

A Thermostable α -Amylase Producing Natural Variant of *Bacillus* spp. Isolated From Soil in Iran

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Abstract: Thermophilic processes appear more stable, rapid and less expensive and facilitate reactant activity and product recovery. Amylases have a quarter of the world enzyme market and thermostable α -amylases possess extensive commercial applications. Since little work has been done on strain isolation, growth and enzyme yield optimization, the level of thermophilic enzyme production remains relatively low. Therefore, large scale exploitation of thermophiles requires further intensive and integrated work. The present study describes isolation of an α -amylase producing *Bacillus* from soil. The isolated *Bacillus* was identified and named as *Bacillus licheniformis* Shahed-07. The strain was cultured in liquid media to produce α -amylase. The enzyme production conditions of the newly isolated *Bacillus* revealed that the maximum enzyme production after 26 h of cultivation at pH 7.0 and 50°C. 0.5% tryptophan in production medium enhanced the enzyme productivity to two fold whereas peptone and lysin at 0.5% level showed a strong repression. Crude α -amylase characterization revealed that optimum activity was at pH 7.5 and 70°C. The crude enzyme was stable for 24 h at pH range of 6-7 at 70°C. Enzyme activity increased with temperature within the range of 40-70°C. The *Bacillus licheniformis* Shahed-07 strain produced thermostable α -amylase with characteristics suitable for application in starch processing and food industries.

Key words: Thermostable α -amylase, *Bacillus licheniformis* shahed-07, media optimization, characterization, starch processing

INTRODUCTION

Starch, a main component of our daily diet, is frequently found not only in food residues on dishes but also in food stains on clothes. Amylases are among the most important enzymes and are of great significance in present-day biotechnology. Amylases having approximately 25% of the enzyme market have almost completely replaced chemical hydrolysis of starch in starch processing industry^[1]. Thermostable α -amylases have had extensive commercial applications in starch processing, brewing and sugar production^[2]. Thermophilic processes appear more stable, rapid and less expensive and facilitate reactant activity and product recovery. Bacteria belonging to the genus *Bacillus* have been widely used for the commercial production of thermostable α -amylases. The most important characteristic of thermophilic organisms is their ability to produce thermostable enzymes with a higher operational stability and a longer shelf-life^[3]. It is desirable that α -amylases should be active at the high

temperatures of gelatinization (100-110°C) and liquefaction (80-90°C) to economize processes; therefore, there has been a need and continual search for more thermophile and thermostable α -amylases^[4]. Thermophilic bacteria are common in soil and volcanic habitats and have a limited species composition. Yet they possess all the major nutritional categories and metabolize the same substrates as mesophilic bacteria. The ability to proliferate at growth temperature optima well above 60°C is associated with extremely thermally stable macromolecules. As a consequence of growth at high temperature and unique macromolecular properties, thermophilic bacteria can possess high metabolic rates, physically and chemically stable enzymes and lower growth but higher end product yields than similar mesophilic species. Thermophilic processes appear more stable, rapid and less expensive and facilitate reactant activity and product recovery. Bacteria belonging to the genus *Bacillus* have been widely used for the commercial production of thermostable α -amylases. The most important

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characteristic of thermophilic organisms is their ability to produce thermostable enzymes with a higher operational stability and a longer shelf-life^[3]. *Bacillus licheniformis* α -amylase (BLA) is among the most extensively thermostable natural enzymes used in starch technology and in the biotechnological processes. Although it is encoded by a nonthermophilic bacteria, it remains active for several h at temperatures over 90°C under conditions of industrial starch hydrolysis^[5]. Very few studies have been performed on strain selection, growth and enzyme yield optimization and the level of thermophilic enzyme production remains relatively low. Since little work has been done on strain selection, growth and enzyme yield optimization, the level of thermophilic enzyme production remains relatively low^[6]. Therefore, large scale exploitation of thermophiles requires further intensive and integrated work. In this study we have made an attempt to isolate and identify a thermostable amylase producing *Bacillus*. The enzyme production conditions of the newly isolated *Bacillus* are also reported here.

