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Production, purification, and characterization of α-amylase by *Bacillus subtilis* and its mutant derivates

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Abstract: The effects of various carbon and nitrogen sources on production of α-amylase by *Bacillus subtilis* and its mutant derivates were investigated. The maximum production of α-amylase by all strains was obtained in the presence of mesoinositol as the carbon source. There was no more significant increase in enzyme yield in the case of the supplementation of nitrogen sources, whereas malt extract and tryptone were preferred nitrogen sources for amylase production by *Bacillus subtilis* and mutant U 2-6 strain, respectively. α-Amylases of *B. subtilis* and its mutant strain (EBUE 5-3) were purified through a series of steps, and characterized. The optimum temperature and pH values of the purified amylases were found to be 45 °C and 6.0, respectively. The enzyme of mutant strain had more stability than the enzyme of the parental strain in alkaline conditions (85% at pH 8.0 for 1 h). The K_m and V_{max} values of both amylases were also compared. Enzymes were strongly inhibited by Cu^{2+}, Hg^{2+}, and Ag^{2+}, but activated by Ca^{2+}, Ba^{2+}, Mg^{2+}, Li^{2+}, and Mn^{2+}. Metal ion concentration of 1 mM had a greater effect on enzyme activities than 5 mM did. The estimated molecular weight of the purified enzymes was 56 kDa. The N-terminal amino acid sequence of amylases produced by the parental and the mutant strain showed homology.

Key words: *Bacillus subtilis*, mutant, α-amylase, production, purification, characterization

Bacillus subtilis ve mutant türevleri tarafından α-amilazın üretimi, saflaştırılması ve karakterizasyonu

Özet: Çalışmada, *B. subtilis* ve onun mutant türevleri tarafından α-amilazın üretimi üzerine çeşitli karbon ve azot kaynaklarının etkisi araştırıldı. Tüm suşlar tarafından maksimum α-amilaz üretimi karbon kaynağı olarak mesoinositol varlığında elde edildi. Azot kaynaklarının ilavesi durumunda enzim veriminde önemli bir atış olmadığı görüldü, ancak malt ekstrat *B. subtilis*, tripton mutant U 2-6 tarafından amilaz üretimi için tercih edilen azot kaynaklarıydı. *B. subtilis* ve mutant suşu (EBUE 5-3)'nun α-amilazları bir seri basamakta saflaştırıldı ve karakterize edildi. Saflaştırılan α-amilazların optimum sıcaklık ve pH değerleri sırasıyla 45 °C ve 6,0 olarak bulundu. Mutant suşun enzimi alkali koşullarda ana suş enziminden daha stabildi (pH 8,0'de 1 saat % 85). Her iki amilazın The K_m ve V_{max} değerleri karşılaştırıldı. Enzimler Cu^{2+}, Hg^{2+} ve Ag^{2+} tarafından güçlü olarak inhibe edildi, fakat enzim Ca^{2+}, Ba^{2+}, Mg^{2+}, Li^{2+} ve Mn^{2+} tarafından aktive edildi. 1 mM metal iyon konsantrasyonu enzim aktivitesi üzerine 5 mM'den daha büyük etkiye sahipti. Saflaştırılan enzimlerin tahmini moleküler ağırlıkları 56 kDa'du. Ana ve onun mutant suşu tarafından üretilen amilazların N-terminal amino asit sekansları homoloji gösterdi.

Anahtar sözcükler: *Bacillus subtilis*, mutant, α-amilaz, üretim, saflaştırma, karakterizasyon

