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RESEARCH ARTICLE

Alpha-Amylase Production by *Bacillus subtilis* RSKK96 in Submerged Cultivation

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Summary

The bacterial strain Bacillus subtilis RSKK96 was shown to produce extracellular α-amylase. Enzyme synthesis occurred at 72 h with an optimum of 37°C. Effects of various carbon, nitrogen, amino acid sources and chemicals on α-amylase production were examined. $FeSO_4$, $ZnSO_4$ and $CuSO_4$ inhibited bacterial growth as a result, amylase production. Maximum α-amylase production (858.6±41.9 U/mg) was obtained in a medium containing 0.5% cotton stalk in 72 h.

Keywords: *Bacillus subtilis RSKK96, α-Amylase, Submerged fermentation, Enzyme production*

Submerged Kültürü ile *Bacillus subtilis* RSKK96'dan α-Amilaz Üretimi

Özet

Bakteriyel tür Bacillus subtilis RSKK96'nın ekstrasellüler α-amilaz ürettiği gözlenmiştir. Enzim sentezi 72. saatte optimum 37°C'de elde edilmiştir. Çeşitli karbon, azot ve aminoasit kaynakları ve kimyasalların α-amilaz üretimi üzerine etkileri incelenmiştir. $FeSO_4$, $ZnSO_4$ ve $CuSO_4$ bakteriyel büyümeyi ve dolayısıyla α-amilaz üretmini inhibe etmiştir. Maksimum α-amilaz üretimi (858.6±41.9 U/mg) %0.5 pamuk sapı bulunan ortamda 72. saatte elde edilmiştir.

Anahtar sözcükler: *Bacillus subtilis RSKK96, α-Amilaz, Submerged fermantasyon, Enzim üretimi*

INTRODUCTION

α-Amylase (EC: 3.2.1.1), hydrolyzes the internal α-1, 4 linkages in starch in a random fashion leading to the formation of soluble maltodextrins, maltose, and glucose [1]. These enzymes account for about 30% of the world's enzyme production and have a great significance with extensive biotechnological applications in bread and baking, food, textile, and paper industries [2,3]. Although amylases can be derived from several sources, including plants, animals and microorganisms, microbial enzymes generally meet industrial demands [4]. Today a large number of microbial amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry [3].

In spite of the wide distribution of amylases, microbial sources, namely fungal and bacterial amylases, are used for the industrial production due to advantages such as cost effectiveness, consistency, less time and space required for production and ease of process modification and optimization [5]. Among bacteria, *Bacillus* sp. is widely used for thermostable α-amylase production to meet industrial needs. *B. subtilis*, *B. stearothermophilus*, *B. licheniformis* and *B. amyloliquefaciens* are known to be good producers of α-amylase and these have been widely used for commercial production of the enzyme for various applications [2].

To meet the demand of industries, low-cost medium is required for the production of α-amylase. Submerged fermentation (SmF) could be used for the production of

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α-amylases. These enzymes have traditionally been obtained from submerged cultures because of ease of handling and greater control of environmental factors such as temperature and pH [2]. The present study describes the effects of culture conditions on the production of α-amylase by *Bacillus subtilis* RSKK96.

MATERIAL and METHODS

Microorganism

α-Amlyase producing *B. subtilis* RSKK96 which was procured from Refik Saydam Hıfzıssıhha Institute, Ankara, Turkey was used as biological material. *B. subtilis* RSKK96 was grown on nutrient agar at 37°C for 24 h for inoculum preparation. A loopful of the growth was transferred to Laura broth (LB) liquid medium (1% yeast extract, 0.5% peptone, 0.5% NaCl, (w/v), pH 7.0).

Enzyme Production

The organism was propagated at 37°C for 5 days in 25 ml of medium with shaking on a shaker (150 rpm). Samples were taken at 12, 24, 48,72, 96 and 120 h. The supernatant of the culture after centrifugation (10.000 rpm, 10 min) at 4°C was used to determine α-amylase activity.

Enzyme Assay

α-Amylase activity was determined by the procedure of Bernfeld using soluble starch as a substrate [6]. The reaction mixture containing 200 µl of 1% substrate (w/v) in 0.1 M phosphate buffer (pH: 7.0) and 150 µl of enzyme solution was incubated for 30 min at 37°C. The reaction was stopped by adding 400 µl of 3,5-dinitrosalicylic acid solution followed by heating in a boiling water bath for 5 min and cooling at room temperature and then 8 ml of deionized water was added. Absorbance of each solution containing the brown reduction product was measured at 489 nm in a UV-visible spectrophotometer.