MATERIALS AND METHODS

Selection, isolation and identification of bacterial strain: *Bacillus licheniformis* Shahed-07, a moderate thermophilic bacterium was isolated in the Department of Microbiology, Shahed University, Tehran, Iran during the year 2007. Two samples from positive growth on nutrient agar medium I consisting of 2% peptone, 1% yeast extract, 1% NaCl and 2% agar at pH 7.0 were selected after 48 h of incubation at 55°C. The bacterial colonies appearing on plate I were transferred to medium II containing 1% soluble starch, 0.2% yeast extract, 0.5% peptone, 0.1% MgSO₄, 0.1% NaCl, 0.02% CaCl₂ and 2% agar at pH 7.0. These cultures were incubated at 55°C for 48 h. Typical cultural and morphological characteristics were observed for *Bacillus* species. Amylase producing colonies were selected by flooding the media II plates with iodine solution. The isolated *Bacillus* was identified by morphological and biochemical examination.

Enzyme production medium: The enzyme production was carried out at 55°C (except temperature optimization experiment) in a rotary shaker at 150 rpm for 144 h in 100 mL of the basal medium of the following composition: Soluble Starch (1%), Maltose (1%), (NH₄)₂SO₄ (0.2%), CaCl₂ (10⁻⁴M), K₂HPO₄ (10⁻¹M), MgCl₂, 6H₂O (0.02%), pH 7. At regular

intervals (2 h), the triplicate samples were harvested and the cells were separated by centrifugation (10,000×g 20 min) at 4°C in a refrigerated centrifuge. The supernatant was used for enzyme assay and characterization studies.

α -amylase assay: The activity of α -amylase was assayed by incubating 0.5 mL enzyme with 0.5 mL soluble starch (1%, w/v) prepared in 0.1 M sodium phosphate buffer (pH 7.0). After incubation at 70°C for 60 min the reaction was stopped by the addition of 2 ML of 3-5-dinitrosalicylic acid reagent^[7] and absorbance was measured in a UV/vis spectrophotometer (Biofuge). One unit (U) is defined as the amount of enzyme which releases 1 μ mol of reducing end groups min⁻¹ in 0.1 M sodium phosphate buffer (pH 7.0) with 0.5% (w/v) soluble starch as substrate at 55°C.

Optimization of medium and culture conditions: The basal medium containing 1% (w/v) soluble starch was supplemented with organic and inorganic nitrogen sources, each at a concentration of 0.5% (w/v). Nitrogen sources were tryptone, proteose peptone and yeast extract. The effect of varying pH values (5-10) and temperatures (30-100°C) on α -amylase production by the bacterium was also investigated. In all cases, 100 mL medium in 500 mL baffled flasks was inoculated with 2% (v/v) of an overnight culture. The culture was incubated under shaking in an incubator shaker at a shake rate of 150 rpm and under stationary conditions. Growth was determined spectrophotometrically by measuring optical density of the culture at 600 nm. The cell dry weight was determined by centrifugating 10 mL culture suspension taken at each time intervals at 10000 g and drying the cell mass overnight at 45°C.

Effect of pH on enzyme activity and stability: The pH optimum of the enzyme was determined by varying the pH of the assay reaction mixture using the following buffers (0.1 M): sodium acetate (pH 5.0-5.5), sodium phosphate (pH 6.0-7.0), Tris-HCl (pH 7.5-8) and glycine-NaOH buffer (pH 9-10). To determine the stability of α -amylase, the enzyme was pre-incubated in different buffers (pH 5-10) for 24 h. The residual enzyme activity was determined.

Effect of temperature on enzyme activity and stability: The temperature optimum of the enzyme was evaluated by measuring the α -amylase activity at different temperatures (30-100°C) in 0.1 M sodium

phosphate buffer (pH 7.5). The effect of temperature on amylase stability was determined by measuring the residual activity after 24 h of pre-incubation in 0.1 M sodium phosphate buffer (pH 7.5), at temperatures ranging from 30-100°C.

