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EXTRACTION AND PURIFICATION OF A THERMOSTABLE α–AMYLASE FROM *BACILLUS COAGULANS* FOUND IN IRANIAN SOIL SAMPLES

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ABSTRACT

Some thermostable α–amylase producing bacteria were isolated from different soil samples collected from various parts of Iran and identified as *Bacillus Coagulans, Bacillus licheniformis and Bacillus brevis*. The optimum temperature and incubation period of the strains for α-amylase production was specified. An extracellular thermostable amylase was obtained from these bacteria. The enzyme was purified by chromatography using a carboxymethylcellulose column and its biological activity measured. Some of the physical and kinetic properties of the enzyme such as optimum pH and temperature and V_{max} were also obtained.

Key words : α-Amylase, *Bacillus Coagulans*, Extracellular enzymes, Optimum temperature, Thermostable bacteria

INTRODUCTION

A wide variety of microorganisms produce, and in most cases secrete extracellularly, amylases with some interesting properties[1-8]. Amylases are enzymes capable of degrading starch and are universally distributed throughout animal, plant and microbial kingdoms. These enzymes, which also act on glycogen and related polysaccharides, catalyze hydrolysis of α–1, 4- and/or α-1,6-glucosidic linkages. From biotechnological viewpoint the more important of these enzymes fall into four groups (Table 1). There are α-amylases, which cause endo-cleavage of substrates; β-amylases, which hydrolyze alternate bonds from the non-reducing end of the substrate; the amyloglucosidases which hydrolyze successive bonds from the non-reducing end of the substrate; and the debranching enzymes which cleave the α-1,6-glucosidic linkages in amylopectin and glycogen.

Table 1. Some commercially important starch degrading enzymes

Trivial Name	Classification Number	Systematic or Scientific Name
α-Amylase	EC3.2.1.1	α-1,4-glucan 4-glucanohydrolase
β-Amylase	EC3.2.1.2	α-1,4-glucan maltohydrolase

Amyloglucosidase	EC3.2.1.3	α-1,4-glucan glucohydrolase
Debranching enzyme	EC3.2.1.68	α-1,6-glucan 6-glucohydrolase

Some important characteristics of these enzymes are summarized in Table 2. Gram-positive bacteria, and particularly the genus Bacillus, are prolific producers of amylases. However, a very few Gram-negative bacteria are found to produce amylases. Some of the bacteria which produce α-amylases are: *Bacillus subtilis, B. cereus, B. amyloliquefaciens, B. coagulans, B. polymyxa, B. stearothermophilus, B. caldolyticus, B. acidocaldarius, B. subtilis var, amylosaccharaticus, B. licheniformis, Lactobacillus, Micrococcus, Pseudomonas, Arthrobacter, Escherichia, Proteus, Thermomonospora,* and *Serratia.* More recently, interest in amylolytic yeasts has increased and several species of yeasts have been reported to utilize starch as a sole carbon source and secrete amylolytic enzymes[9].

Table 2. Characteristics of starch degrading enzymes

	α-Amylase	β-Amylase	Amyloglucosidase	Debranching enzyme
Hydrolyse α-1,4 glucosidic bonds	Yes	Yes	Yes	No
Hydrolyse α-1,6 glucosidic bonds	No	No	Yes	Yes
Ability to by-pass α-1,6 branch points	Yes	No	Bounds Cleaved	Bonds Cleaved
Configuration of C_1 of product	α	β	β	—
Mechanism of substrate attack	endo	exo	exo	—
Viscosity reduction*	Fast	Slow	Slow	—
Production of reducing sugars	Slow	Fast	Fast	—
Iodine staining power	Decreased quickly	Decreased slowly	Decreased Slowly	Increases

* Relative to bond hydrolysis

Some α-amylase-producing fungi are from the genera *Aspergillus, Penicillium, Cephalosporium, Mucor, Candida, Neurospora, and Rhizopus. Bacillus* amylases are used much more extensively than those of *Aspergillus.* The most important applications of these two enzymes are shown in Table 3.

