract 3; $CaCl_2{\cdot}H_2O$ 0.5; $MgSO_4{\cdot}7H_2O$ 0.3; and K_2HPO_4 1, and incubated at the same condition as preculture medium. The production of the amylase was examined in various times of cultivation to determine the optimum time of the enzyme production. The effect of various pH values (3.0–9.0) on the amylase production was also studied.

2.4. Determination of enzyme activity and protein concentration

α-Amylase activity was determined at room temperature in a 1.0 ml reaction mixture that contained 0.5 ml of a 1.0% (w/v) potato starch solution in 20 mM Tris–HCl, pH 7.4. The concentration of reducing sugars obtained from the catalyzed reaction for 3 min was measured by the dinitrosalicylic acid method according to Bernfeld [12]. 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. α-Amylase was also assayed using *ρ*-nitrophenyl α-D-moltoheptaoside-4-6-*O*-ethylidene as the absolutely specific substrate for α-amylase and the excess of α-glucosidase as a coupling enzyme in the same conditions [13,14]. The released *ρ*-nitrophenol was recorded by monitoring the absorption at 405 nm. Protein concentration was determined by the Bradford method [15].

2.5. Purification procedures of α-amylase

After 48 h of cultivation in production medium, the culture medium was centrifuged at 3000 × *g* for 10 min at 4 °C, and the supernatant was collected and adjusted to 1 mM PMSF (phenylmethylsulfonyl fluoride). Ammonium sulfate was added to the crude culture supernatant to 85% saturation at 4 °C for 2 h. The precipitates were centrifuged at 10,000 × *g* for 15 min at 4 °C and the pellets were dissolved in minimum volume of 20 mM Tris buffer, pH 7.4. The concentrated enzyme was dialyzed against the same buffer overnight at 4 °C. This solution was applied to DEAE-Sepharose column (10 mm × 100 mm) at a flow rate of 1 ml/min, previously equilibrated with 20 mM Tris, pH 7.4. Proteins were eluted with a linear gradient of 0–0.5 M NaCl in the same buffer and flow rate. The active fractions were combined and applied to the next step of the purification onto HiPrep^{TM}16/10 phenyl FF (high sub) phenyl-Sepharose column (Amersham biosciences, Uppsala, Sweden), previously equilibrated with 20 mM Tris, pH 7.4 containing 0.4 M ammonium sulfate. The enzyme solution was also adjusted to 0.4 M ammonium sulfate before it was applied. The column was washed with a linear gradient of 0.4–0 M ammonium sulfate in the same buffer at flow rate of 1 ml/min. The active fractions were pooled and concentrated by ultrafiltration (Amicon, Beverly, MA, USA). During these experiments we used an AKTA FPLC system (Amersham biosciences, Uppsala, Sweden) with both DEAE- and phenyl-Sepharose columns.

2.6. Polyacrylamide gel electrophoresis

SDS-PAGE was carried out using a 12% polyacrylamide gel by the method of Laemmli [16], and protein bands were detected by coomassie brilliant blue R250.

2.7. Activity and stability studies on purified α-amylase

The pH profile and pH optima were determined at room temperature in various pH of 25 mM phosphate-glycine buffer. The pH stability was studied for the purified α-amylase by incubation of the enzyme in 25 mM phosphate-glycine buffer, pH 3.5 for a series of time intervals at room temperature, and then reached to pH 7.0 using 50 mM phosphate-glycine buffer and finally the residual activity was determined under assay conditions.

The activity of purified α-amylase was determined at several temperatures (from 20 to 80 °C) in 20 mM Tris, pH 7.4. To determine the thermal stability, the purified α-amylase was incubated at 70 °C 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, and finally the residual activity was determined under assay conditions. The purified enzyme was also dialyzed twice against 20 mM Tris, pH 7.4 contain-

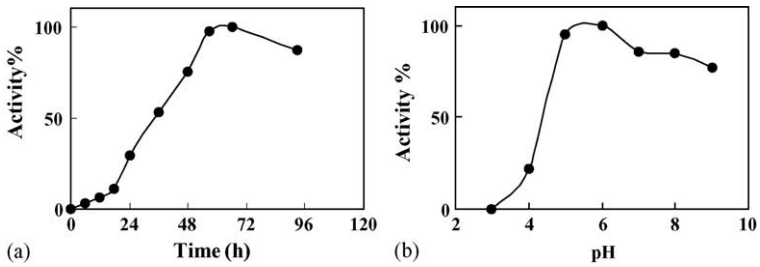

Fig. 1. Effect of time (a) and pH (b) on amylase production.

ing 10 mM EDTA and then aforementioned experiment was carried out at this buffer.

