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Purification and Characterization of a Novel Thermostable and Acid Stable α-Amylase from *Bacillus Sp.* Iranian S1

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ABSTRACT

This study describes the purification and biochemical characterization of a novel thermostable and acidic pH stable α -amylase from *Bacillus sp.*Iranian S1 which isolated from the desert soil (Gandom-e-Beryan in Lut desert, Iran). Maximum enzyme production was in exponential phase with activity 2.93 U ml^{-1} at 50°C and pH 5. The enzyme was purified by isopropanol sedimentation, ion-exchange chromatography on DEAE cellulose DE-52 and gel filtration on Sephadex G-100. The purification procedure performed for α-amylase enzyme achieved about 98.9-fold purification with 25% yield and specific activity 375.9 U mg^{-1} of protein. The molecular weight of purified α-amylase was estimated around 70 KDa by SDS-PAGE and it was stable in a wide range of temperature and pH between 30°C-110°C and 3-9, respectively. Assayed with soluble starch as substrate, the enzyme displayed optimal activity at 90°C and pH 5. The purified α-amylase was acid- and thermo-stable with novel properties making it suitable for many industrial purposes.

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1. INTRODUCTION

α-Amylase (EC3.2.1, 1,4-α-D-glucan glucanohydrolase and endoamylase) hydrolyzes the starch by randomly cleaving internal α-1,4-glucosidic linkages and produces reducing sugar. Amylases have potential application in different industrial processes such as textile, paper, food and detergent industries [1-4].

Liquefaction of the starch at high temperature using thermostable amylases has certain benefits over the other ways of thinning starch. The advantages to use thermostable enzymes in biocatalysis are their high stability, improved transfer rates, lower viscosity and a reduced risk of contamination during the processes [5]. Microbial thermostabile α-amylases play an important role in food, chemical, and pharmaceutical industries [3, 4, 6]. Among the bacterial sources, thermophilc bacilli were potentially exploited for thermostable α-amylase production to fulfill industrial requirements [2, 3, 5, 7]. Thermostability, acidic pH and long-term stability are desired characteristics for amylase to fit potentially in starch-processing industry [1, 2, 6-8]. There are a few reports about commercially available thermostable α-amylases capable of working at pH 4.5-5.5 [6, 8].

Therefore isolation and characterization of new thermophilic bacilli able to produce amylases with high stability at extreme conditions is still required [1, 2, 4, 6-9]. Microorganisms which are adapted to grow optimally at high temperatures have been isolated from high temperature terrestrial and marine habitats. The most common biotopes are hot springs and deserts [5, 6, 8-13]. In this research the purification and biochemical characterization of starch-digesting thermostable α-amylase from *Bacillus sp.* Iranian S1 isolated from the desert soil (Gandom-e-Beryan in Lut desert, Iran) have been reported.

2. MATERIALS AND METHODS

2. 1. Isolation and α-Amylase Production

Bacillus sp. Iranian S1 was isolated from geothermal soil of Gandom-e-Beryan in Lut desert of Iran and defined for extracellular amylase production [11]. The isolate was screened for amylase production on starch-agar plates containing (g l^{-1}) soluble starch, 10.0; peptone, 2.0; yeast extract, 5.0; NaCl, 1.5;

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$MgSO_4.7H_2O$, 0.5; KH_2PO_4, 0.5; $CaCl_2$, 0.1; agar, 20.0; pH 7.0. After 16 hour growth at 50°C, the plates were flooded with iodine solution to check the zone of clearance.

For evaluating the growth and amylase production the same liquid medium was inoculated with a cell suspension of optical density 0.5 and incubated at 50°C in rotary shaker at 160 rpm. The optical density of culture broth and amylase production was monitored at different intervals of time. The growths were followed by spectroscopic measurements of the optical density of the cultures at 550 nm. Samples were centrifuged at 8000×g for 10 min and cell free supernatant was applied as enzyme source.

The effect of temperature and pH on enzyme production, using the same liquid medium as described above, was studied at 25-110°C and 3.5–9.5, respectively.

2. 2. Enzyme Assay α-Amylase activity was determined by estimating the reducing sugar from 1% (w/v) starch as substrate at 50°C and pH 5.0. The amount of released reducing sugars was determined by dinitrosalicylic acid method [14]. The D-glucose was used as a standard. One unit of amylase activity was defined as the amount of enzyme that released 1.0 µM of glucose per min under assay conditions. In all tests the enzyme activity was measured as the average of three autonomous sets of tests and the standard deviation in all cases was found in significant. Total protein content was estimated by the Bradford method using bovine serum albumin as the standard [15].

