Effects of Carbon Sources and Various Chemicals on the Production of a Novel Amylase from a Thermophilic Bacillus sp. K-12

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Received: 16.04.2004

Abstract: The amylase producer thermophilic Bacillus sp. K-12 was isolated from soil samples from Zeytinli hot spring in Kahramanmaraş. Enzyme synthesis occurred at 20-55 °C with an optimum of 42 °C. There was a slight variation in amylase synthesis within the pH range 4.5-10.5. Effects of various carbon sources and chemicals on α-amyrase production were examined and maximum α-amyrase production was obtained in a medium containing 1% starch in 60 h. MnSO₄, ZnSO₄ and EDTA inhibited α-amyrase production of Bacillus sp. K-12.

Key Words: Thermostable, Amylase, Bacillus

Introduction

Starch, the primary storage polysaccharide in plants, is degraded by amylolytic enzymes from numerous microorganisms (1). Amylases from plants, animals and microorganisms have been studied since enzymes were first discovered (2). Amylases are among the most important enzymes and are of great significance in present day biotechnology. Enzymes from microbial sources generally meet industrial demands. The spectrum of amylase application has widened in many other fields, such as clinical, medical and analytical chemistry, as well as their wide separate applications in starch saccharafication, textile industry, and the food, brewing and distilling industries (3).

Several Bacillus spp. and thermostable Actinomycetes like thermomonospora and thermoactinomyces are versatile producers of the enzyme (4). The genus Bacillus produces a large range of extracellular enzymes, of which amylases and proteases are of significant industrial importance. An extremely thermostable α-amylases is available from the mesophile Bacillus licheniformis (5).

Recent research with thermostable α-amylase has concentrated on the enzymes of thermophiles and extreme thermophiles (Bacillus licheniformis, Bacillus amyloliquifaciens) (6) and little is known about the properties of the enzymes produced by these organisms. α-amyrases have had many commercial applications for several decades. These enzymes are used in the textile and paper industries, in starch liquefaction, as a food adhesive, and in sugar production (4). The present study deals with the isolation of a bacterium and describes the effects of culture conditions on the activity of α-amyrase.
Materials and Methods

Organism and culture conditions: *Bacillus* sp. K-12 was isolated from soil samples collected in Kahramanmaraş. Gram-positive spore-forming bacteria *Bacillus* sp. soil was pasteurized at 60 °C for 30 min (7). This organism was found to produce an amylase on M9 agar plates containing peptone 0.5% g, yeast extract 0.3% g, 1% (w/v) soluble starch, NaCl 0.3% g, K2HPO4 0.1% g, MgSO4·7H2O 0.02% g and agar 1.5% g (8). The organism was propagated at different temperatures (20-55 °C) and pH values (4.5-10.5) (8). Amylase production was detected after flooding the plates with iodine solution (9).

Enzyme production: The organism was propagated at 42 °C for 3 days in 100 ml of medium with shaking on a shaker (100 rpm). Samples were taken at 12-h intervals (12, 24, 36, 48, 60, 72 h). The supernatant of the culture after centrifugation (6000 rpm, 20 min) at 4 °C was used to determine extracellular amylase activity (8,10,11).

Enzyme Assay: Saccharolytic activity was determined (12). The reaction mixture contained 1 ml of substrate solution [2% soluble starch in 40 mM potassium phosphate buffer (pH 6) including 1 mM CaCl2] and 1 ml of the enzyme solution (10). After 10 min of incubation at 70 °C, the reaction was stopped by the addition of 2 ml of dinitrosalicylic acid solution (1,12). The mixture was heated at 100 °C for 5 min and measured at 540 nm (1). The enzyme activities were calculated using a calibration curve prepared with D-glucose as standard by following the same procedure above. One unit of activity was defined as the amount of enzyme that catalyzed the liberation of reducing sugar equivalent to 1 mmol of D-glucose per min under the assay conditions.

Protein Assay: The protein concentrations determined using bovine serum albumin as standard (13).

Effects of temperature and initial pH: The effect of temperature on enzyme production was determined by measuring activity at 20, 30, 37, 42, 50 and 55 °C (1,12). The effect of initial pH on amylase production was performed at pH 4.5-10.5. The buffers used were 0.2 M sodium citrate (pH 4-6), 0.2 M sodium phosphate (pH 6-8), 0.2 M glycine-NaOH (pH 8.5-10.5) (14).

Effects of carbon sources: To determine the effect of carbon sources, the starch in complete medium was replaced with 1% sucrose, 1% lactose and 1% dextrose. Total enzyme activity (U), total protein amount (mg), and specific activity (U/mg) were analyzed at 12-h intervals during incubation.

Results and Discussion

In industry, bacterial alpha amylases are produced mainly from cultures of *Bacillus subtilis* var. amyloliquefaciens (15,16). *Bacillus stearothermophilus* and *Bacillus licheniformes* alpha amylases are well characterized and heavily used in the starch processing industry. Since thermostability is an important factor in the use of amylolytic enzymes in starch processing, amylases from thermophilic and hyperthermophilic bacteria are of special interest as a source of novel thermostable enzymes (17).

Effects of MnSO4, ZnSO4 and EDTA: Effects of MnSO4, ZnSO4 and EDTA on bacterial growth and enzyme production were examined with the addition of these chemicals to the growth medium. Total enzyme activity (U), total protein amount (mg), and specific activity (U/mg) were analyzed at 12-h intervals during incubation.
growth of *Bacillus licheniformis* TCRDC-B13 occurred at pH 3-11, and it was observed that growth of TCRDC-B13 decreased with increasing pH. Initial pH was 5 for enzyme synthesis of *Bacillus licheniformis* TCRDC-B13 and enzyme synthesis continued until pH 10 and maximum activity occurred at pH 6-9 (4). Boyer and Ingle (2) found that the optimum pH was 9.2 for amylase activity.