3. Results and discussions

3.1. Identification of bacteria

All strains were spore forming, Gram positive, rod shaped and identified as *Bacillus* species. They were screened for the α-amylase activity at various pH and temperatures. From a large collection, six strains from Karaj including KR-8101–8106 and 12 strains from Hamadan including HS-01-12 were selected for the further experimental works. Finally the *Bacillus* sp. KR-8104 strain was selected as the best potent producer of α-amylase. It was catalase and oxidase positive. This strain was able to use sodium citrate and sodium propionate. Furthermore, it was also able to grow on 7% NaCl and its lecitinase activity test and indol productions were negative. It was facultative anaerobic and anaerobically broke down L(+) arginine. It was also able to grow at 45, 50 and 55 °C and acid compounds were produced from glucose, mannitol, xylose and arabinose. These combinations of morphological, physiological, and biochemical data suggest that the KR-8104 strain is a *Bacillus* species.

3.2. α-Amylase production and purification

Total amylase production by *Bacillus* sp. KR-8104 started from about 20 h after inoculating of the production medium. The maximum production was obtained at 60–65 h and pH 5.0–6.0 (Fig. 1). After the concentration of crude culture medium, Anion-exchange chromatography on DEAE-Sepharose was used. The fractions of the largest peak from the elution profile (Fig. 2a) that had amylase activity were pooled, adjusted to 0.4 M ammonium sulphate, and applied to phenyl-Sepharose column. After the elution, the second peak that eluted immediately at the end of the ammonium sulphate

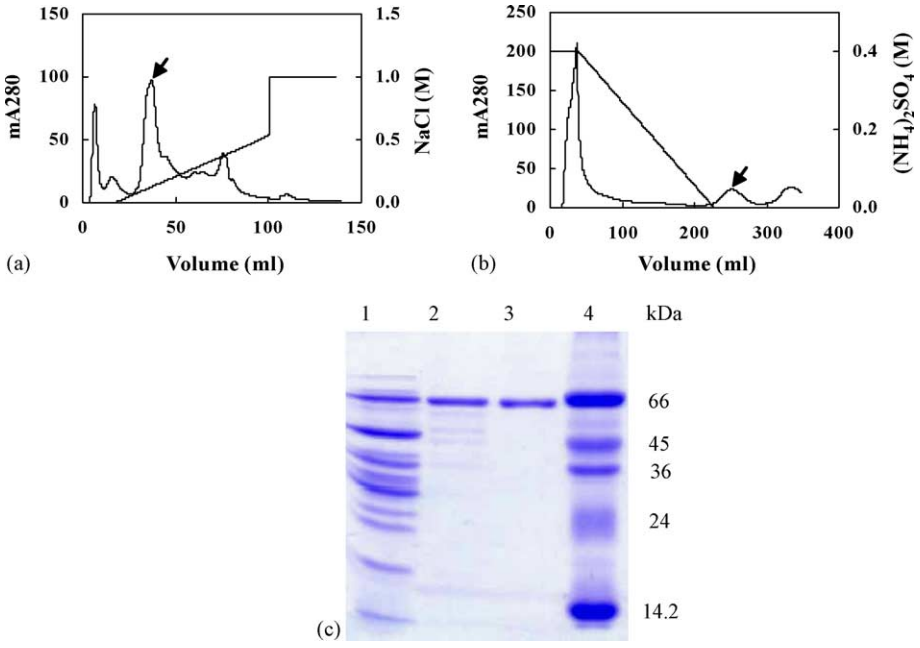

Fig. 2. Elution profiles of strain KR-8104 α-amylase on DEAE-Sepharose (a) and phenyl-Sepharose (b) column as described under Section 2. The active peaks are indicated. (c) SDS-PAGE illustrates different steps of α-amylase purification. Lane 1 (30 μg), crude enzyme after precipitated with $(NH_4)_2SO_4$; Lane 2 (2.5 μg), after ion exchange chromatography using DEAE-Sepharose column; Lane 3 (1 μg), after hydrophobic interaction chromatography using phenyl-Sepharose column (purified enzyme); and Lane 4, molecular size markers.