2. 3. Enzyme Purification The supernatant was gathered by centrifugation at 10,000×g for 10 min at 4°C. Two volumes of cold isopropanol were added smoothly to the cold supernatant fluid with steady stirring, preserving the temperature at 4-8°C for 40 min. The chemical sediment was gathered by centrifugation at 10,000×g for 10 min at 4°C, dissolved in minimal volume of 0.01 M citrate buffer (pH 5) and centrifuged again. The clear solution was dialyzed against the similar buffer for 18 h at 5-8°C. The dialysate was used to a column (1.6 x 45 cm) of CM-Cellulose and DEAE cellulose (DE52, Whatman) balanced with the dialysis buffer, pH 5 containing 0.02% NaN_3 as preservative [16]. After washing the column with 50 ml of the same buffer, elution was performed with a linear gradient of NaCl concentrations from 0.1 to 1.0 M in 0.01 M citrate phosphate buffer (pH 5). The volume of NaCl gradient was maintained 100 ml. The flow rate was regulated to 20 ml h^{-1}, 2.5 ml fractions were gathered and analyzed for protein and amylase activity. The active fractions were pooled, concentrated by isopropanol chemical sediment and dissolved in minimum quantity of similar buffer, pH 5 and used on Sephadex G-100 (Merck, Germany) column (1.6 × 50 cm) and fractioned as above.

2. 4. Enzyme Molecular Mass Determination The determination of molecular weight was done by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gel according to the method of Laemmli [17]. The molecular mass was determined by using a low molecular weight marker (ColorBrust Marker SIGMA.USA). After electrophoresis, protein bands were visualized by staining with coomassie brilliant blue G 250.

2. 5. Effec of pH and Temperature on α-Amylase Activity The temperature profile to find maximal activity was determined by incubating the enzyme for 30 min in the temperature range of 30-110°C. After incubation the enzyme was kept at 4°C and the activity was measured by assaying the reducing sugar at different temperatures. The effect of pH was determined at 90°C in the pH ranges of 3.0–9.0 with appropriate buffers. The enzyme was incubated with these buffers at 90°C for 30 min without substrate, and then activity was determined on soluble starch by measuring the reducing sugars produced [14]. All the experiments were performed in triplicates.

3. RESULTS AND DISCUSSION

Bacillus sp. Iranian S1 wild strain producing thermostable α-amylase was isolated from desert soil (Gandom-e-Beryan in Lut desert, Iran) and the enzyme was partially characterized.

Bacillus sp. Iranian S1 was found to be potent amylase producer which showed the zone of clearance on starch plate when flooded with iodine solution (see Figure 1).

Amylase production was determined to be growth associated. It was shown earlier that the amylase production could be in parallel with growth for many bacilli strains [18].

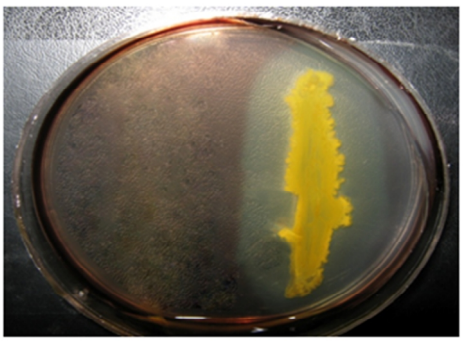

Figure 1. *Bacillus sp.* Iranian S1 showing the zone of clearance with iodine solution on starch plates

The isolate was able to grow and produce amylase in the temperature range from 15 to 80 °C and pH 3.5 to 9.5. The maximal production of amylase was observed in exponential phase with activity 2.93 U ml^{-1} at its optimal growth temperature 50°C and pH 5 (see Figure 2).

Most of *Bacillus* strains used for the commercial production of α-amylase has an optimum pH between 6.0 and 9.0 for growth and enzyme production, respectively [4, 6, 8]. *Bacillus sp.* Iranian S1 showed optimum growth and enzyme production at 50°C temperature and pH 5. This suggests that strain *Bacillus sp.* Iranian S1 could be called moderate thermophilic and typically acidophilic.