**Effects of carbon sources:** To investigate the effects of various carbon sources, *Bacillus* sp. K-12 strain was incubated in the medium containing starch, sucrose, lactose or dextrose for 72 h and samples were analyzed at 12, 24, 36, 48, 60, and 72 h. Growth and enzyme production were different for each medium. The results of α-amylase enzyme activity (U/ml per min) of *Bacillus* sp. K12 at various time intervals are shown in Figure 2. Among the carbon sources tested, starch was found to support α-amylase synthesis, whereas dextrose, lactose and sucrose suppressed enzyme production. The maximum enzyme level was obtained in the medium containing starch at 60 h. Teodoro and Martins (18) found that α-amylase synthesis was diminished when glucose (0.5%) was added to the culture medium. Bajpai and Bajpai (4) concluded that the synthesis of α-amylase was greatly suppressed when the bacterium was grown on sucrose, glucose, or fructose; amylase production was enhanced when the bacterium was grown on starch and dextrin. Albayrak et al. (11) 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 (8). Therefore, our results are in good agreement with the findings of these studies.

Table 1 shows total enzyme activity, total protein amount and specific activity for dextrose, lactose and sucrose. Among the dextrose, lactose and sucrose α-amylase was suppressed by sucrose much more than by the others. In media containing dextrose and sucrose, total α-amylase activity reached 65.34 U and 42.60 U, respectively, whereas in the medium containing sucrose total α-amylase activity only reached 17.88 U.

**Effects of MnSO₄, ZnSO₄ and EDTA:** The effects of various chemicals on amylase production were investigated by growing strain K-12 in complete medium supplemented with MnSO₄, ZnSO₄ and EDTA. Total protein amount, total activity and specific activity were analyzed at 12, 24, 36, 48, 60, and 72 h. The results are shown in Table 2.

Maximum total activity was 8.88 U, in medium containing MnSO₄.H₂O; after 36 h activity decreased. Sarikaya (19) reported that Mn favored the synthesis of amylase. Kadrekar and Ramasarma (20) found that Mn³⁺ supported amylase synthesis. Aguilar et al. (21) and Lin et al. (8) observed that Mn³⁺ had no effect on α-amylase activity. However, Shatta et al. (22) observed that amylase production decreased from 570 U to 425 U in medium containing Mn³⁺.

With the addition of ZnSO₄.7H₂O to the medium, α-amylase production decreased after 48 h. Zn²⁺ addition to the medium inhibited the production of α-amylase. Early studies showed that the effect of Zn²⁺ varied between amylases. Shatta et al. (22) tested the effect of Zn²⁺ and they found that amylase production decreased from 570...
to 415 U. The results are also confirmed by Kadrekar and Ramasarma (20), who stated that the presence of \(\text{Zn}^{2+}\) had a potent inhibitory effect on the amylases from \(\text{Schwanniomyces alluvius}\) and \(\text{Bacillus cereus NY 14}\). Igarashi et al. (23) found that \(\text{Zn}^{2+}\) strongly inhibited the enzymatic activity (91%) of alkaliphilic \(\text{Bacillus sp.}\). As for the thermostable \(\alpha\)-amylase from a thermophilic \(\text{Bacillus sp.}\), 46% and 13% inhibition were reported, suggesting that the inhibition with \(\text{Zn}^{2+}\) determines the thermostability of the enzyme (24). Arıkan et al. (14) found that \(\text{Zn}^{2+}\) showed 37% inhibition on enzyme production from \(\text{Bacillus sp. Ant-6}\) and inhibition with the addition of \(\text{Zn}^{2+}\) was also reported by Aboud-zeid (25).

It was observed that in medium containing 10 mM EDTA, maximum total amylase activity occurred in 12 h (3.52 U); after 12 h, total activity decreased. Twelve percent inhibition was reported in 20 mM EDTA containing medium by Boyer and Ingle (2). Albayrak et al. (11) found that amylase activity rapidly decreased with more than 0.3 mM EDTA. Five percent inhibition was reported in containing 10 mM medium EDTA by Arıkan et al. (14). Similarly, EDTA has been found to be a potent inhibitor of amylases from \(\text{Myxococcus coralloides}\) and \(\text{S. alluvius}\) (8). According to our results and early studies, the inhibitory effect of the chelating agent EDTA, which binds metal ions, demonstrated the ion requirement of the amylase (26). However, saccharifying amylases from \(\text{Bacillus sp. strain A-40-2}, \text{Bacillus sp. strain NRRL B-3881}\) and \(\text{Bacillus alkathermophilis A3-8}\) are all stable in response to EDTA treatment (2,27,28).

### Acknowledgment

This research was supported by the Kahramanmaraş Sütçü İmam University Research Fund (2001.7/4).

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**Table 1.** Effect of carbon sources on total \(\alpha\)-amylase production, total protein and specific activity of amylase from \(\text{Bacillus sp. K-12}\).

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<tr>
<th>Time (h)</th>
<th>Sucrose (U)</th>
<th>Lactose (U)</th>
<th>Dextrose (U)</th>
<th>Sucrose (mg)</th>
<th>Lactose (mg)</th>
<th>Dextrose (mg)</th>
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**Table 2.** Effect of \(\text{MnSO}_4\), \(\text{ZnSO}_4\) and EDTA on total \(\alpha\)-amylase production, total protein and specific activity of amylase from \(\text{Bacillus sp. K-12}\).

<table>
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<th>(\text{ZnSO}_4) (U)</th>
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<th>(\text{MnSO}_4) (mg)</th>
<th>(\text{ZnSO}_4) (mg)</th>
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