Assay of Protein Concentration

The protein concentration was determined by the Lowry's method using bovine serum albumin used as standard [7].

Effect of Incubation Period

The effect of incubation period was determined by incubating production medium for different incubation periods (12, 24, 48, 72, 96, and 120 h) at 37°C. Taking other conditions into consideration.

Effect of Carbon Sources

Different carbon sources such as soluble starch, wheat starch, patato starch, corn starch, wheat flour, rice flour, corn flour, glycerol, mannose, xylose, lactose, galactose, arabinose, glucose, sucrose, and fructose were employed to find the suitable carbon source for α-amylase production by *B. subtilis* RSKK96. All these sources were studied at 1% (by mass per volume) initial concentrations.

Effect of Nitrogen Sources

Two categories, viz. organic nitrogen sources and inorganic nitrogen sources were employed. The growth medium was initially supplemented with different organic nitrogen sources, i.e. yeast extract, tryptone, beef extract, peptone, casein, urea, soy flour each at 1% (by mass per volume). Among the inorganic nitrogen sources, ammonium nitrate, ammonium chloride, ammonium sulphate, and sodium nitrate again at 1% (by mass per volume) were tested.

Effect of Amino acid Sources

To study the effect of amino acid sources on production of α-amylase glycin, L-lysin, L-tryosine, L-cysteine, glutamic acid, L-alanine, L-phenylalanine, L-isoleusine, L-valine and L-metionine selected as amino acid source. All these sources were studied at 0.01% (by mass per volume) initial concentrations.

Effect of Metal Salts

The effect of metal salts on α-amylase production is determined by adding different metal salts in the fermentation medium. The metal salts selected for present study are $FeSO_4$, $MgSO_4$, $CaCl_2$, $CuSO_4$, and $ZnSO_4$, at 0.1% concentration.

Effects of $CaCl_2$

The effect of metal salts on growth and α-amylase secretion was studied by varying the $CaCl_2$ concentrations (0.25, 0.5, 0.75, 1, 1.25 and 1.5%. w/v) . Growth and enzyme production were quantified after incubation for 72 h at 37°C under shaking at 150 rpm.

Effect of Cotton Stalk (Cheap Source)

The effect of agroindustrial residue on α-amylase production is determined by adding such as cotton stalk in the fermentation medium. This source was studied at 0.25, 0.5, 1 and 2% (w/v).

RESULTS

Data presented here show that *Bacillus subtilis* RSKK96 produces α-amylase. The optimal conditions for α-amylase production have been determined under submerged fermentation conditions. Our results indicated that the optimum incubation period for α-amylase production was 72 h *(Fig. 1)*. Enzyme production gradually declined beyond the 72 h.

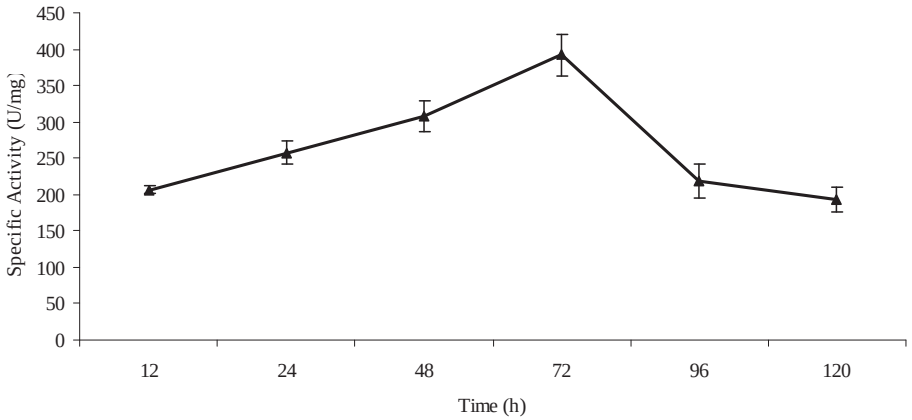

Fig 1. Effect of incubation time on the production of *Bacillus subtilis* RSKK96 α-amylase

Şekil 1. *Bacillus subtilis* RSKK96'nın α-amilaz üretimi üzerine inkübasyon zamanının etkisi

Among the all carbon sources showed a repressive effect on α-amylase production. *Table 1* shows specific activity of amylase for carbon sources. Among all carbon sources α- amylase was suppressed by arabinose, glycerol and fructose much more than by the others. Comparison with the control in media containing arabinose, glycerol and fructose, production of α-amylase supressed range 92, 96 and 84%, respectively.

