National Academy of Sciences of RA
Electronic Journal of
NATURAL SCIENCES

ՀՀ Գիտությունների Ազգային Ակադեմիա
Էլեկտրոնային ամսագիր
ԲՆԱԿԱՆ ԳԻՏՈՒԹՅՈՒՆՆԵՐ

Национальная Академия наук РА
Электронный журнал
ЕСТЕСТВЕННЫЕ НАУКИ

2(21), 2013

Biotechnology *Կենսատեխնոլոգիա* *Биотехнология*

PRODUCTION OF THERMOSTABLE ALPHA-AMYALSE BY *BACILLUS* SP. IRANIAN S2 USING SOLID STATE FERMENTATION

S. Sharifi Alghabpoor, H. Panosyan, Yu. Popov, A. Trchounian

Department of Microbiology and Plant and Microbe Biotechnology, Yerevan State University, Alex Manoogian 1, 0025, Yerevan, Armenia; e-mail: sorour.sharifi@ymail.com

ABSTRACT

Production of α-amylase under solid-state fermentation by *Bacillus* sp. Iranian S2 has been studied using wheat bran producing waste as substrates. The influences of incubation time, inoculum size, incubation temperature and pH, additional carbon and nitrogen sources on the production of α-amylase were investigated. The highest enzyme production (96±3 U/g) expressed as units per mass of dry substrate was observed after 72 h incubation. The optimum temperature for α-amylase production was observed at 55°C and pH 5.5. Production parameters were optimized as inoculums size 10 % (volume per mass) and substrate: moisture ratio 1:1. Among the defined carbohydrates, the addition of glucose (0.05 g/g dry substrate) has significantly improved the production of α-amylase (128±5 U/g). Supplementation of different nitrogen sources (0.02 g/g) showed decline in enzyme production.

Key words: *α-amylase, Bacillus sp. Iranian S2, solid state fermentation, wheat bran.*

INTRODUCTION

α-Amylases (endo-1, 4-α-D-glucan glucohydrolase, E.C. 3.2.1.1) are extracellular and endo-acting enzymes that randomly cleave the 1, 4-α-D-glucosidic linkages between adjacent glucose units in the linear amylose chain [5, 9]. The amylases can be derived from several sources, such as plants, animals and microorganisms. Because of their short growth period, the enzymes from microbial sources generally meet industrial demands. Representatives of *Bacillus* and related genera have been found to be the best candidate for commercial production of thermostable α-amylases [3, 4]. A large number of microbial α-amylases have applications in food, textile, paper and detergent industries and many other fields such as clinical, medicinal and analytical chemistry [2].

The production of α-amylases has traditionally been carried out using submerged fermentation (SMF) because of the ease of handling and greater control of temperature and pH, but solid state fermentation (SSF) systems appear as a promising alternative technology [1, 6, 8]. SSF is preferred to SMF because of simple technique, low capital investment, lower levels of catabolic repression and end product inhibition, low waste water output, better product recovery, and high quality production [6, 8]. The fermentation takes place in the absence or near absence of free water. Many substrates have been used as SSF substrate; wheat bran however, holds the key and has most commonly been used, in various processes. Application of these agro-industrial residues in bioprocesses also solves pollution problems, which their disposal may otherwise cause [1]. In this present study thermostable α-amylase production from *Bacillus* sp. S2 Iranian under SSF has been studied using wheat bran producing waste as substrate.

MATERIALS AND METHODS

The strain *Bacillus* sp. Iranian S2 used in the research was isolated from geothermal soil samples collected from Gandom-Beryan in Lut desert, Iran [10]. The isolate screened for amylase production on starch-agar plates containing (g/L) soluble starch, 10.0; peptone, 2.0; yeast extract, 5.0; NaCl, 1.5; $MgSO_4 \cdot 7H_2O$, 0.5; KH_2PO_4, 0.5; $CaCl_2$, 0.1; agar, 20.0; pH 7.0. After 16 hour growth at 55°C, the plates were flooded with iodine solution to check the zone of clearance. The isolate was maintained on medium contained (g/L) sodium chloride, 5; glucose, 20; yeast extract, 5; peptone 10; $CaCO_3$ 6; agar-agar 20; pH 7.2, and was stored at 4°C.