Introduction

Microbial enzymes are widely used in industrial processes and α-amylase is one of the most important industrial enzymes, having applications in industrial processes such as brewing, baking, textiles, pharmaceuticals, starch processing, and detergents. α-Amylases are some of the most versatile enzymes in the industrial enzyme sector and account for approximately 25% of the enzyme market (1). α-Amylase (E.C.3.2.1.1) catalyzes the endo-hydrolysis of 1,4-alpha-D-gylcosidic linkages in polysaccharides containing 3 or more 1,4- α-linked glucose units. The enzyme acts on starches, glycogen and oligosaccharides in a random manner, liberating reducing groups (2). Strains of *Bacillus* have been some of the workhorses of enzyme production for decades, mainly because of their ability to overproduce amylase (3). *B. subtilis*, *B. stearothermophilus*, *B. licheniformis*, and *B. amyloliquefaciens* are known to be good producers of α-amylase, and they have been widely used for commercial production of the enzyme for various applications (4). Most of the strains used for enzyme production have been improved through classical selection (5). UV and chemicals such as ethyl methyl sulfonate (EMS), nitrous acid, and N-methyl—N′-nitro-N-nitrosoguanidine (NTG) were found to be suitable mutagens for the improvement of α-amylase production by *Bacillus* and obtained mutants have a higher capacity for amylase production (1,6,7). To obtain maximum yield of an enzyme, development of a suitable medium and culture conditions is obligatory (8,9). Starch or other sugars as a carbon source and ammonium salts or complex organic compounds as a nitrogen source are needed for bacterial growth and enzyme production (10,11). Most amylases are known to be metal ion-dependent enzymes, particularly with regard to divalent ions like Ca^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} (12,13).

A series of experiments were carried out to study the effect of various carbon and nitrogen sources on the growth and the production of α-amylases by *B. subtilis* and its mutant derivates. A mutant of high enzyme activity was purified and characterized.

Materials and methods

Materials

A new strain of *B. subtilis* was isolated from Turkish soil and identified by ORBA Biochemicals plant (İstanbul, Turkey). Mutants of *Bacillus subtilis* were obtained by ethidium bromide (EtBr), ultraviolet radiation (UV) and ethyl methyl sulfonate (EMS) treatments and their combinations and given the names U 2-6 and EBUE 5-3 (14).

Culture conditions

Bacteria were grown in growth medium for 18 h. Then overnight cultures with OD_{600} = 0.3 were inoculated at 1% in the defined enzyme production medium (15) and allowed to grow for 88 h. Cultivation was carried out on a rotary shaker at 150 rpm and at 30 °C at 16, 24, 40, 48, 64, 72, and 88 h. The cells were removed by centrifugation (6000 rpm, 10 min) and the supernatants were used to determine the enzyme activity while the pellets were used to quantify the total protein mass. The total protein was determined using the method of Biuret (16). α-Amylase activity was assayed using the starch-iodine method (17). One enzyme unit was defined as the amount of enzyme that hydrolyzed 1 mg of starch (0.1% w/v) in 10 min at 37 °C and pH 5.9 (unit/mL).

Enzyme production

To study the efficacy of various carbon sources on α-amylase production, maltose, lactose, inositol, and raffinose were selected as carbon sources. Starch (1% w/v) was replaced in the defined production medium with equal amounts of the various carbon sources to be tested. Beef extract, malt extract, tryptone, and ammonium phosphate $(NH_4)_2HPO_4$ were examined as alternative nitrogen sources to the peptone and ammonium sulfate in the production medium (1.3% w/v). A volume of 150 mL of production media containing test carbon and nitrogen sources was inoculated at 1% and placed in 500-mL Erlenmeyer flasks for culture.

Enzyme purification and characterization

α-Amylases produced from the parental and its mutant strain (EBUE 5-3) were purified by a series

of precipitation with 80% ammonium sulfate, TSK Toyopeal column chromatography, ultrafiltration, dialysis, and SP Sepharose column chromatography. Purification was confirmed by sodium dodecyl sulfate (SDS) gel electrophoresis (18). The effects of pH, temperature and presence of various metal ions on the activity of α-amylase were determined. Enzyme samples were incubated for 10 min between 30 and 90 °C. Thermal stabilities were determined to be 45 and 50 °C for 1 h. The optimum pH for the enzymes was determined in a Britton and Robinson buffer system between pH 4.0 and 9.0 pH. pH 5.0 and 8.0 were used for pH stabilities at 2 h.

