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

Özlem KIRAN[1], Uğur ÇÖMLEKÇİOĞLU[1], Burhan ARIKAN[2]

[1]Kahramanmaraş Sütçü İmam University, Faculty of Arts and Sciences, Department of Biology, Kahramanmaraş - TURKEY

[2]Çukurova University, Faculty of Arts and Sciences, Department of Biology, Adana - TURKEY

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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 ºC with an optimum of 42 ºC. 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-amylase production were examined and maximum α-amylase production was obtained in a medium containing 1% starch in 60 h. $MnSO_4$, $ZnSO_4$ and EDTA inhibited α-amylase production of *Bacillus* sp. K-12.

Key Words: Thermostable, Amylase, *Bacillus*

Termofilik *Bacillus* sp. K-12'nin, Yeni Bir Amilaz Üretimi Üzerine Karbon Kaynakları ve Çeşitli Kimyasalların Etkisi

Özet: Amilaz üreticisi termofilik *Bacillus* sp. K-12 suşu Kahramanmaraş'ta bulunan Zeytinli Ilıcası'ndan alınan toprak örneklerinden izole edilmiştir. Enzim sentezi 20-55 ºC sıcaklıkları arasında gerçekleşirken optimum sıcaklık 42 ºC olarak bulunmuştur. Amilaz sentezinde 4,5-10,5 pH aralıklarında değişimler görülmüştür. Amilaz üretimi üzerine çeşitli karbon kaynakları ve kimyasalların etkisi incelenmiştir. Maksimum α-amilaz üretimi %1 nişasta içeren besiyerinde 60. saatte elde edilmiştir. $MnSO_4$, $ZnSO_4$ ve EDTA, *Bacillus* sp. K-12'in amilaz üretimini inhibe ettiği görülmüştür.

Anahtar Sözcükler: Termal Kararlı, Amilaz, *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 amyloliquefaciens*) (6) and little is known about the properties of the enzymes produced by these organisms. α-amylases 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-amylase.

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, K_2HPO_4 0.1% g, $MgSO_4.7H_2O$ 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 $CaCl_2$] 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.

Effects of $MnSO_4$, $ZnSO_4$ and EDTA: Effects of $MnSO_4$, $ZnSO_4$ 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.

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 temperature and initial pH: Enzyme production of *Bacillus* sp. K-12 was analyzed at different temperatures and pH values. The production of enzyme was determined at 20, 30, 37, 42, 50 and 55 °C. Optimal production was observed at 42 °C. The maximum production was around 93% at pH 6-8, but alkaline activity was around 54% at pH 8.5-9.5. The experiments were repeated 3 times and mean values were used.

Enzyme synthesis and bacterial growth occurred between 20 and 55 °C, with an optimum of 42 °C. Lin et al. (1) found that enzyme synthesis of *Bacillus* sp. TS-23 occurred between 42 and 60 °C, with an optimum of 55 °C. Bajpai and Bajpai (4) reported that the enzyme synthesis and growth temperature of *Bacillus licheniformis* TCRDC-B13 strain was 25-50 °C and maximum enzyme production was obtained at 35 °C.

Enzyme synthesis and bacterial growth of K-12 strain was observed at pH 4.5-10.5. The maximum enzyme production was obtained at pH 6-8. Enzyme production was decreased at pH 8.5 and there was a slight variation in amylase synthesis at pH 8.5-9.5 (Figure 1). Bacterial

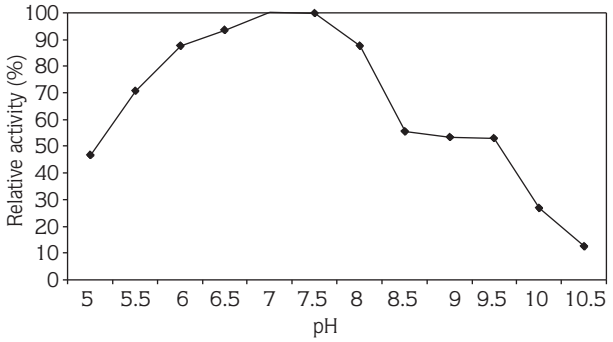

Figure 1. Effect of the initial pH of the culture medium and relative α-amylase production by *Bacillus* sp. K-12.

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 showed a repressive effect on α-amylase production. Starch favored α-amylase production, but sucrose, lactose and dextrose 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.

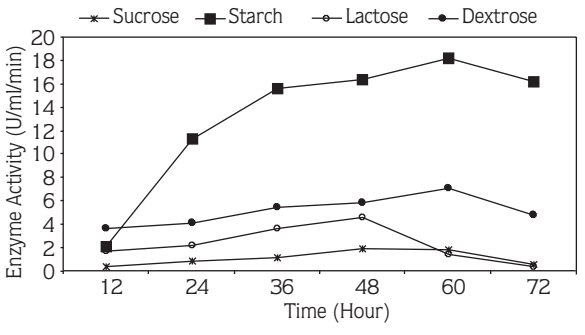

Figure 2. Effects of carbon sources on α-amylase enzyme activity (U/ml per min) of *Bacillus* sp. K-12 at various time intervals.

