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Research / Araştırma

PRODUCTION of AMYLASE by A NOVEL *Bacillus* sp. ZBP10 in SUBMERGED FERMENTATION

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Abstract

Bacillus sp. ZBP10 is an amylase producing strain isolated from a soil sample collected from a potato cultivation field in Sakarya. In this study, culture conditions were optimized for *Bacillus* sp. ZBP10 in order to increase amylase production using submerged fermentation. The effects of temperature (30-40°C), fermentation time (24-72 h), initial medium pH (6.0-9.0), carbon sources (soluble, wheat, rice and corn starches) and substrate concentration (5-30 g/L) on the production of amylase were determined. According to the results, the bacterium produced maximum amount of amylase, when the initial pH of the medium was 7.0, at 33°C, and within 48 h. Soluble starch was the best substrate among the starches tested. Optimum substrate concentration was 20 g/L for enzyme production with which 3.57±0.19 U/mL enzymatic activity was obtained.

Key Words: Amylase, *Bacillus*, enzyme production, starch.

Bacillus sp. ZBP10 SUŞU ile DERİN KÜLTÜR FERMANTASYONUNDA AMİLAZ ÜRETİMİ

Özet

Bacillus sp. ZBP10, Sakarya'da patates yetiştirilen bir alandan alınan topraktan izole edilmiş olan amilaz üreticisi bir suştur. Bu çalışmada, derin kültür fermantasyonu ile *Bacillus* sp. ZBP10'un amilaz üretimini arttırmak için üretim koşullarının optimizasyonu yapılmıştır. Amilaz üretimine sıcaklık (30-40°C), fermantasyon süresi (24-72 saat), besiyeri başlangıç pH'sı (6.0-9.0), karbon kaynakları (çözünür, buğday, pirinç ve mısır nişastaları) ve substrat konsantrasyonunun (5-30 g/L) etkisi belirlenmiştir. Elde edilen sonuçlara göre, bakteri en iyi amilaz üretimini başlangıç pH'sı 7.0 olan besiyerinde 33°C inkübasyon sıcaklığında, 48 saatte gerçekleştirmiştir. Çalışmada kullanılan nişasta kaynaklarından amilaz üretiminde en etkili olanının çözünür nişasta olduğu belirlenmiştir. Optimum substrat konsantrasyonunun ise 20 g/L olduğu belirlenmiş ve bu konsantrasyonda 3.57±0.19 U/mL amilaz aktivitesine ulaşılmıştır.

Anahtar kelimeler: Amilaz, *Bacillus*, enzim, nişasta.

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INTRODUCTION

Amylases (EC. 3.2.1.1) are extracellular enzymes that catalyze the hydrolysis of starch into dextrins, maltose and glucose (1). They are secreted by plants, animals and microorganisms. However, commercial production is dominated by microbial processes due to thermostability and higher enzyme yields and productivities. Moreover precise control of process conditions (e.g. temperature and pH) is more convenient and microorganisms can be manipulated more easily to increase the efficiency of the enzyme (2-4). Amylases command 25-30% of the global enzyme market, which is estimated to be 6 billion dollars in 2016 (5-7). The major industrial application of amylases is for starch hydrolysis, which has largely replaced chemical hydrolysis processes (8). The food industry is the main user of amylases where they are applied for glucose syrup production and by the brewing and baking industry. In addition, the textile, leather, detergent, paper and pulp industries also utilize amylases in their processes (9-13).

Industrial amylases are produced using *Aspergillus oryzea* and *Bacillus* species (8, 12, 14). *Bacillus* species are considered superior for production of amylases (15). The bacteria are also notable for their use in production of a variety of industrially important extracellular enzymes (5). Amongst *Bacillus* strains, *B. subtilis, B. amyloliquefaciens, B. licheniformis, B. stearothermophilus B. megaterium* and *B. circulans* are widely used for amylase production (4). Each produces amylase with different properties. In the development of fermentation processes, optimization of the cultural conditions is necessary in order to improve the enzyme yield for eventual industrial application (3, 8). Solid-state fermentation or submerged fermentation can be applied for the production of amylases (8). Submerged fermentation is often preferred for industrial microbial based processes because of the ability to efficiently control culture conditions including temperature and pH. In addition, it facilitates sterilization and has lower labor costs (16, 17). The objective of this study, to investigate the relation of the environmental conditions, such as temperature, pH, time, substrate concentration, with the amylase production by *Bacillus* sp. ZBP10 that was isolated from soil.