The crude enzyme sample obtained by isopropanol sedimentation and further dialysis was purified by ion-exchange chromatography on DEAE cellulose DE-52 and gel chromatography on Sephadex G-100. The dialyzed fraction calculated for a total activity of 1520 U and a specific activity of 157.7 U mg^{-1}. The elution pattern showed a major peak of amylase activity (see Figure 3). This fraction showed a total activity of 696 U and specific activity of 289.33 U mg^{-1}. The total purification increased to 88.41 fold.

The concentrated active fractions were purified by gel chromatography on Sephadex G-100. A single peak of amylase activity was obtained (see Figure 4). The purification procedure performed for α-amylase enzyme achieved about 99-fold purification with 25% yield. In this research, the enzyme provision obtained at the end of purification process had a certain activity of 375.9 U mg^{-1}. A summary of the purification is given in Table 1.

Molecular weights of α-amylases are usually between 50-60 kDa but differences in molecular weights ranging from 10 to 210 kDa are reported in the literature [7]. The molecular mass of the studied α-amylase was found to be 70 kDa on 10% SDS-PAGE (see Figure 5).

TABLE 1. Purification of the extracellular α-amylase of *Bacillus sp. Iranian S1*

Purification steps	Isopropanol chemical sediment	DEAE cellulose column	Sephadex G-100 column
Total activity (U)	1520	696	288
Total protein (Mg)	9.8	4.2	0.84
Specific activity (U mg^{-1})	157.77	89.33	375.9
Yield %	7	55	25
Purification flod	43.2	88.41	98.9

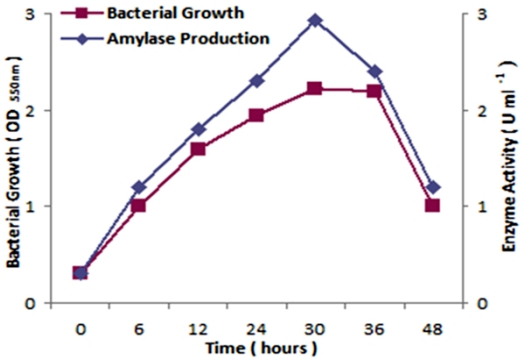

Figure 2. Growth of *Bacillus sp.* Iranian S1 and α-amylase production at 50°C and pH 5

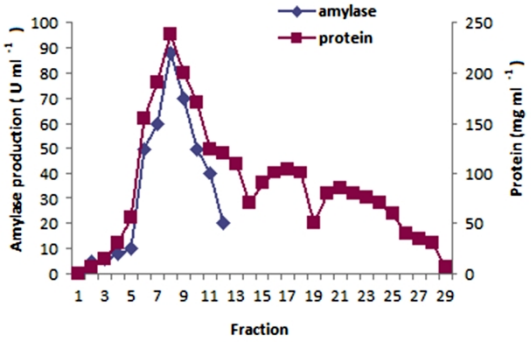

Figure 4. Elution profile of *Bacillus sp.* Iranian S1 α-amylase from Sephadex G-100 column

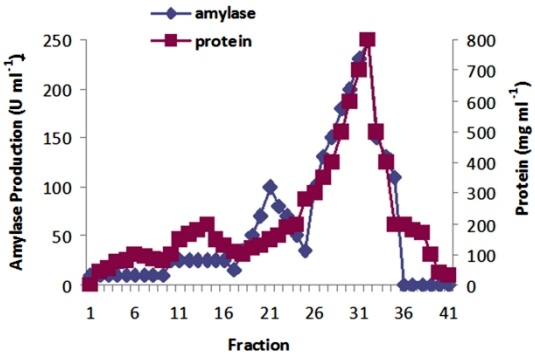

Figure 3. Elution profile of *Bacillus sp.* Iranian S1 α-amylase from DEAE cellulose column

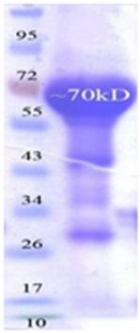

Figure 5. SDS-PAGE of the purified α-amylase: Left: molecular weight marker; Right: α-amylase purified by Sephadex G-100

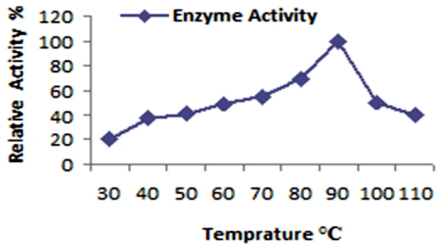

Figure 6. Effect of temperature on α-amylase activity at different ranges 30-110°C