RESULTS AND DISCUSSION

The isolated *Bacillus* strain was characterized by various parameters and it was confirmed to be *Bacillus licheniformis* (Table 1). The *Bacillus* strain was named as *Bacillus licheniformis* Shahed-07. The results on the time-course studies on α -amylase production and cell growth of *Bacillus licheniformis* Shahed-07 grown in basal medium supplemented with 1% soluble starch as inducer substrate in both shake flasks and stationary condition are shown in Fig. 1 and 2 respectively. It was observed that maximum α -amylase production by *B. licheniformis* Shahed-07 occurred when cell population reached the peak in shake flasks (Fig. 1). In the stationary cultures the peak enzyme activity was noted at the stationary phase of growth (Fig. 2). The pH and the optimum temperature for growth and enzyme production were 7.0 and 50°C respectively (Fig. 3, 4). There was a stimulation of enzyme synthesis with an increase in pH from 5-7 and higher enzyme synthesis at pH 7.0 is a result of enhanced bacterial growth. The pH change observed during the growth of the organism also affects product stability in the medium. Enzyme synthesis occurred at temperatures between 30 and 80°C. The bacterium could grow satisfactorily at all temperatures tested but the maximal α -amylase activity in the growth medium was achieved at 50°C (Fig. 4). A reduction in enzyme activity was observed at temperatures above 55°C. α -amylase production peaked at 26 h and was found to decline gradually after 30 h. The influence of various carbon and nitrogen sources on α -amylase production was quantified in batch fermentation in shake flasks. Starch at 2% level had positive impact on the enzyme productivity. Among defined carbohydrates, tested starch and maltose supported good growth and amylase production, with the highest productivity recorded in the presence of starch. The productivity remained constant up to 8% starch level after which it gradually declined (Fig. 5). Tryptophan was found to enhance the enzyme productivity to 202% as compared to the basal medium whereas peptone and lysin at 0.5% level showed a strong repression (Fig. 6).

Most of the *Bacillus* strains used commercially for the production of α -amylases have an optimum pH between 6.0 and 9.0 for growth and enzyme production^[4]. Neutral pH was found to be optimal for

Table 1: Characterization of *B. licheniformis* Shahed-07 and comparison with the characteristics of standard *B. licheniformis*

Tartrate characteristics	<i>B. licheniformis</i>	<i>B. licheniformis</i> Shahed-07
Spores round	-	-
Parasporal crystals	-	-
Catalase	+	+
Anaerobic growth	+	+
Voges-Proskauer test	+	+
pH in V-P broth	+	+
<6		
>7	-	+
Acid from		
D-Glucose	+	+
L-Arabinose	+	+
D-Mannitol	+	+
Gas from glucose	(-) ^P	-
Hydrolysis of		
Casein	+	+
Gelatin	+	+
Starch	+	+
Utilization of citrate	+	-
Degradation of tyrosine	-	+
Deamination of phenylalanine	-	-
Egg-yolk lecithinase	-	-
Nitrate reduced to nitrite	+	+
Formation of		
Indole	-	-
Dihydroxyactone	ND	-
NaCl and KCl required	-	-
Growth at pH		
6.8 nutrient broth	+	+
5.7	+	+
Growth at NaCl		
2%	+	+
5%	+	+
7%	+	+
10%	ND	-
Growth at		
5°C	-	-
10°C	+	-
30°C	+	+
40°C	+	+
50°C	+	+
55°C	+	+
65°C	+	-
Oxidase	d	+
Arginine dihydrolase	+	+
Lysine decarboxylase	-	+
Lipase (olive oil)	d	+
Hydrolysis of		
Tween 80		+
Urea	-	+
Gas from nitrate	d	-
Growth at pH 5		+
Growth factors required	-	-
Reduction of methylene blue		
Acid from		
Fructose		+
Galactose		+
Glycerol		+
Lactose		-
Maltose		+
Sucrose		+

Table 1: Continued

Formation of poly- β -hydroxybutyrate or other storage products	+
Utilization of	
Acetate	+
Glycerol	+
Glycine	+
Mannitol	+

-: Ninety percent or more of strains are negative; +: Ninety percent or more of strains are positive; (-)^P: Few gas bubbles may be formed; ND: No data available; NG: No growth; D: Eleven to eighty nine percent of strains are positive