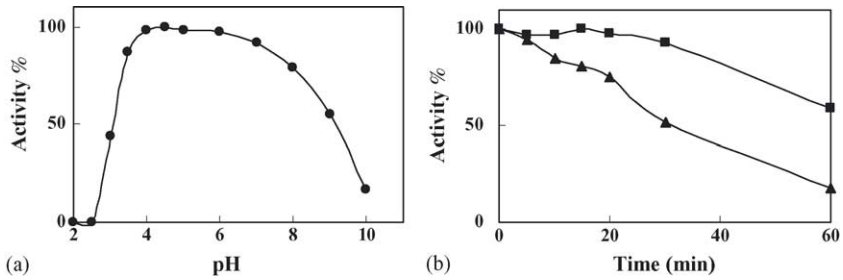

Fig. 3. (a) Effect of pH on activity of *Bacillus* sp. KR-8104 α-amylase, (b) pH stability at pH 3.5 for α-amylase from *Bacillus* sp. KR-8104 (■) and BAA (▲).

gradient showed α-amylase activity (Fig. 2b) and thus there is no need to desalting extra step by the dialysis or the gel filtration chromatography. The fraction corresponding to the active peak that combined and concentrated by the ultrafiltration showed homogeneity and appeared as a single band on SDS-PAGE (Fig. 2c). The specific activity was improved from 11 U/mg in the concentrated crude culture enzyme to 330 U/mg in purified enzyme (30-fold). The apparent molecular weight of the purified enzyme was also estimated to be 59 kDa by SDS-PAGE (Fig. 2c).

Since the blocking group prevents hydrolysis of the blocked EPS substrate by the exo-acting enzymes such as β-amylase, α-glucosidase, and amyloglucosidase [14] and purified α-amylase from *Bacillus* sp. KR-8104 is able to act on this substrate, therefore, we can suggest that this is an α-amylase.

3.3. Effect of pH on α-amylase activity and stability

The influence of pH on the enzyme activity (pH profile) is shown in Fig. 3a. The pH profile of this enzyme shows a very broad pH range of the activity at room temperature, so that it has the 90% of its maximum activity in the range of pH 3.5–7.0 with its optimum pHs at pH 4.0–6.0. The reported α-amylases produced by several bacterial sources, including *Bacillus* sp., has a variety of pH profiles. The maximum activity of the most of these earlier reported enzymes is in the range of pH 6.0–8.0 [17–19] or pH 5.0–7.0 [20–22]. In spite of earlier reports on the activity at low pH for some α-amylases, such as those produced by *Bacillus subtilis* X-23 [23] and *Lactobacillus manihotivorans* LMG 18010^T [24], there are few reports on α-amylases which have the maximum activity at pH lower than 4.0. For example, the α-amylase produced by *Alicyclobacillus acidocaldarius* has the maximum activity at pH 3.0 [25] and one by *Pyrococcus furiosus* has about 70% of its maximum activity at pH 3.5 without a wide pH profile [26].

On the other hand, the pH stability of this enzyme was measured at pH 3.5, as shown in Fig. 3b. These results reveal that our enzyme retained its full activity after 30 min and 80% of its original activity after 1 h at pH 3.5, while the residual activity of BAA at the same condition is nearly 25% after 60 min (Fig. 3b).

The broad range of pH activity profile and its optimum activity at lower pH values make this enzyme highly attractive for both the basic research studies and the industrial processes. Currently, there are several industrial applications which take place at low pH and consequently there is a great deal of interest to improve low pH activity for α-amylases. Achieving this goal is very difficult task at the present time [27].