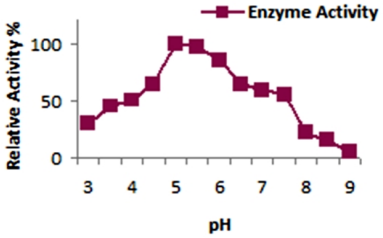

Figure 7. Effect of pH on α-amylase activity. The buffers used were glycine-HCl (3.0-4.0), acetate (5.0-6.0), phosphate (6.0-8.0) and glycine-NaOH (8-9)

The optimal temperature for α-amylase was 90°C, with 20 and 40% relative activities at 30 and 110°C, respectively (Figure 6). The amylase showed activity within a broad range of pH values from 3.0 to 9.0 with an optimum at pH 5.0, retaining 45% of the initial activity at pH 3.5 and 20% at pH 8 (Figure 7). Data of pH and temperature effect on α-amylase activity were shown in Figures 6 and 7.

Thermophilic and hyperthermophilic microbes serve as a source of novel thermostable enzymes. Many thermostable α-amylase microbial producers are known: among the bacterial sources species of *Bacillus* and related genera such as *B. amyloliquefaciens*, *B. licheniformis*, *B. stearothermophilus*, *Geobacillus sp. IIPTN* and *G. thermoleovorans subsp. stromboliensis* were potentially exploited for thermostable α-amylase production to fulfill industrial requirements [4, 8, 9].

Thermostable enzymes are optimally active at temperatures above the host organism's optimal growth temperature [6]. On the other hand, it has been reported that the optima pH for α-amylases varies from (2-12), [19]. It was reported that very few thermostable amylases obtained from bacilli can work up to 100°C temperature and at pH 5.0. Recently Dheeran et al. [8] isolated *Geobacillus sp. IIPTN* novel α-amylase which worked up to temperature of 120°C and at pH 5.5. It was a bacilli strain isolated from hot springs in Jordan that produced a novel, highly acid- and thermo-stable amylase with optimum pH 4.4 and 90°C temperature [19]. The α-amylase *Bacillus sp.* Iranian S1 can also efficiently work up to 110°C at pH 5.0. Ability to work at acidic pH conditions indicates that enzyme is slightly acid stable. The α-amylase reported in this research seems to be different from formerly reported enzymes in terms of adaptability for extreme temperature and pH conditions.

4. CONCLUSION

The *Bacillus sp.* Iranian S1 strain produced thermostable α-amylase. The optimal temperature and pH for the activity of the α-amylase was 90°C and pH 5.0, respectively. The enzyme remained stable in a range of temperature and pH between 30°C- 110°C and 3-9, respectively. Besides thermostability, the novelty of the enzyme is the resistance at slightly acidic pH conditions. Further optimization of enzyme production and purification conditions *Bacillus sp.* Iranian S1 α-amylase can be commercialized.

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چکیده

این مطالعه درباره تلخیص و تعیین خصوصیات بیوشیمیایی آنزیم آلفا – آمیلاز مقاوم به حرارت و pH اسیدی از *Bacillus.Sp.*Iranian S1 جدا شده از خاک بیابان (منطقه گندم بریان درکویر لوت، ایران) است. حداکثرفعالیت و تولید آنزیم در فاز نمایی معادل $U\ ml^{-1}$ ۲.۹۳ در دمای ۵۰ درجه سانتی گراد و pH ۵ بود . این آنزیم توسط رسوب ایزوپروپانول، کروماتوگرافی تبادل یونی در DEAE سلولز DE-52 و ژل فیلتراسیون در سفادکس G-100 خالص سازی شد. در روش انجام شده برای خالص سازی آنزیم آلفا-آمیلاز مقدار ۹۸.۹ برابر با ۲۵٪ عملکرد و فعالیت ویژه $U\ ml^{-1}$ ۳۷۵.۹ از پروتئین به دست آمد .وزن مولکولی آلفا–آمیلاز خالص توسط SDS-PAGE در حدود ۷۰ کیلو دالتون تخمین زده شد و آنزیم در طیف گسترده ای از دما و pH به ترتیب بین ۳۰ تا ۱۱۰°C و pH ۳ تا ۹ پایدار بود. سنجش با محلول نشاسته به عنوان سوبسترا، فعالیت بهینه آنزیم را در دمای ۹۰ درجه سانتی گراد و pH برابر ۵ به معرض نمایش گذاشت . این آلفا– آمیلاز خالص شده و مقاوم به حرارت و اسیدیته برای بسیاری از اهداف صنعتی مناسب است ..

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