MATERIALS AND METHODS

Materials

3,5 Dinitrosalicylic acid (DNS) was purchased from Sigma-Aldrich (MO, USA), soluble starch, nutrient agar, nutrient broth, yeast extract, peptone, Tris-base were purchased from Merck (Darmstadt, Germany). Wheat and corn starches were purchased from a local vendor. All the chemicals used were analytical grade.

Bacterial strain and seed culture

The strain was isolated from a soil sample collected from Sakarya district and identified as *Bacillus* sp. ZBP10 according to standard morphological and biochemical tests. The culture was stored at -18°C in 50% (v/v) glycerol. It was revived by growing on a nutrient agar plate incubated at 33°C for 24 h. Seed cultures were prepared by inoculating single colonies, from the plates, into 100 mL Erlenmeyer flasks containing 30 mL of nutrient broth. Growth was carried out at 33°C for 24 h on a shaking incubator (Benchmark Incu-Shaker/mini, NJ, USA) set at 120 rpm.

Enzyme production

A basal medium containing soluble starch, 10 g; yeast extract, 5 g; peptone from meat, 5 g; K_2HPO_4, 1 g; $MgSO_4.7H_2O$, 0.2 g; NaCl, 0.5 g is prepared in one liter of deionized water and pH was adjusted to 7.0 by using 2N HCl. Media were filled into 100 mL flasks as 30 mL portions and sterilized at 121°C for 15 min. Five percent (v/v) fresh seed culture was used for inoculation. Unless otherwise indicated incubations were carried out at 33°C for 24 h on a shaking incubator at 120 rpm. In order to study the effect of temperature on the enzyme production, conditions mentioned above were used except for the incubation temperature, which were held at 30; 33; 37 and 40°C. For the pH experiments, initial pH of the basal media were adjusted to 6;7; 8 and 9 by using either 2N NaCl or 2N HCl. Effect of substrate concentration on the enzyme production was determined using 5; 10; 15; 20 and 30 g/L soluble starch in the basal media. Production of enzyme from different substrates was tested by replacing soluble starch in the basal media with starch from rice, wheat or corn (each 10 g/L). Samples were taken at the end of the incubations and centrifuged at 10000 rpm for 10 min by using

Hettich Universal 320 R (Tuttlingen, Germany) centrifuge, and the resulting supernatants were analyzed for amylase activity. Experiments were carried out in duplicate.

Analysis

For the determination of amylase activity, soluble starch (2%; w/v) was prepared in 100 mM Tris-HCl buffer, pH 7.0 and boiled until the starch gelatinized and then cooled to ambient temperature. A 0.1 mL of supernatant was added to the reaction mixture and incubated in a water bath (JSR, JSSB-30T, Gongju-City, Korea) at 60°C for 15 min. Released glucose was measured as reducing sugar content as determined by the DNS (3,5 dinitrosalycylic acid) method. Absorbance was measured at 540 nm using UV-VIS spectrophotometer (Shimadzu UV Mini-1240, Kyoto, Japan). A glucose standard curve was constructed to estimate the reducing sugar content. One unit of amylase activity was defined as the amount of enzyme that liberates 1 µmol glucose per min under the reaction conditions (18).

RESULTS AND DISCUSSION

Bacillus is a workhorse of the enzyme industry, especially for production of amylases (5, 15). Eighty isolates from various environments were screened for amylase production using the starch iodine assay on agar plates. A total of 12 isolates were identified as amylase producers. *Bacillus* sp. ZBP4 was one of the best, thus it was chosen for the further studies. *Bacillus* sp. ZBP10 is Gram-positive, spore-forming, catalase positive, rod shaped bacterium. It was isolated from the soil taken from a potato cultivation area. Cultural conditions were optimized for amylase production. Maximizing enzyme production requires optimizing pH, temperature, substrate type and concentration (5).