In order to progress of inoculum, volume of 100 mL of medium described above taken in a 250-mL Erlenmeyer flask was inoculated with a loop full of cells from a 24-hour-old slant and kept at 50°C in a rotary shaker with 140 rpm. Cells were harvested up to late exponential phase and culture broth used as inoculum. SSF was carried out by taking 5 g of dry substrate (wheat bran) in a 250-mL Erlenmeyer flask to which mineral salt solution containing (in g/L): KH_2PO_4 1; K_2HPO_4 1; $CaCl_2$ 0.02; NH_4NO_3 1; NaCl 1; $MgSO_4 \cdot 7H_2O$ 2 and distilled water was added to adjust the required moisture level. The contents of the flasks were mixed and autoclaved at 121 °C for 20 min. The flasks were inoculated

using culture broth (10 % inoculum (volume per mass)) and incubated at 50 °C for 24 h. The enzyme production was controlled after every 24 h for 4 days.

Enzyme was extracted in 50 mL of 0.1 M phosphate buffer (pH 7) on a rotary shaker at 200 rpm for 30 min. The content was filtered through muslin cloth, filtrate was centrifuged at 8000 × *g* at 4 °C for 10 min and the supernatant was carefully collected and used as crude enzyme for enzyme assay. α-Amylase activity was determined by the spectrophotometric method in an assay mixture, consisted of 0.5 mL of crude enzyme 2.5 ml of 1 % soluble starch and 1ml 0.1 M phosphate buffer (pH 7). After 15 min of incubation at 50 °C, the liberated reducing sugars (glucose equivalents) were estimated by the 3, 5-dinitrosalicylic acid (DNS) method [7]. The reducing sugar released was measured at 540 nm. One unit (U) of α-amylase activity was defined as the amount of enzyme that releases 1 µmol of reducing sugar as glucose per minute, under assay conditions and expressed as U/g of dry substrate. All the experiments were performed in triplicates and the standard error has been reported.

Effect of different inoculum size (volume per mass 10, 20, 30 and 40 %) was studied. The ratio of substrate:moisture was maintained as 1:1.5, 1:2, 1:2.5 and 1:3 and the enzyme production was checked using wheat bran as substrate and mineral salt medium as moistening agent. To study the efficacy of various inducers the SSF medium was supplemented independently with different carbon sources (0.05 g/g dry substrate) as glucose, maltose and sucrose, soluble starch and nitrogen sources (0.02 g/g dry substrate) as casein, yeast extract, NH_4Cl and urea. Controls contained mineral salt solution with wheat bran.

To study effect of temperature on enzyme activity, assay was done at 35, 45, 55, 65, 75 and 85°C. For definition of suitable pH range for enzyme activity, pH of enzyme assay buffers was varied as 5.5 (acetate buffer), 7 and 9 (phosphate buffer).

RESULTS AND DISCUSSION

Production of α-amylase using *Bacillus* sp. Iranian S2 has been studied under SSF using wheat bran producing waste as substrate. Maximum enzyme production (96±3) U/g) was observed after 72 h, which decreased with further incubation (*Table 1*). The results indicate that further incubation did not show any increase in activity. α-Amylase appeared to be growth related since the cell mass growth kinetics as exactly similar to the enzyme production rate. Cell mass was also increased up to 72 h, then decreased along with cell growth; the results suggested that enzyme production has direct relationship with cell growth.

Table 1. Production of a-amylase by *Bacillus* sp. Iranian S2 on wheat bran substrate by SSF.

Incubation time (h)	Enzyme activity (U/g)
24	24±2
48	75±2
72	96±3
96	50±3

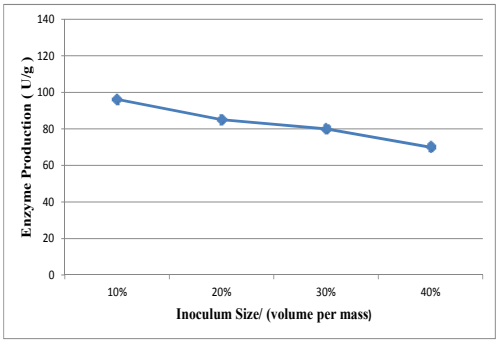

Fig. 1. Effect of inoculum size on the production of a-amylase by *Bacillus* sp. Iranian S2.

It was shown that 10 % (volume per mass) inoculum was optimum for the enzyme production during the fermentation (*Fig. 1*). Higher inoculums decrease enzyme production. This may be due to the limiting nutrients at higher inoculum size. Thus 10 % (volume per mass) inoculum was used as inoculum for further studies.