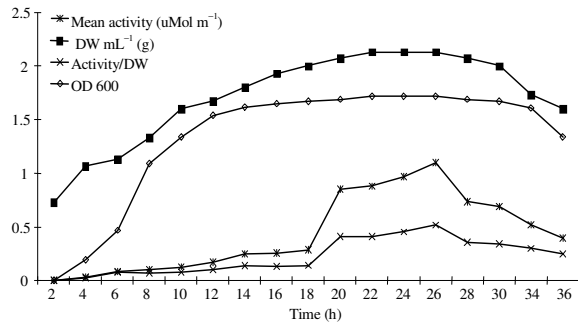


Fig. 1: Microbial growth and α -amylase production in shake flasks

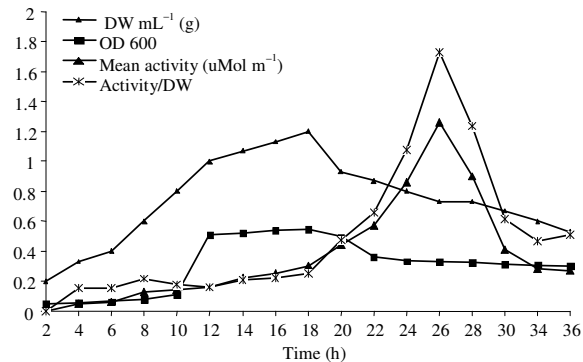


Fig. 2: Microbial growth and α -amylase production in stationary flasks

amylase production as also reported in *B. thermooleovorans* NP54^[8], *B. coagulans*^[9], *B. licheniformis*^[10], *B. subtilis* JS-2004⁶ and *B. brevis*^[11]. The strain *Bacillus licheniformis* Shahed-07 possessed the ability to produce α -amylase and hydrolyze starch. Among the physical parameters, the pH of the growth medium plays an important role by inducing morphological change in the organism and in enzyme secretion. The influence of temperature on amylase production is related to the growth of the organism. A wide range of temperature (35-80°C) has been reported for optimum growth and α -amylase

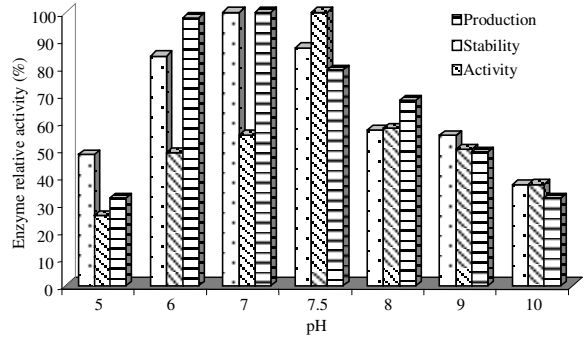


Fig. 3: Effect of pH on relative productivity, stability and activity of α -amylase

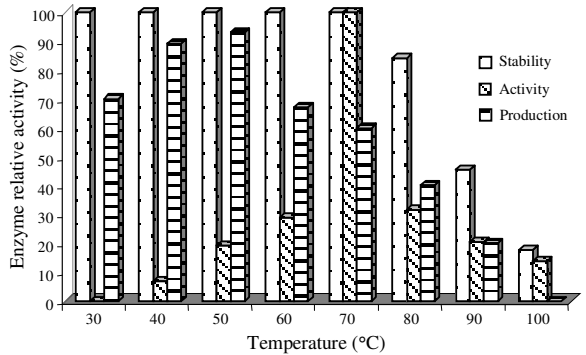


Fig. 4: Effect of temperature on relative productivity, activity and stability of α -amylase

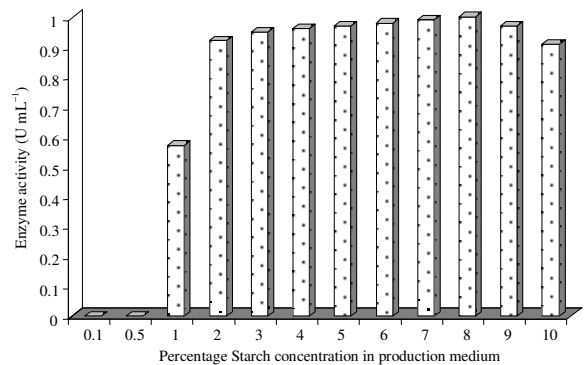


Fig. 5: Effect of substrate concentration on α -amylase productivity of *B. licheniformis* Shahed-07

production in bacteria^[4]. Konsula and Liakopoulou-Kyriakides^[12] reported that a thermophilic *B. subtilis* strain, isolated from fresh sheep's milk, produced maximum extracellular thermostable α -amylase at 40°C in a medium containing low starch concentration.