Kinetic constants as K_m and V_{max} were measured by estimating hydrolysis with a starch-iodide method and using several starch concentrations. K_m and V_{max} values were determined using the Michaelis-Menten equation.

Enzymes samples were incubated with some metal ions at a concentration of 1 and 5 mM. Relative activities were expressed as a percentage of the activity of the untreated control taken as 100%.

SDS-Polyacrylamide gel electrophoresis (10%) of purified parental and mutant *Bacillus subtilis* amylases were performed for the determination of molecular weight (18). α-Amylase activities were localized by running the enzyme in a native-PAGE. The gel was immersed in soluble starch (1% w/v) for 1 h at room temperature. The gel was then kept in the same buffer, followed by staining with iodine solution (0.02% (w/v) iodine and 4% (w/v) KI) for 10 min.

N-terminal sequencing of purified amylases was performed on a protein sequencer 492 A (Applied Biosystems). The first 20 residues of the N-terminal sequence were determined.

Results and discussion

Effects of carbon and nitrogen sources on enzyme production

Mutant strains of *Bacillus* have better ability for the production of α-amylase (19,20). For α-amylase production a carbohydrate source is required, where this may be starch or one of a variety of sugars. Nitrogen can be supplied in the form of ammonium salts or as a complex organic source.

A series of experiments were carried out to study the effect of carbon and nitrogen sources. To study the effect of carbon sources, the soluble starch was substituted with various carbon sources (1% w/v) including maltose, lactose, mesoinositol, and raffinose. In addition, different nitrogen sources, including tryptone, malt extract, beef extract, and $(NH_4)_2HPO_4$ were used instead of peptone and $(NH_4)_2SO_4$ in the original medium (1.3% w/v).

Maximum enzyme production in all carbon and nitrogen sources by the parental strain and mutant EBUE 5-3 strain was achieved by 72 h. However, maximum production of the enzyme for the mutant U 2-16 strain was reached at 48 h. It was also determined that *B. subtilis* and its mutants, which produce mucous material during the incubation period, did not produce any mucous material in the presence of mesoinositol.

The results showed that mesoinositol was the best carbon source for enzyme production for all strains and as shown in Figure 1 in comparison with the control (starch) there was a significant increase in the enzyme yield in the presence of mesoinositol. The mutant U 2-16 strain produced more amylase in the presence of any of the carbon sources than the parental and mutant EBUE 5-3 strains did. For the parental, mutant U 2-16, and mutant EBUE 5-3

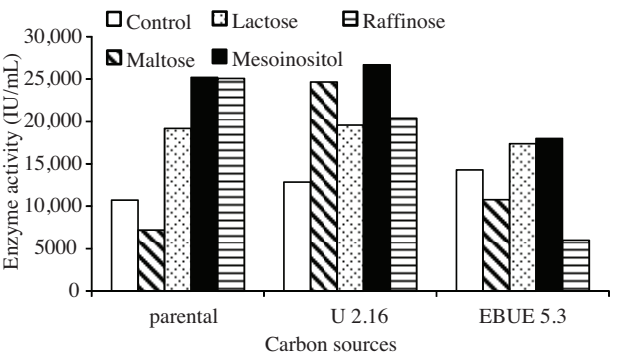

Figure 1. The effects of various carbon sources on amylase production by *B. subtilis* (parental) and its mutant strains. Values are shown as means of triplicates.