The moisture is a critical factor for the production of enzymes under SSF. Increase in inoculum size was seen to adversely affect the enzyme production. During fermentation the moisture capacity of the medium is changes as a result of evaporation and metabolic activities. Therefore to adjust the optimum moisture level of substrate during SSF is important [8]. During SSF higher moisture level decreases porosity, changes wheat bran particle structure, promotes development of stickiness reduces gas volume and decreases diffusion, which results in lowered oxygen transfer. In contrast, the low moisture content leads to the decreased solubility of nutrients of the solid substrate, lower degree of swelling and higher water tension [1, 6]. In our study, high enzyme production was obtained when the substrate:moisture ratio was maintained as 1:2.5 in comparison with that of low or high moisture levels.

α-Amylase is an inducible enzyme, which is generally induced in the presence of starch or its hydrolytic product maltose [5, 9]. Supplementation of carbon sources in the form of different carbon sources (glucose, maltose, sucrose and soluble starch) as an effector molecules resulted in marginal increase in α-amylase production. Results are shown in *Fig. 2*. Glucose gives the highest enzyme yield (128±5 U/g), followed by soluble starch (118 U/g), then maltose (110 U/g) and sucrose (100 U/g).

In our studies, as shown in *Fig. 3* in comparison with the control, there was no significant increase in enzyme yield in the case of the supplementation of either inorganic or organic nitrogen sources. Moreover adding of organic nitrogen and especially inorganic nitrogen sources to the medium resulted in outstanding decrease in α-amylase production by *Bacillus* sp. Iranian S2.

Optimum production of enzyme was effected by incubation at different temperatures 35℃-85℃. The results in *Fig. 4* indicate that incubation temperature of 55℃ was found to be the best for enzymes activity (96±3U/g) of *Bacillus* sp.

Iranian S2. In addition, high temperature might have reduced the moisture contents of the fermentation medium and growth of the organism resulting in the decreased enzyme. However, in our study at 80 °C, 90 % activity was showed compared to the optimum enzyme activity at 55°C.

Among the physicochemical parameters, the pH of growth medium plays an important role by inducing morphological changes in the organism and in enzyme secretion. In our study the optimum of amylase production was found to be pH 5.5 (*Fig. 5*). Results show that enzyme production was generally stable from pH 4-7.0, which indicates excellent buffering property of the agroresidues used for solid-state fermentation.

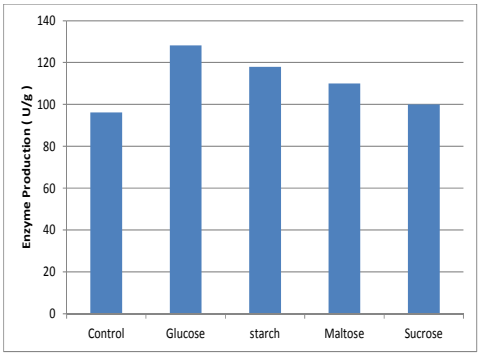

Fig. 2. Effect of carbon source (0.05 g/g) supplementation on α-amylase production by *Bacillus* sp. Iranian S2 under SSF using wheat bran as substrate.

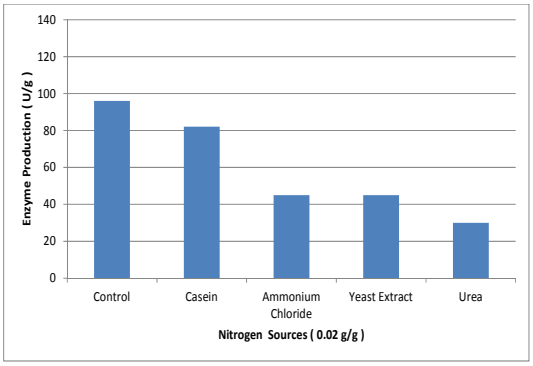

Fig. 3. Effect of nitrogen source supplementation on α-amylase production by *Bacillus* sp. Iranian S2 under SSF using wheat bran as substrate.

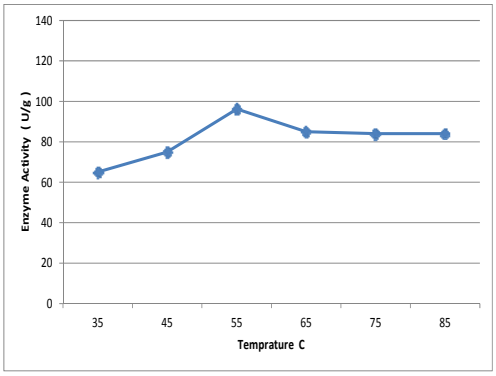

Fig. 4. α-amylase production by *Bacillus* sp. Iranian S2 at different temperatures.