The maximum activity of the enzyme at low pH values with a good stability at pH 3.5 is very important from the application point of view. Majority of the α-amylases are unstable at low pH [28] and on the other hand the liquefaction step in the starch process is currently constrained to operate at pH 5.8–6.2, which is around the optimum pH of the α-amylase in use [8]. Since both the prior and post process steps take place at pH 4.5, therefore, if the α-amylase is stable and active at low pH values one can omit the pH adjustment steps,

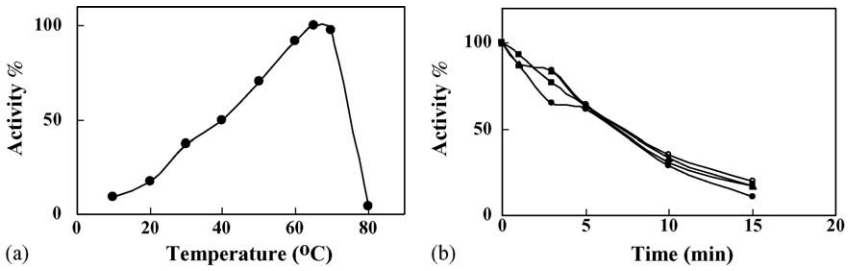

Fig. 4. (a) Effect of temperature on activity of *Bacillus* sp. KR-8104 α-amylase, (b) thermal stability of this α-amylase at 70 °C in the absence of 10 mM Ca^{2+} and 5 mM EDTA (■), in the presence of 10 mM Ca^{2+} (●), in the presence of 5 mM EDTA (▲), and in the presence of 10 mM EDTA after dialyzing against 10 mM Tris, pH 7.4 containing 10 mM EDTA (◯).

which is very important in the processing. Consequently, the need for low pH α-amylase is very clear.

3.4. Effect of temperature and Ca^{2+} on α-amylase activity and stability

The influence of temperature on the enzyme activity is shown in Fig. 4a. The optimum activity temperature is between 70 and 75 °C and the enzyme activity will decline sharply at 80 °C. The temperature activity profile of this enzyme is similar to that of BAA.

The irreversible thermoinactivation of the enzyme at 70 °C was recorded in four conditions: the absence of Ca^{2+} and EDTA, the presence of 10 mM Ca^{2+}, the presence of 5 mM EDTA, and after dialysis against 10 mM EDTA. The thermal stability of this enzyme is not influenced by the addition of Ca^{2+} and EDTA as shown in Fig. 4b. EDTA and chloride salts of Ca^{2+}, Mg^{2+}, K^+, and Na^+ at 5 mM have no effect on this α-amylase activity (data not shown). Thus, *Bacillus* sp. KR-8104 α-amylase does not require Ca^{2+} ions for its activity and thermostability and both of them are Ca-independent. Ca^{2+} ion generally alters activity and/or thermal stability in the α-amylases as mentioned in earlier reports [29,30] and thermal stability is usually increased in the presence of Ca^{2+} ion [17,31]. There are few reports for α-amylases of non-*Bacillus* sp. and there is none for α-amylases obtained from *Bacillus* sp. with Ca-independency properties of both the thermal stability and the enzyme activity [32]. Today, $CaCl_2$ is added to stabilize the used α-amylase in liquefaction step of starch process. This added Ca^{2+} ions should be removed by using ion exchange process prior to the isomerization of glucose to fructose due to the inhibitory effect of Ca^{2+} ions on the glucose isomerase [8]. However, for the Ca-independent α-amylase, the addition/removal of $CaCl_2$ steps could be eliminated, which is a great advantage. On the other hand we should mention that the relative low thermostability of our enzyme provides a handicap for this potential application, which should be improved in future.

4. Conclusion

To conclude our discussion we have to add that *Bacillus* sp. KR-8104 produces α-amylase, which is active and stable at low pH. Furthermore, it is active at a wide pH range. The α-amylase of *Bacillus* sp. KR-8104 is Ca-independent (both its activity and thermal stability) too. These two features of the α-amylases are very important in starch industry, which is lacking in the majority of reported α-amylases up to now. Since the α-amylase of this strain has a broad pH range of activity, moderate thermostability, and appropriate temperature profile, therefore, it can be suitable candidate to be used as an additive for detergent industries. Due to the importance of our recent finding, further enzymatic studies, structural analysis, and determination of encoded gene sequence of this α-amylase can be carried out in order to understand the mechanism of its activity at low pH and its Ca-independent features. This has been the focus of many research groups in this field in last 5 years.

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