strain, the α-amylase production was affected by the various carbohydrate sources in the following order: mesoinositol = raffinose > lactose > maltose; mesoinositol > maltose > raffinose > lactose; mesoinositol > lactose > maltose > raffinose, respectively. The increase in enzyme production was parallel to the growth rate. In the present study, maltose did not increase amylase production in the parental and EBUE 5-3 strains; however, it was found to be an inducer for the mutant U 2-16 strain. Some researchers have previously reported that maltose is a good inducer of amylase production (8,21-23). On the other hand, some reports suggest that the highest production was observed with glucose (24), while others have observed that glucose represses the production of amylase (25). The monosaccharides repressed the enzyme production, whereas inositol and D-sorbitol favored amylase production (11). Carbon sources such as galactose, glycogen and inulin have been reported as suitable substrates for the production of amylases by *B. licheniformis* and *Bacillus* sp. I-3 (26). Starch and glycerol were known to increase enzyme production in *B. subtilis* IMG22, *Bacillus* sp. PS-7, and *Bacillus* sp. I-3 (27-29). Soluble starch has been found to be the best substrate for the production of α-amylase by *B. stearothermophilus* (10). *Bacillus thermooleovorans* is reported to prefer starch, glucose, lactose, maltose, and maltodextrins as carbon sources for α-amylase secretion (8,30). Of the various 1% soluble sugars, amylase production was highest in the sucrose medium. Nonmetabolizable sugars like arabinose, raffinose, mesoinositol, sucrose, and galactose did not support amylase production (22). The influence of carbon sources including glucose, sucrose, starch, carboxymethyl cellulose, fructose, sorbitol, xylose, galactose, and dextrin were tested. Starch, sucrose, dextrin, and galactose were good carbon sources for amylase production (31).

The effects of different organic and inorganic nitrogen sources on the cell density and amylase activity were studied. The effects of nitrogen sources on the production of amylase by *B. subtilis* and its mutant derivates are shown in Figure 2. It was found that the optimal nitrogen sources were different for each strain. Maximum enzyme production and biomass of the parental strain were malt extract > $(NH_4)_2HPO_4$ = tryptone > beef extract; enzyme production for mutant U 2-16 was tryptone > malt extract = $(NH_4)_2HPO_4$ > beef extract, and for mutant EBUE 5-3 strain was beef extract > malt extract = $(NH_4)_2HPO_4$ > tryptone. Maximum enzyme activity and biomass for all strains were obtained at 72 h. However, as shown in Figure 2 in comparison with the control, there was no effective increase in enzyme production in the presence of inorganic and organic nitrogen sources, especially with mutant EBUE 5-3 strain. Similar findings have been reported by other researchers (32-34). In the present study, tryptone was found to be much better for amylase production by the parental and mutant strains amylase production. In fact, tryptone has been observed to be the ideal nitrogen source for amylase production (35). $(NH_4)_2HPO_4$ was found to be the best nitrogen source for amylase production (22,36). Among the nitrogen sources peptone and yeast extract produced maximum amylase (23,31). It has been reported that peptone increased enzyme activity, while yeast extract exhibited no effect on α-amylase production (28). Strains of *B. stearothermophilus* and *B. amylolyticus* secreted maximum α-amylase in a medium supplemented with peptone, yeast extract, and maltose under vigorous shaking conditions (37). Peptone has been reported to be a better nitrogen source than ammonium hydrogen phosphate, normally the best among inorganic nitrogen sources

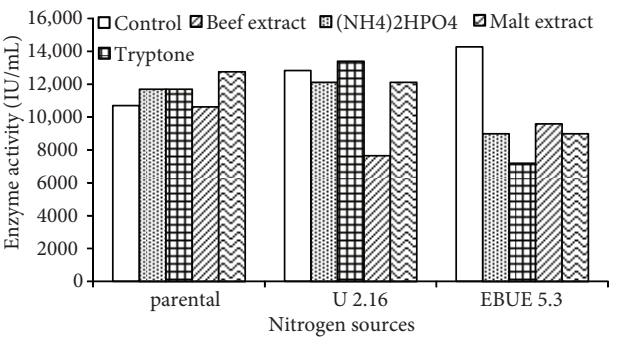

Figure 2. The effects of various nitrogen sources on amylase production by *B. subtilis* (parental) and its mutant strains. Values are shown as means of triplicates.

for enzyme production by *B. licheniformis* SPT 278 (22). Soya bean meal was found to be the best nitrogen source for α-amylase production by *Bacillus* sp. I-3 (26,29). Addition of organic nitrogen sources such as casein, yeast extract, and urea, and inorganic nitrogen sources such as ammonium chloride to the medium resulted in considerable decrease in α-amylase production by *B. cereus*. In general, supplementation of additional nitrogen sources in general has been reported to be inhibitory for α-amylase production by microorganisms (24). *B. brevis* produced more amylase in the presence of beef extract as nitrogen source in comparison to other organic nitrogen sources (peptone, yeast extract, and casein), while asparagine, potassium nitrate, ammonium sulfate, ammonium nitrate, and urea reduced the enzyme activity (9).