Table 3. Important applications of α-amylases

Industry	Source		Application
	Bacillus	Aspergillus	
Starch	+		Liquefaction of starch for production of glucose, fructose, maltose
Milling			Modification of α-amylase-deficient flour
Alcohol	+	+	Liquefaction of starch before the addition of malt

		+	for saccharification
Baked goods	+	+	Increase the proportion of fermentable carbohydrates
Brewing			Barley preparation, Liquefaction of additives Improved fermentability of grains, modification of beer characteristics
Paper	+		Liquefaction of starch without sugar production for sizing of paper
Textiles	+		Continuous desizing at high temperatures
Feed industry	+		Improvement of utilization of enzymatically treated barley in poultry and calf raising
Sugar	+		Improvement of filterability of cane sugar juice via breakdown of starch in juice
Laudary and detergent	+		Increase in cleansing power for laundry soiled with starch, additive in dishwasher detergents

α-amylase (EC 3.2.1.1, 1,4-α-glucan-glucanohydrolase, endo-amylase) is an extracellular enzyme, which hydrolyzes α-1,4-glycosidic bonds. α-Amylases are amylolytic enzyme of industrial importance, particularly in the food and detergent industries[10]. The molecular weights of various α-amylases do not differ considerably (Table 4). They all contain a large proportion of tyrosine and tryptophan in the enzyme protein and most require calcium as a stabilizer. Several characteristics and amino acid sequences of the α-amylase have been reported[11-13]. In this piece of research work, some thermostable alpha-amylase producing bacteria were isolated from different soil samples collected from various parts of Iran. The optimum temperature and incubation period of the strains for alpha-amylase production was specified. The isolates were identified by Gram and Spore staining as *Bacillus Coagulans, Bacillus licheniformis and Bacillus brevis.* An extracellular thermostable amylase was obtained from these bacteria. The enzyme was purified by chromatography using a carboxymethylcellulose column and its biological activity measured. Some of the physical and kinetic properties of the enzyme such as optimum pH and temperature, and V_{max} were also studied.

Table 4. Molecular weight of some α-amylase from different microorganisms

Organism	Molecular weight × 10^3
Aspergillus oryzae	51-52
Aspergillus niger	58-61
Bacillus acidecalarius	68
Bacillus amyloliquefaciens	49
Bacillus subtillis	24-100
Thermomonospora curvata	62

EXPERIMENTAL

Soil samples from different parts of Iran (including North and Central regions) were collected. Suspensions were prepared by mixing one gram of each sample with 10 mL water. 0.5 mL of each suspension was cultured on a selected medium consisted of 2% agar, 0.3% yeast extract and 1% starch at 50 °C for at least 12 hours. The following bacteria had grown under these conditions; Ard A3, Ard A4, Ard A6, Rao A1, Ras A2, Shi A1, Shi A2, Ima A1, Gho A1, Cot A1, Jas A1, Arg A1, The A1, Sad A1, Sha A1, Low A1 and XA1. When the soil samples were cultured on the same medium at 60° C for 12 hours, only three bacteria (Sha B1, Cot B1 and The B1) were grown. The isolates were grown further on a solid medium containing 2% agar, 0.2% yeast extract and 1% starch. It was found that the bacteria grown at 60° C did not grow further on this later medium. On the other hand most of bacteria grown at 50° C, had a good growth on the second medium. Their culture was continued at 50 °C and the following isolates showed the most growth on agar plate; Jon A1, Sha A1, Jas A1, Gho A1, Low A1, Sad A1, Arg A1, Cot A1 and Sha A2. These isolates were cultured at 57° C and it was found that Arg A1, Sha A2 and Low A1 had the highest growth at this condition, although the following bacteria were also grown to a lower degree; Sha A1, Jon A1, Gho A1, Jas A1, Cot A1 and Sad A1. Both group were cultured again at 57° C for 12 hours on the same medium and only the following isolates (Sha A2, Low A1, Sad A1, Sha A1 and Gho A1) showed a considerable growth and, therefore, transferred to a liquid cultural medium. The constituents of the liquid medium were similar to the solid except that agar was eliminated and the concentration of malt extract was varied between 0.1 to 0.3%. The cells grown at 47° C after 12 hours were separated by centrifugation (4000 rpm/10 min).