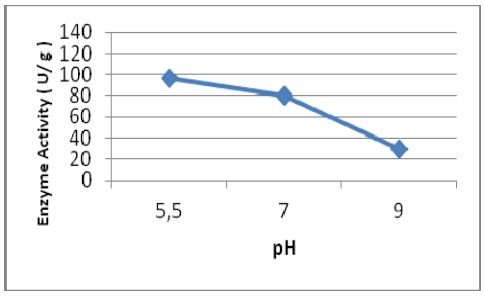

Fig. 5. α-amylase activity of *Bacillus* sp. Iranian S2 at different pH values.

CONCLUSION

Study on evaluation of wheat bran as a substrate and effect of inoculum size, fermentation period, temperature and pH of the medium, inorganic and organic nitrogen sources for the production of α-amylase by *Bacillus* sp. Iranian S2 under SSF was carried out. The results of the present study proved that the isolate can be successfully used for the production of α-amylase employing wheat bran in a mass ratio of 1:1 within a relatively shorter time interval of three days. The optimum temperature for α-amylase production was observed at 55°C and pH 5.5. Among the defined carbohydrates, the addition of glucose (0.05 g/g dry substrate) has significantly improved the production of α-amylase (128±5 U/g). Since the enzymes of these strains have moderate acid- and thermostability, and could be produced on cheap substrates, therefore, it can be suitable candidate to be used as an additive for starch, biofuel and detergent industries.

ACKNOWLEDGEMENT

We appreciate all the colleagues from Shahid Bahonar University of Iran for technical support and advise.

REFERENCES

[1] *Anto H., Trivedi U.B., Patel K.C.,* **2006.** Glucoamylase production by solid-state fermentation using rice flake manufacturing waste products as substrate / Bioresour. Technol., 97: 1161–1166.

[2] *Das S., Singh S., Sharma V., Soni M.L.*, **2011**. Biotechnological applications of industrially important amylase enzyme / *Inter. J. Phar. Bio. Sci.*, 2(1): 486–496.

[3] *Dheeran P., Kumar S., Jaiswal Y.K., Adhikari D.K*., **2010.** Characterization of hyperthermostable α-amylase from Geobacillus sp. IIPTN / Appl. Microbiol. Biotechnol., 86: 1857-1866.
[4] *Finore I., Kasavi C., Poli A., Romano I., Oner E.T., Kirdar B., Dipasquale L., Nicolaus B., Lama L.,* **2011.** Purification, biochemical characterization and gene sequencing of a thermostable raw starch digesting α-amylase from **Geobacillus thermoleovorans** subsp. **stromboliensis** subsp. nov. / World J. Microbiol. Biotechnol., 27: 2425-33.
[5] *Gupta R., Gigras P., Mohapatra H., Goswami V.K., Chauhan B.,* **2003.** Microbial α-amylase: a biotechnological perspective / Proc. Biochem., 38: 1599-1616.
[6] *Ibrahim S.E., El Amin H.B., Hassan E.N., Sulieman A.M.E.,* **2012**. Amylase Production on Solid State Fermentation by *Bacillus* spp. / Food and Public Health, 2(1): 30-35.
[7] *Miller G.L.,* **1959**. Use of dinitrosalicylic acid reagent for determination of reducing sugar / *Anal. Chem.,* 31 (3): 426-8.
[8] *Pandey A., Soccol C.R., Rodriguez-Leon J.A., Nigam P*., **2001.** Solid-State Fermentation in Biotechnology: Fundamentals and Applications / Asiatech Publishers Inc., New Delhi, 221.
[9] *Sivaramakrishnan S., Gangadharan D., Nampoothiri M.K., Soccol C.R., Pandey A.,* **2006** α-Amylases from microbial sources-an overview on recent developments / Food Technol. Biotechnol., 44: 173-184.
[10] *Sharifi Alghabpoor S.F.* **2012**. Thermophilic bacilli isolated from geothermal soils of Gandom Beryan in Lut desert, Iran and their identification based on phenotypic and phylogenetic approaches / Biolog. J. Armenia, 64 (4): 6-12.