Effect of temperature on the amylase production

The bacterium was grown at 30-40°C to determine the optimal temperature (Figure 1). Enzyme production was detected throughout this range. Maximum enzyme production was at 33°C and the minimum at 30°C with a 41% spread in activities between them. Fermentation temperature affects cell growth rate and metabolite production (5, 19). Reported optimal temperatures for prorogating *Bacillus* strains vary widely. *Bacillus* sp. VS.4 (6) and *B altudinis* (11) each had maximum production of amylase at 40°C, and *B. amyloliquefaciens* had its maximum at 42°C (5). On the other hand, there are also thermophilic *Bacillus* strains producing amylase optimally at 55-60°C (20, 21, 22). *Bacillus* sp. KR-8104 has produced amylase at 37°C (23). Ravindar and Elangovan (12) reported maximum amylase production at 32°C with a *B. subtilis* strain, which is quite similar to our finding.

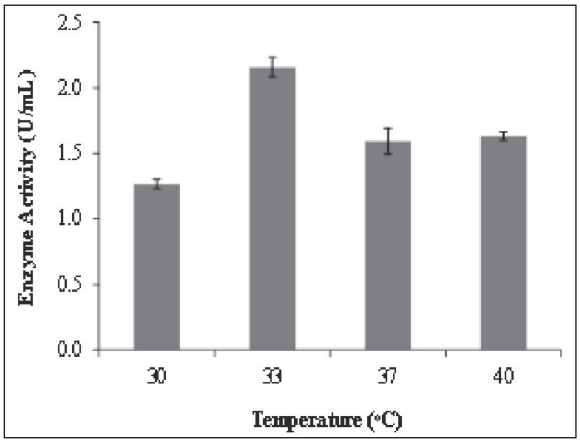

Figure 1. Effect of incubation temperature on amylase production by *Bacillus* sp. ZBP10 in 24 h.

Effect of pH on the amylase production

The results obtained when the bacterium was grown at varying pH values are shown in Figure 2. The pH of the medium alters cell morphology and enzyme secretion in microorganisms, and additionally affects the stability of the enzyme once secreted. Culture pH affects growth and enzyme production and optimal pH varies widely among bacteria (5, 24, 25). Generally, *Bacillus* strains produce the maximum amount of enzyme when the initial pH of the medium is between 6.0 and 9.0. In our case, the optimum pH was pH 7.0. Incubation at pH 6.0 decreased enzyme production by 30%. However, more alkaline pH values supported enzyme production. Measured amylase activities at pH 8.0 and 9.0 were only 3.5 and 7.1% lower than that observed at pH 7.0, respectively. This indicates that the amylase can be produced in a broad pH range by *Bacillus* sp. ZBP10. Various other *Bacillus* strains have also been reported to have an optimal growth pH value of 7.0 (11, 12, 24, 25).

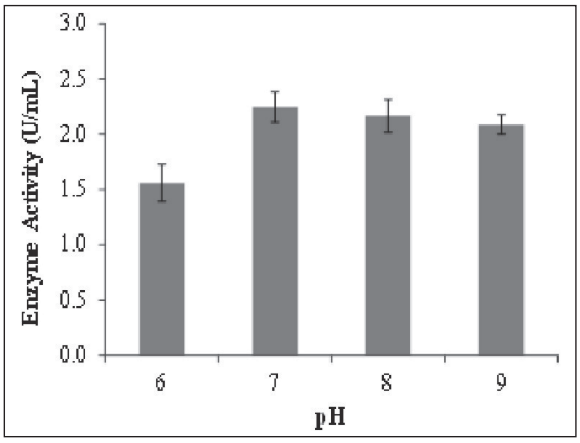

Figure 2. Effect of pH on amylase production by *Bacillus* sp. ZBP10 at 33°C in 24 h.