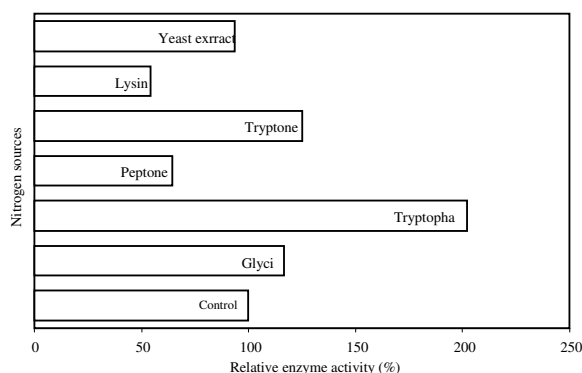


Fig. 6: Effect of various nitrogen sources on α -amylase production

Members of the genus *Bacillus* produce a large variety of extracellular enzymes of which amylases are of particularly significant industrial importance. Showing similarity to *B. thermooleovorans* NP54^[8], *B. licheniformis* Shahed-07 showed to produce α -amylase maximally at its optimal growth temperature (50°C). A thermostable extracellular amylase from a *bacillus* isolate had an optimum temperature and pH of 60°C and 6.5, respectively^[13]. Our strain showed higher thermal activity of 70°C which being an advantage is comparable to that described for other *Bacillus* α -amylases^[4]. α -amylase production from *B. subtilis* JS-2004 was reported to be highest at 48 h declining gradually up to 96 h^[6]. Effective induction may not occur until the stationary phase has been reached and the readily available carbon source was depleted^[14]. Culture conditions have been found to have a profound influence on amylase production. The enzyme production was maximal when the cell population entered into stationary phase, suggesting that enzyme secretion is not growth associated. α -amylase from *Bacillus licheniformis* has been produced during the growth phase and not at the onset of the stationary phase^[15]. The results of this study do not favor this report. A newly isolated *B. licheniformis* Shahed-07 strain was cultured in liquid media containing soluble starch to produce α -amylase. The maximum amylase production has been reported to be achieved with maltose as carbon source. Among the nitrogen sources, peptone and yeast extract produced maximum amylase^[13]. Barley and corn flour have been reported to result in maximum amylase production compared to starch^[16]. Amylase production by this strain is inducible since the enzyme synthesis took place only in presence of starch. The effect of glycine, tryptophan, peptone, tryptone, lysine and yeast extract supplementation of

the production medium on enzyme production was studied. Starch and tryptone have been reported to be the ideal carbon and nitrogen sources, respectively^[17]. The amylase synthesis by several microorganisms has been correlated to the presence or absence of different nitrogen sources and various amino acids in the growth medium. Organic sources like yeast extract, peptone usually have stimulating effects^[18]. The differences in nutritional requirements of various α -amylase producing organisms or microbial strains could be attributed to the difference in their genetics. Studies on crude α -amylase characterization revealed that optimum activity was at pH 7.5 and 70°C (Fig. 3 and 4). The crude enzyme was stable for 24 h at pH range of 6-7 at 70°C (Fig. 3). The enzyme was quite stable at 70°C, while at 80 and 90°C, 16 and 54% of the original activities were lost, respectively (Fig. 4). Enzyme activity increased with temperature within the range of 40-70°C. A reduction in enzyme activity was observed at temperatures above 70°C. Thermostability for 4 h at 100°C have been reported for α -amylase from *B. licheniformis* CUMC 305^[10]. *Bacillus* sp. ANT-6 α -amylase was stable after overnight (85.5%) and 24 h (55%) incubation at 100°C and pH 10.5^[4]. A strain of *Bacillus stearothermophilus* isolated from the samples of a potato processing industry had a highly thermostable α -amylase. The temperature optimum for the activity of this enzyme was 70°C but pH optimum for activity was relatively low, in the range 5.5-6.0^[19]. α -amylases from *Bacillus* genus are heat stable and this is a desirable property for industrial starch liquefaction. Higher operational stability and a longer shelf-life of the enzyme produced by *B. licheniformis* Shahed-07 could be encouraging factors to consider further studies on its industrial application.

CONCLUSIONS

The *B. licheniformis* Shahed-07 strain produced thermostable α -amylase with characteristics suitable for application in starch processing and other food industries. Further optimization for enhanced enzyme production for commercialized process is needed.

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