Table 2 in comparison with the control (521.0±31.8 U/mg), there was no significant increase in enzyme yield in the case of the supplementation of either inorganic or organic nitrogen sources. A marginal increase was noted with the addition of casein (554.1±57.4 U/mg), indicating that any of the sources can be alternatively used. The other nitrogen sources inhibited the enzyme production.

Table 1. *Effect of carbon sources on the production of Bacillus subtilis RSKK96 α-amylase*

Tablo 1. *Bacillus subtilis RSKK96'nın α-amilaz üretimi üzerine karbon kaynaklarının etkisi*

Carbon Source 1% (w/v)	Specific Activity(U/mg)
Control	521.0±31.8
Soluble starch	419.3±38.5
Wheat starch	389.0±32.8
Patato starch	248.1±47.9
Corn starch	370.4±18.5
Wheat flour	314.6±22.3
Rice flour	344.6±52.7
Corn flour	444.6±40.0
Glycerol	51.7±38.4
Mannose	141.4±48.1
Xylose	237.4±10.6
Lactose	86.1±41.8
Galactose	327.9±25.2
Arabinose	21.1±3.9
Glucose	253.0±40.8
Sucrose	365.6±47.7
Fructose	279.9±23.8

Table 2. *Effect of nitrogen sources on the production of Bacillus subtilis RSKK96 α-amylase*

Tablo 2. *Bacillus subtilis RSKK96'nın α-amilaz üretimi üzerine azot kaynaklarının etkisi*

Nitrogen Source 1% (w/v)	Specific Activity(U/mg)
Control	521.0±31.8
Yeast extract	346.0±45.9
Tryptone	261.6±54.3
Beef extract	284.2±29.7
Peptone	285.4±37.8
Ammonium nitrate	205.6±48.4
Ammonium chloride	95.1±42.0
Ammonium sulphate	42.9±31.3
Sodium nitrate	10.4±11.7
Urea	398.7±43.5
Casein	554.1±57.4
Soy flour	174.2±20.2

Among the amino acids, glutamic acid, L-lysine and phenilalanine was found to support α-amylase synthesis, whereas others showed a repressive effect on α-amylase production *(Table 3)*. Comparison with the control in media containing glutamic acid, L-lysine and phenilalanine, enhanced production of α-amylase range 20, 18 and 3%, respectively.

The effect of various metal salts in the cultivation medium was studied for the optimal production of amylase from *B. subtilis* RSKK96 at 0.1% (w/v), $FeSO_4$, $CuSO_4$, and $ZnSO_4$ completely inhibited bacterial growth, and, as a result, amylase production *(Table 4)*. The other metal salts tested ($MgSO_4$ and $CaCl_2$), at 0.1% (w/v) decreased enzyme production by 41 and 8% repectively.

Addition of various concentrations (0.25, 0.5, 0.75, 1, 1.25 and 1.5% (w/v) $CaCl_2$ to the fermentation media, there was no significant increase in enzyme production *(Fig. 2)*. Comparison with the control (669.2±36.1 U/mg), marginal increase (679.3±57.9 U/mg) was noted with the addition at 1.5% (w/v) $CaCl_2$.

Table 3. *Effect of amino acid sources on the production of Bacillus subtilis RSKK96 α-amylase*

Tablo 3. *Bacillus subtilis RSKK96'nın α-amilaz üretimi üzerine aminoasit kaynaklarının etkisi*

Amino Acid 0.01% (w/v)	Specific Activity (U/mg)
Control	521.0±60.1
Glysine	382.0±56.9
L-lysine	619.2±59.5
L-tyrosine	358.5±73.9
L-cysteine	335.5±64.3
Glutamic acid	629.0±61.5
L-alanine	268.4±53.2
L-tryptophane	415.8±55.1
L-phenylalanine	538.4±60.8
L-isoleusine-	510.0±39.7
L-valine	183.1±58.2
L-metionine	445.3±63.8

Supplementtaion with agroindustrial residue such as cotton stalk various concentrations [0.25, 0.5, 1 and 2% (w/v)] to the fermentation media for production α-amylase by *B. subtilis* RSKK96. Comparison with the control (669.2±36.1 U/mg), significant increase (858±41.9 U/mg) was observed with the addition at 0.5% (w/v) cotton stalk *(Fig. 3)*.