Effects of various starches on the amylase production

It has been observed that amylase production is induced in the presence of starch (2). For this reason, various starches (e.g. corn, wheat, rice and soluble) were tested to identify which starch is best for amylase production by *Bacillus* sp. ZBP10. Amylase activities obtained with these substrates are presented in Figure 3. The highest enzymatic activity was observed using soluble starch (2.25±0.14 U/mL). Activities using wheat, corn and rice starches were 41, 49 and 59% less than that observed with soluble starch, respectively. According to the literature, substrate requirement for growth of *Bacillus* strains vary widely. For instance, Divakaran et al (4) tested the production of amylase from soluble starch, rice and ground wheat by *B. licheniformis* MTCC strain and observed maximum production on starch (2.59 U/mL). However, they obtained very similar activities with rice and ground wheat to result obtained with starch, which is not what was observed here.

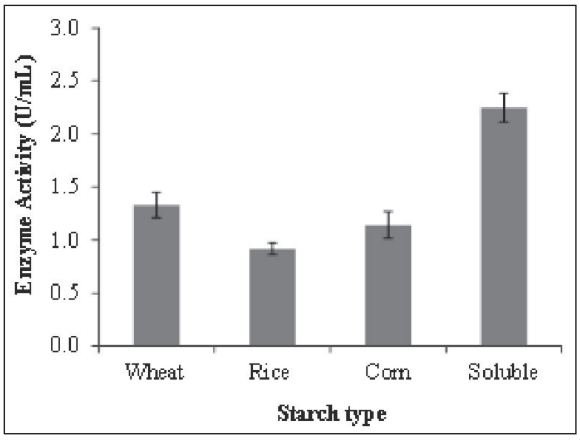

Figure 3. Effects of different carbon sources on the amylase production by *Bacillus* sp. ZBP10.

Effect of substrate concentration on the amylase production

Soluble starch in varying amounts (5-30 g/L) was used to determine the optimal substrate concentration for enzyme production. Enzyme production gradually increased with greater substrate concentration and the maximum amount of enzyme (3.57±0.19 U/mL) was reached using 20 g/L soluble starch concentration (Figure 4). The lowest enzyme production was obtained using 5 g/L substrate concentration and there was a 5-fold increase in maximum enzymatic activity when the concentration was increased from 5 to 20 g/L. However, at 30 g/L soluble starch, the activity declined to 3.13±0.56 U/mL. Our finding are in agreement with those reported by Vishmu et al (6), and Mishra and Behera (26) who reported maximum amylase production at 2% starch concentrations. On the other hand, Saxena et al. (22) reported maximum amylase activity at 0.6% starch concentration.

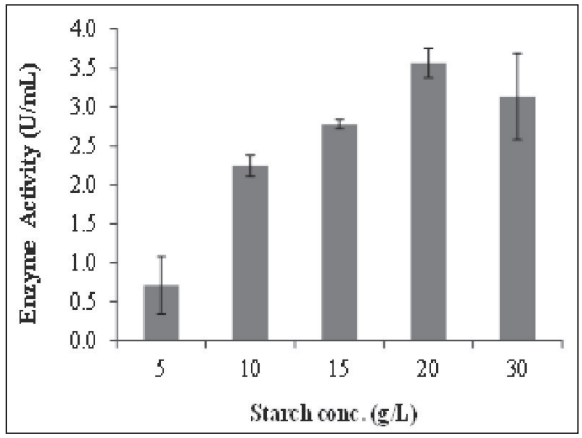

Figure 4. Effect of starch concentration on the production of amylase by *Bacillus* sp. ZBP10.

Effect of incubation time on the amylase production

Bacillus sp. ZBP10 was incubated in the basal medium at 33°C for 72 h and periodically sampled for profiling enzyme production (Figure 5). The strain produced the maximum amount of enzyme (3.4±0.1 U/mL) after 48 h of growth and enzymatic

activity declined at 72h. Enzyme production is growth related and in general reaches maximum at stationary phase (27). Minimizing culture time is important for enzyme production, because prolonged incubations increase the cost of production as well as allowing for enzyme degradation by proteases secreted by the microorganism (12). Most *Bacillus* species studied for amylase production also have been observed to achieve maximum activities at 48 h (11, 16). On the other hand, some *Bacillus* strains produce the maximum amount of amylase in 72 h (8, 28).