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 $MnSO_4$, $ZnSO_4$ and EDTA: The effects of various chemicals on amylase production were investigated by growing strain K-12 in complete medium supplemented with $MnSO_4$, $ZnSO_4$ 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 $MnSO_4.H_2O$; after 36 h activity decreased. Sarıkaya (19) reported that Mn favored the synthesis of amylase. Kadrekar and Ramasarma (20) found that Mn^{2+} supported amylase synthesis. Aguilar et al. (21) and Lin et al. (8) observed that Mn^{2+} 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 Mn^{2+}.

With the addition of $ZnSO_4.7H_2O$ to the medium, α-amylase production decreased after 48 h. Zn^{2+} addition to the medium inhibited the production of α-amylase. Early studies showed that the effect of Zn^{2+} varied between amylases. Shatta et al. (22) tested the effect of Zn^{2+} and they found that amylase production decreased from 570

Table 1. Effect of carbon sources on total α-amylase production, total protein and specific activity of amylase from *Bacillus* sp. K-12.

	Total Activity (U)			Total Protein (mg)			Specific Activity (U/mg)		
Time (h)	Sucrose	Lactose	Dextrose	Sucrose	Lactose	Dextrose	Sucrose	Lactose	Dextrose
12	3.74	16.08	33.33	20.53	28.83	31.02	0.18	0.56	1.07
24	8.29	20.16	37.70	21.22	29.89	32.34	0.39	0.67	1.17
36	10.57	33.65	50.36	22.99	32.39	29.33	0.71	1.04	1.72
48	17.88	42.60	53.72	25.47	38.80	31.05	0.80	1.48	1.73
60	16.39	13.72	65.34	24.11	30.89	30.93	0.68	0.44	2.11
72	5.19	3.94	44.19	24.54	28.06	29.65	0.21	0.14	1.49

Table 2. Effect of $MnSO_4$, $ZnSO_4$ and EDTA on total α-amylase production, total protein and specific activity of amylase from *Bacillus* sp. K-12.

	Total Activity (U)			Total Protein (mg)			Specific Activity (U/mg)		
Time (h)	EDTA	$MnSO_4$	$ZnSO_4$	EDTA	$MnSO_4$	$ZnSO_4$	EDTA	$MnSO_4$	$ZnSO_4$
12	3.52	6.52	2.74	15.38	27.67	18.22	0.23	0.24	0.15
24	3.45	7.55	3.20	15.54	28.33	18.06	0.22	0.27	0.18
36	2.27	8.88	2.13	17.70	25.45	16.01	0.13	0.35	0.13
48	2.04	6.44	1.81	16.23	22.73	18.48	0.13	0.28	0.10
60	2.39	6.18	1.68	14.95	20.75	19.30	0.16	0.30	0.09
72	2.55	5.09	1.89	16.15	19.27	16.95	0.16	0.26	0.11

to 415 U. The results are also confirmed by Kadrekar and Ramasarma (20), who stated that the presence of Zn^{2+} had a potent inhibitory effect on the amylases from *Schwanniomyces alluvius* and *Bacillus cereus* NY 14. Igarashi et al. (23) found that Zn^{2+} strongly inhibited the enzymatic activity (91%) of alkaliphilic *Bacillus* sp. As for the thermostable α-amylase from a thermophilic *Bacillus* 46% and 13% inhibition were reported, suggesting that the inhibition with Zn^{2+} determines the thermostability of the enzyme (24). Arıkan et al. (14) found that Zn^{2+} showed 37% inhibition on enzyme production from *Bacillus* sp. Ant-6 and inhibition with the addition of 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 *Myxococcus coralloides* and *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 *Bacillus* sp. strain A-40-2, *Bacillus* sp. strain NRRL B-3881 and *Bacillus alkalothermophilis* A3-8 are all stable in response to EDTA treatment (2,27,28).

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Corresponding author:

Özlem KIRAN

Kahramanmaraş Sütçü İmam Üniversitesi,

Fen Edebiyat Fakültesi,

Biyoloji Bölümü Kahramanmaraş - TURKEY

E-mail: ozkiran@ksu.edu.tr

References

1. Lin LL, Hsu WH, Chu WS. A gene encoding for an α-amylase from thermophilic *Bacillus* sp. Strain TS-23 and its expression in *Escherichia coli*. J. Appl. Microbiol., 82: 325-334, 1997.
2. Boyer EW, Ingle M. Extracellular alkaline amylase from a *Bacillus* species. J. Bacteriology, 110: 992-1000, 1972.
3. Pandley A, Nigam P, Soccol CR et al. Advances in microbial amylases. Biotechnol. Appl. Biochem, 31: 135-152, 2000.
4. Bajpai P, Bajpai PK. High-temperature alkaline α-amylase from *Bacillus licheniformis* TCRDC-B13. Biotechnol Bioengineering, 33: 72-78, 1989.
5. Morgan FJ, Priest FG. Characterization of thermostable α-amylase from *Bacillus licheniformis* NCTB 6346. J. Appl. Bacteriol, 50: 74-80, 1981.
6. Teodoro CES, Martins MLL. Culture conditions for the production of thermostable amylases by *Bacillus* sp. Brazilian Journal of Microbiology, 31: 1-9, 2000.
7. Kiran O. Doğal ortamdan izole edilen alkalifilik *Bacillus sp.* Su?larında a- amilaz üretimi, enzimin pH ve sıcaklık stabilitesinin araştırılması, PhD Çukurova Üniversitesi Fen Bilimleri Enstitüsü, 2001.
8. Lin LL, Chyau CC, Hsu WH. Production and properties of a raw-starch-degrading amylase from the thermophilic and alkaliphilic *Bacillus* sp. TS-23. Biotechnol. Appl. Biochem, 28: 61-68, 1998.
9. Hols P, Ferain T, Garmyn D et al. Use of expression secretion signals and vector free stable chromosomal integration in engineering of *Lactobacillus plantarum* for α-amylase and levanase expression. Appl Environ Microbiol, 60: 1401-3, 1994.
10. Aira S, Kilal K, Imanaka A. Cloning and expression of thermostable α-amylase gene from *Bacillus stearothermophilus* in *Bacillus stearothermophilus* and *Bacillus subtilis*. Appl Environ Microbiol, 1059-1065, 1983.
11. Albayrak N, Donmez S, Balk M. Toprak ve su örneklerinden amilolitik bakterilerin izolasyonıu ve bazı enzimatik özelliklerinin belirlenmesi. Tr. J. of Biology, 20: 47-54, 1996.
12. Bernfeld P. Amylases α and β. Methods Enzymol, 1: 149-158, 1955.
13. Lowry OH, Rosebrough NJ, Forr AC et al. Protein measurement with the folin phenol reagent. J. Biol. Chem, 193: 265-275, 1951.
14. Arikan B, Unaldi N, Coral G et al. Enzymatic properties of novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic *Bacillus* sp. isolate ANT-6. Process Biochemistry, 38: 1397-1403, 2003.
15. Aunstrup K. Production, isolation and economics of extracellular enzymes, In. Wingard JR, Kattchalski-Katzir LB, Golstein L (Eds.). Enzyme Technology, Appl Biochem Bieoeng, vol. 2, Academic Press, NY/San Francisco/London, 1979.
16. Uhlig H. Industrial Enzymes and their Application. PhD, Translated and Updated by Elfride M. Linsmaier-Bednar, Wiley, 1998.
17. Leveue E, Janacek S, Haye B et al. Thermophilic archeal amylolytic enzymes. Trends in Biotechnol, 7: 49-53, 1989.
18. Teodoro CES, Martins MLL. Culture conditions for the production of thermostable amylases by *Bacillus* sp. Brazilian Journal of Microbiology, 31: 1-9, 2000.
19. Sarıkaya E. α-Amilaz üreten bazı *Bacillus* suşlarının gelişme parametreleri, enzim özellik ve üretim koşullarının optimizasyonu, PhD Ankara Üniversitesi Fen Bilimleri Enstitüsü, 1995.
20. Kadrekar SN, Ramasarma GB. Amylase and protease production by *Bacillus subtilis*: Studies on improvement of strain and medium. J. Fd. Sci. Technol, 27: 4-6, 1990.
21. Aguilar G, Guyot MJ, Aguilar TB et al. Purification and characterization of an extracellular a-amilase produced by *Lactobacillus manihotivorans* LMG 18010^T amylolytic lactic acid bacterium. Enzyme and Microbial Technology, 27: 406-413, 2000.
22. Sahatta AM, El-Hamahmy AF, Ahmed FH et al. The influence of certain nutritional and environmental factors on the production of amylase enzyme by *Streptomyces aureofaciens* 77. Journal of Islamic Academy of Sciences, 3:2, 134-138, 1990.
23. Igarashi K, Hatada Y, Hagihara H et al. Enzymatic properties of a novel liquefying α-amylase from an alkaliphilic *Bacillus* isolate and entire nucleotide and amino acid sequences. Appl Environ Microbiol, 64: 9, 3282-3289, 1998.
24. Mamo G, Gessesse A. Purification and characterization of two raw-starch-digesting thermostable α-amylase from a thermophilic *Bacillus*. Enzyme Microb Technol, 25: 433-8, 1999.
25. Aboud-zeid AM. Production and characterization of an extracellular α-amylase enzyme from *Aspergillus flavus*. Microbios. 89: 55-66, 1997.
26. Özcelik S. Genel Mikrobiyoloji Uygulama Kılavuzu. Süleyman Demirel Üniversitesi Ziraat Fakültesi Yayınları No.2, 92s, 1995.
27. Horikoshi K. Alkaliphiles–from an industrial point of view. FEMS Microbiology Reviews, 18: 259-270, 1996.
28. Oxaki A, Tanaka A. Heat stable alkaline amylase from *Bacillus.* Japanase Kokai Patent, 9, 049, 584, 1990.