Inorganic nitrogen source was as effective as organic ones (Figure 2). Similar results were obtained in the case of other *Bacillus* spp., that is, *B. licheniformis* (22), *B. subtilis* (38,39), *B. thermooleovorans* (8), and *B. coagulans* (33). In contrast, it has been reported that maximum α-amylase production by *B. subtilis* DM-03 is obtained by using ammonium chloride as the nitrogen source (40).

Enzyme characterization

The amylases obtained from *B. subtilis* and mutant EBUE 5-3 strains were purified by a series of treatments. After purification, each enzyme showed a single band on SDS-polyacrylamide gel electrophoresis.

The optimum temperature and pH of both α-amylases were found to be 45 °C and 6.0, respectively (Figures 3 and 4). The mutant enzyme was more stable in the alkaline pH than acidic pH (85% at pH 8.0 and 33% at pH 5.0 for 1 h) than the parental strain. The mutant enzyme was more thermostable (36% at 45 °C for 1 h) than the parental type. It was observed that when the temperature was increased up to 43 °C, the amylase activity of *B. amyloliquefaciens* UNG-16 mutant strain markedly declined (41). It has previously been reported that amylases of *B. amyloliquefaciens* and its mutant strain were more active in alkaline pH than in acidic pH and also that the mutant enzyme was more thermostable (70% at 50 °C for 4 h) than the parental strain (42).

Enzymes showed Michaelis-type kinetics when hydrolyzing soluble starch. Kinetic parameters were calculated as 1.08 and 1.43 (mg/mL) for K_m and 100 and 151 (U/mL) for V_{max} for the parental and mutant strains, respectively.

The effect of metal ions on α-amylase activity was measured in the presence of various metal ions at a concentration of 1 mM and 5 mM (Table). The 1 mM concentration was more effective than the 5 mM. Activities of both enzymes were stimulated

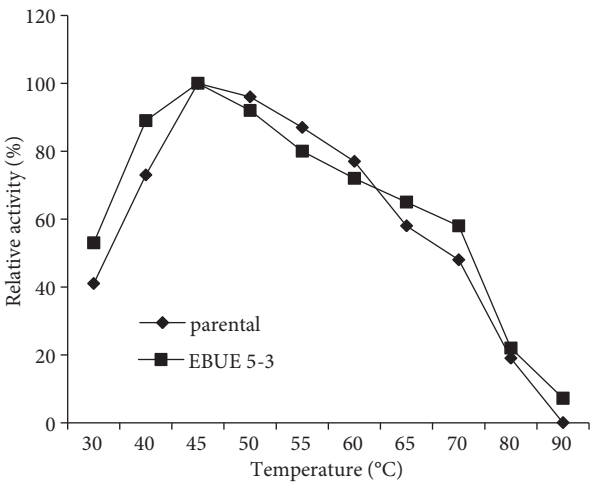

Figure 3. Effect of temperature on the enzyme activities.

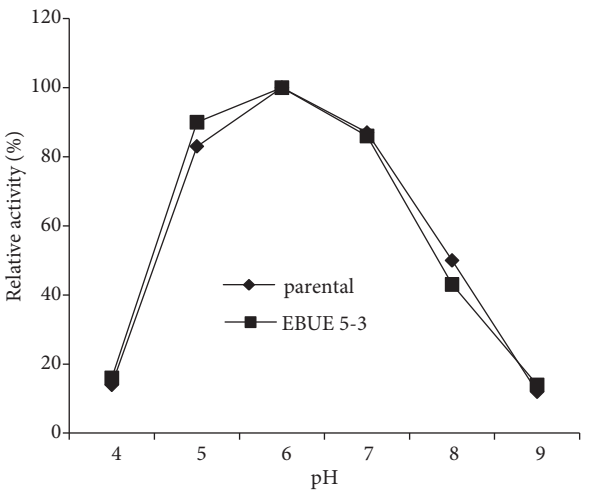

Figure 4. Effect of pH on enzyme activities.