RESULTS

The results of Gram and Spore staining used for identification of the strains are presented in Table 5. It is demonstrated from this table that the target bacterium was a Spored Gram positive bacillus. Therefore, some biophysical and biochemical methods such as growth rate at 30, 50, 55 and 60° C, lactose fermentation and MR were performed in order to identify isolated bacteria. Some of these results are demonstrated in Table 6.

Table 5. The morphology of isolates and their behavior to Gram and Spore stainings.

Bacterium	Morphology	Gram reaction	Presence of Spore
Gho A1	Bacillus	+	+
Sha A1	Bacillus	+	+
Sha A2	Cocobacillus	+	-
Low A1	Bacillus	+	+
Sad A1	Short bacillus	+	+

Table 6. Biochemical and biophysical tests for identification of Low A1 compared to three bacilli[14]

Test	Low A1	B. brevis	B. coagulans	B. licheniformis
Citrate	+	±	11-89%	+
MR	+	+	+	ND
VP	-	-	+	+
Lactose	+	+	+	+
Sucrose	+	+	+	+
Anaerobiosis	+	-	+	+
Growth at 30° C	+	±	+	+
Growth at 55° C	+	80%	+	+
Growth at 65° C	-	-	-	-
Gas from glucose	-	-	-	-
Acid from glucose	+	+	+	+

It was shown that the Low A1 isolate was resistant to high temperature and the biological activity of the enzyme produced by this bacterium was similar at 60° C and 70° C. The biological activity of the enzyme was measured using iodine method described by Mcneely[15]. The method was based on the decrease in the absorption at 660 nm of a solution containing starch and iodine due to the hydrolytic activity of amylase on starch. Table 7 shows the optimum temperature for the activity of α-amylase produced by each bacterium.

Table 7. Measurement of optimum temperature for the α-amylses obtained from five selected bacteria.

Bacteria	Sad A1	Sha A2	Sha A1	Low A1	Gho A1
Temperature (°C)	α-amylase activity (μ/mL)				
30	55.23	83.81	164.91	0	0
40	45.45	65.36	37.5	21.2	30.85
50	14.72	52.46	25.17	23.82	25.68
60	16.5	0	22.35	35.73	8.56
Optimum	≤30	≤30	≤30	≤60	40

It is clear from the results in Table 7 that Low A1 shows maxium α-amylase activity, especially at high temperatures. Therefore, the enzyme produced by this isolate was partially purified by precipitation with ammonium sulfate and acetone followed by chromatography on CM-cellulose. The total protein in each fraction (elute, wash and effluent) was measured using the UV absorption at 280 nm. The specific activity of the enzyme was then calculated from the total protein and the biological activity of the enzyme (Table 8).

Table 8. Partial purification of α-amylase by CM-cellulose and the results of its activity and specific activity in each fraction

Sample	Volume (mL)	Acitivity (m/mL)	Yield (%)	Protein (mg/mL)*	Specific activity
Original	1	92.36	100	6.36	14.5
Effluent	1	44.95	46.66	0.034	132.2
Wash	1	15.27	16.53	0.091	167.80
Eluate (0.1M NaCl)	1	9.68	10.44	1.243	7.87
Eluate (1.0M NaCl)	1	0	0	2.067	0

*obtained from UV absorption at 280 nm.

To obtain optimum pH, the reaction mixture was prepared in buffers of different pH values (4.4-10.0) and the enzyme activity was measured at 60° C. Figure 1 shows the activity of α-amylase in different pH conditions at its optimum temperature 60° C (according to the results shown in Table 7). It is seen in Figure 1 that the optimum pH for this α-amylase is 6.0.

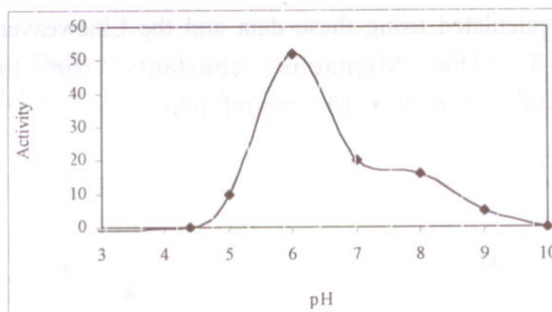

Figure 1. The effect of pH on the activity of α-amylase purified from Low A1 at 60° C. The unit of activity is μ/mL.