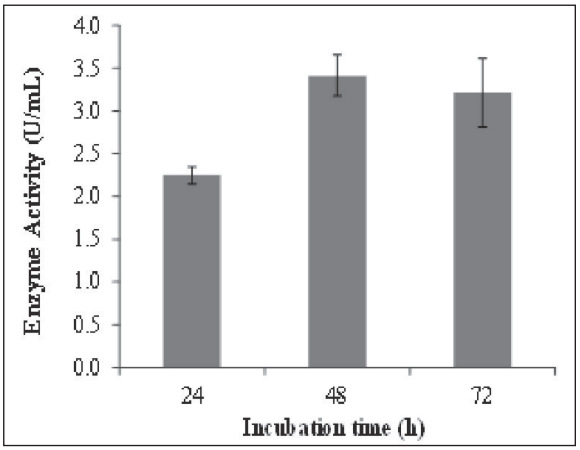

Figure 5. Time course of amylase production by *Bacillus* sp. ZBP10.

CONCLUSIONS

Culture parameters for amylase production by *Bacillus* sp. ZBP10 have been optimized in submerged culture. It produced maximum amylase with an initial pH of the medium at 7.0 after 48 h growth. Culturing on soluble starch led to higher amylase production than using wheat, rice and corn starches. The optimum substrate concentration was 20 g/L.

REFERENCES

1. Rajagopalan G, Krishnan C. 2008. α-amylase production from catabolite depressed *Bacillus subtilis* KCC103 utilizing sugarcane bagasse hydrolysate. *Bioresour Technol*, 99, 3044-3050.

2. Thippeswamy S, Girigowda K, Mulimani VH. 2006. Isolation and identification of a- amylase producing *Bacillus* sp. from dhal industry waste. *Indian J Biochem Biophys*, 43, 295-298.

3. Souza PM, Magalhães PO. 2010. Application of microbial α-amylase in industry: A review. *Braz J Microbiol*, 41, 850-861.

4. Divakaran D, Chandran A, Chandran PR. Comparative study on production of α-amylase from *Bacillus licheniformis* strains. *Braz J Microbiol,* 42, 1397-1404.

5. Deb P, Talukdar SA, Mohsina K, Sarker PK, Abu Sayem SM. 2013. Production and partial characterization of extracellular amylase enzyme from *Bacillus amyloliquefaciens* P-001. *SpringerPlus*, 2, 154.

6. Vishnu TS, Soniyamby AR, Praveesh BV, Hema TA. 2014. Production and optimization of extracellular amylase from soil receiving kitchen waste isolate *Bacillus* sp. VS 04. *World Appl Sci J*, 29 (7), 961-967.

7. Popovic MK, Senz M, Bader J, Skelac L, Schilf W, Bajpai R. 2014. Positive effect of reduced aeration rate on secretion of alpha-amylase and neutral proteases during pressurized fermentation of thermophilic *Bacillus caldolyticus*. *N Biotechnol*, 31 (2), 141-149.

8. Xie F, Quan S, Liu D, Ma H, Li F, Zhou F, Chen G. 2014. Purification and characterization of a novel α-amylase from a newly isolated *Bacillus methylotrophicus* strain P11-2. *Process Biochem*, 49, 47-53.

9. Ma Y, Yang H, Chen X, Sun B, Du G, Zhou Z, Song J, Fan Y, Shen W. 2015. Significantly improving the yield of recombinant proteins in *Bacillus subtilis* by a novel powerful mutagenesis tool (ARTP): Alkaline α-amylase as a case study. *Protein Express Purif*, 114, 82-88.

10. Roy JK, Rai SK, Mukherjee AK. 2012. Characterization and application of a detergent-stable alkaline α-amylase from Bacillus subtilis strain AS-S01a. *Int J Biol Macromol*, 50, 219-229.