Table 4. *Effect of metal salts sources on the production of Bacillus subtilis RSKK96 α-amylase*

Tablo 4. *Bacillus subtilis RSKK96'nın α-amilaz üretimi üzerine metal tuzu kaynaklarının etkisi*

Metal salts 0.1% (w/v)	Specific Activity(U/mg)
Control	669.2±50.2
$FeSO_4$	no growth
$MgSO_4$	401.2±40,9
$CaCl_2$	618.5±29,2
$CuSO_4$	no growth
$ZnSO_4$	no growth

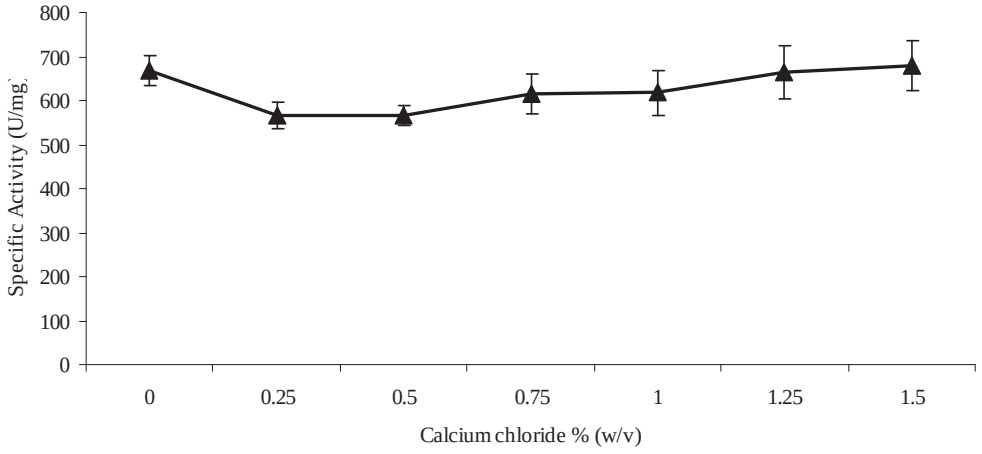

Fig 2. Effect of different calcium chloride ($CaCl_2$) concentrations on the production of *Bacillus subtilis* RSKK96 α-amylase

Şekil 2. *Bacillus subtilis* RSKK96'nın α-amilaz üretimi üzerine farklı kalsiyum klorür ($CaCl_2$) konsantrasyonlarının etkisi

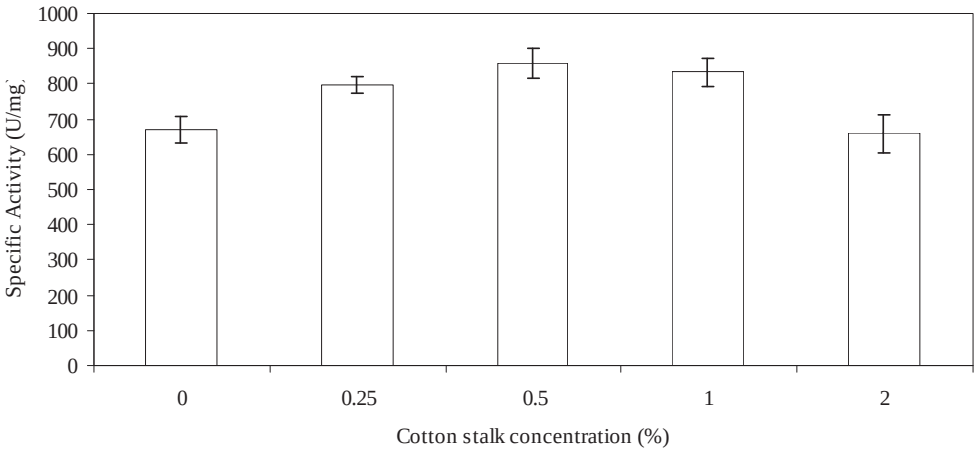

Fig 3. Effect of cotton stalk concentrations on the production of *Bacillus subtilis* RSKK96 α-amylase