Thermal stability of the enzyme was studied by storing it in a known temperature for one, two and three hours. The results showed that although the enzyme remained active after one hour at 60° C, but its activity decreased to 86% and 50% after two and three hours, respectively. At 90° C the decrease in loss of activity was much higher. Figure 2 shows the thermal stability of α-amylase stored for 60, 120 and 180 minutes at different temperatures. It is evident from the data in this figure that the only 10% of the enzymes have remained active after 180 minutes at 90° C. It is also shown in this figure that the stability of α-amylase is similar at 60° and 70° C, while higher temperatures cause some loss of activity.

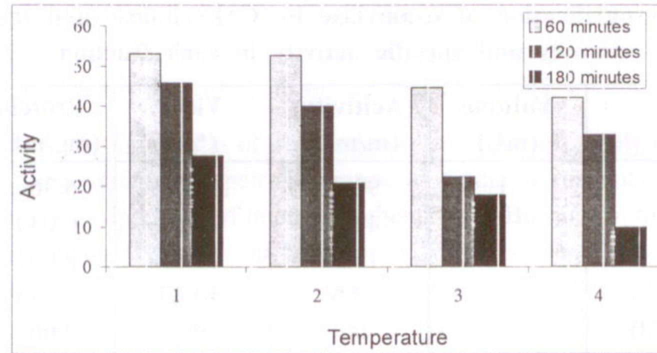

Figure 2. Thermal stability of α-amylase stored at different temperature; 60, 70, 80 and 90° C from left to right. The activity is expressed in μ/ml.

Since the optimum pH for the enzyme activity was 6.0, this was used in order to obtain Michaelis constants, K_m and V_{max}. A reaction mixture was prepared with starch (substrate) concentrations in the range of 0.2-2.0 mg/mL. The purified enzyme was added to the reaction mixture at 60° C and the decrease in absorption at 660 nm was monitored. The rate of the enzymatic reaction was calculated using these data and the Lineweaver-Burk plot obtained is shown in Figure 3. The Michaelis constants for this enzyme were: K_m = 0.625 mg/mL and V_{max} = 6.26 x 10^{-3} mg/mL/min.

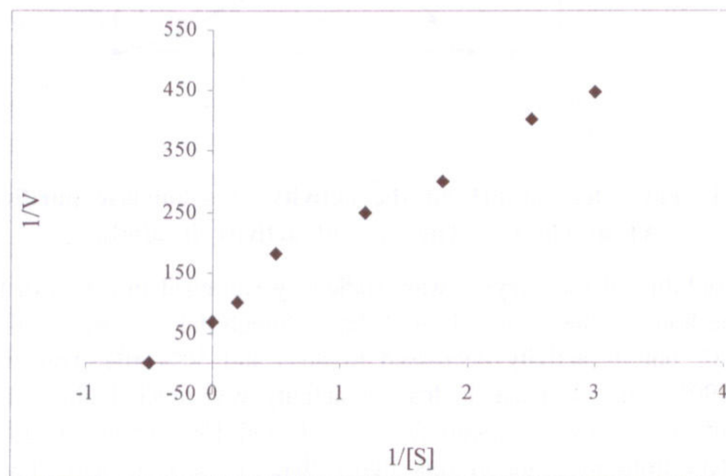

Figure 3. Lineweaver-Burk plot of Low A1 amlyase activity at pH 6.0

DISCUSSION

Based on the results obtained in this study, it was clear that most of the isolates were able to grow at 50° C, therefore, they were all thermotolerant bacteria. The Low A1 bacterium was at the boarder of thermotolerant and thermophil. The absence of yeast between isolates was expected for two reasons. Firstly, that thermotolerant yeasts are very rare, and secondly due to the nearly neutral pH of the medium, which is not suitable for the growth of yeasts. The aim of this research was to find isolates that are able to produce α-amylase at neutral pHs. The α-amylase obtained in this study had a good thermal stability compared to the amylases used in industry. It is suggested, therefore, if the gene responsible for the enzyme is isolated from Low A1 and cloned in an overproducer, it could be a good replacement for the available enzymes used in industry and it has the advantage of being easy to purify, high thermal stability and relatively low cost.

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