Table. Effect of various metal ions on α-amylase activity. Residual activity (%) at indicated test reagent concentration (mM). Values are shown as means of triplicates.

	Parental amylase		EBUE 5.3 amylase	
	1 mM	5 mM	1 mM	5 mM
None	100	100	100	100
$MgSO_4$	105	98	90	100
$CaCl_2$	122	69	102	109
$LiSO_4$	111	62	98	95
$CuSO_4$	49	37	57	43
$Ba(C_2H_3O_2)_2$	117	111	100	104
$HgCl_2$	0	0	0	7
$MnSO_4$	110	98	87	98
$AgNO_3$	270	0	0	
$FeCl_2$	89	0	67	0
$ZnSO_4$	78	67	64	36

in the presence of Ca^{2+}, Ba^{2+}, Mg^{2+}, Li^{2+}, and Mn^{2+} ions. On the other hand, a strong inhibitory effect was observed in the presence of Cu^{2+}, Hg^{2+}, and Ag^{2+} ions. The amylase did not require any specific ion for catalytic activity, but Ca^{2+} independent α-amylase from *Bacillus* spp. has been reported by some authors (34,39,43,44).

Molecular weights of purified enzymes were found to be approximately 56 KDa (Figure 5). Activity staining of amylases showed white bands in

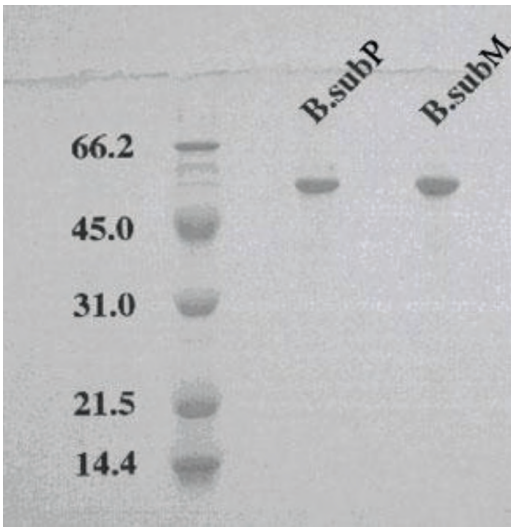

Figure 5. Photographic representation of SDS-PAGE pattern of the purified enzymes. B. subP: *B. subtilis* parental strain; B. subM: *B. subtilis* mutant EBUE 5-3.

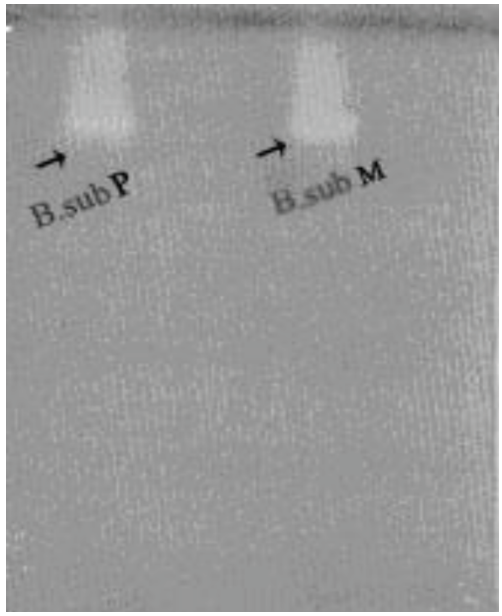

Figure 6. Detection of α-amylase activity by native PAGE.

the dark colored gel, which confirmed the enzyme activity (Figure 6).

A comparison of the N-terminal amino acid sequence of amylases produced by the parental and the mutant type showed homology. The 20 residues of the amino-terminal sequence were determined to be VNGTLMQYFEWYTPNDGQHW.

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