11. Kumar Y, Singh PK, Singh AK, Masih H, Peter KJ, Benjamin JC, Rath S. 2014. Production optimization of alpha amylase from *Bacillus altitudinis*. *Int J Sci Eng Technol Res*, 3 (4), 0564-0573.

12. Ravindar DJ, Elangovan N. 2013. Molecular identification of amylase producing *Bacillus subtilis* and detection of optimal conditions. *J Farm Res*, 6, 426-430.

13. Hmidet N, Ali NEH, Haddar A, Kanoun S, Alya SK, Nasri M. 2009. Alkaline proteases and thermostable α-amylase co-produced by *Bacillus licheniformis* NH1: Characterization and potential application as detergent additive. *Biochem Eng J*, 47, 71-79.

14. Sahnoun M, Bejar S, Sayari A, Triki MA, Kriaa M, Kammoun R. 2012. Production, purification and characterization of two α-amylase isoforms from a newly isolated *Aspergillus oryzae* strain S2. *Process Biochem*, 47, 18-25.

15. Anto H, Trivedi U, Patel K. 2006. Alpha amylase production by *Bacillus cereus* MTCC 1305 using solid-state fermentation. *Food Technol Biotechnol*, 44 (2), 241–245.

16. Sankaralingam S, Shankar T, Ramasubburayan R, Prakash S, Kumar C. 2012. Optimization of culture conditions for the production of amylase from *Bacillus licheniformis* on submerged fermentation. *American-Eurasian J Agric Environ Sci*, 12 (11), 1507-1513.

17. Benjamin S, Smitha RB, Jisha VN, Pradeep S, Sajith S, Sreedevi S, Priji P, Unni KN, Sarath Josh MK. 2013. A monograph on amylases from *Bacillus* spp. *Adv Biosci Biotechnol*, 4, 227-241.

18. Miller GL. 1959. Use of dinitrosalycylic acid reagent for determination of reducing sugar. *Anal Chem*, 31, 662-666.

19. Naidu MA, Saranraj P. 2013. Bacterial amylase: A review. *Int J Pharm Biol Sci Arch*, 4 (2), 274-287.

20. Haki GD, Anceno AJ, Rakshit SK. 2008. Atypical Ca^{2+} independent, raw-starch hydrolyzing alpha-amylase from *Bacillus* sp. GRE1: Characterization and gene isolation. *World J Microbiol Biotechnol*, 24, 2517-2524.

21. Raj V, Hemashenpagam N. 2012. Production and medium optimization of amylase by using fermentation methods. *J Microbiol Biotech Res*, 2 (4), 481-484.

22. Saxena RK, Dutt K, Agarwal L, Nayyar P. 2007. A highly thermostable and alkaline amylase from a *Bacillus* sp. PN5. *Bioresour Technol*, 98, 260-265.

23. Hashemi M, Mousavi SM, Razavi SH, Shojaosadati SA. 2013. Comparison of submerged and solid state fermentation systems effects on the catalytic activity of *Bacillus* sp. KR-8104 α-amylase at different pH and temperatures. *Ind Crop Prod*, 43, 661-667.

24. Asgher M, Javaid Asad M, Rahman SU, Legge RL. 2007. A thermostable α-amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing. *J Food Eng*, 79, 950-955.

25. Viswanathan S, Rohini S, Rajesh R, Poomari K. 2014. Production and medium optimization of amylase by *Bacillus* spp using submerged fermentation method. *World J Chem*, 9 (1), 01-06.

26. Mishra LS, Behera N. 2008. Amylase activity of a starch degrading bacteria isolated from soil receiving kitchen wastes. *Afr J Biotechnol*, 7, 3326-3331.

27. Liu XD, Xu Y. 2008. A novel raw starch digesting a-amylase from a newly isolated *Bacillus* sp. YX-1: Purification and characterization. *Bioresour Technol*, 99, 4315-4320.

28. Vidyalakshmi R, Paranthaman R, Indhumathi J. 2009. Amylase production on submerged fermentation by *Bacillus* spp. *World J Chem*, 4 (1), 89-91.