Şekil 3. *Bacillus subtilis* RSKK96'nın α-amilaz üretimi üzerine pamuk sapı konsantrasyonlarının etkisi

DISCUSSION

Members of the genus *Bacillus* produce a large variety of extracellular enzymes of which amylases are of particularly significant industrial importance [8]. The production of α-amylase by submerged fermentation (SmF) investigated and is affected by a variety of physicochemical factors. Our results indicated that the optimum incubation period for amylase production was 72 h. This result is in complete accordance with finding of many investigators [9,10]. Incubation beyond 72 h was undesirable as this resulted in decreased enzyme yields. The reason for this might have been due to the denaturation of the enzyme caused by the interaction with other components in the medium [11] and probably due to depletion of nutrients available to microorganism.

The nature of carbon source in culture media is important for the growth and production of extracellular amylase in bacteria. It was expected that the α-amylase production by the bacteria would be enhanced by growing the organisms on the medium with the addition of carbon sources. However, the synthesis of α-amylase by the *B. subtilis* RSKK96 was seriously suppressed when the bacteria was grown on arabinose, glycerol and fructose. Albayrak et al.[12] also found that glucose, fructose, saccharose, and maltose affected enzyme activity in a negative manner. According to previous studies, carbohydrate degrading enzymes in most species of the genus *Bacillus* are subject to catabolite repression by readily metabolizable substrates [13,14]. Therefore, our results are in good agreement with the findings of these studies.

Among the different nitrogen sources tested, casein was found to be a good nitrogen source for both bacterial growth and α-amylase production. Other nitrogen containing compounds such as organic did not support α-amylase production. It has been reported that more α-amylase was produced when organic nitrogen compounds were used [15,16]. Inorganic nitrogen sources inhibited amylase synthesis. This finding is in accordance with Aiyer et al.[17]. The effect of amino acids supplementation of the production medium on enzyme production was studied. Glutamic acid and L-lysine were found to be the ideal amino acids sources, respectively. 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. The differences in nutritional requirements of various α-amylase producing organisms or microbial strains could be attributed to the difference in their genetics [8]. At 1%, $FeSO_4$, $CuSO_4$, and $ZnSO_4$ completely inhibited bacterial growth, and, as a result, amylase production. The result are also confirmed by Kıran et al.[14], who stated that the presence of $ZnSO_4$ had a potent inhibitory effect on the production α-amylase from *Bacillus* sp. K-12. The other metal salts tested decreased enzyme production. The inhibitory effects of some of the salts may be related to the pH changes associated with their use in the medium. Ca^{+2} had significant effects on the metabolism and physiology of bacteria, and there was also found to be an effect on enzyme activity and stabilization in the defense against proteases [18]. We did not find a high increase with $CaCl_2$, but Prakash et al., suggest that there is higher production with $CaCl_2$ [19]. The importance of Ca^{+2} ions has been reported by many researchers [19,20]. Similarly in our study, Sarıkaya and Gürgün reported that, Ca^{+2} did not have significant effect on enzyme production [21].

Cotton stalk was good inducer for α-amylase production in fermentation medium. Cost and efficiency are major characteristics of enzymes for industrial application. Hence, it is desirable for the material used in the fermentation medium to be of low cost, available in large amounts, and continually renewable, which is exactly the case of agroindustrial residues, in addition to making microbial growth possible. Thus, it should present in its composition carbon, nitrogen, and mineral sources, and, in the case of enzyme production, the presence of inducers is essential [22]. For the production of α-amylase, substrates that contained proteins in its composition were chosen, and a nutrient solution was incorporated with the aim of making it suitable for the microorganism to grow in the desired fermentation [23].

The best condition for high α-amylase production by *B. subtilis* RSKK96 was using cotton stalk in SmF. In spite of the fact that cotton stalk was the best substrate for α-amylase production, it should be noted that the inclusion of inducers such as the ones used in this work (glutamic acid, and lysine) might be beneficial as additives to the suitable solid substrate and should be further studied.

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