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## Optimization of the growth and $\alpha$ -amylase production of *Bacillus subtilis* IP 5832 in shake flask and laboratory fermenter batch cultures

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**Abstract:** Cell growth and the level of  $\alpha$ -amylase in response to the carbon and nitrogen sources used for the growth of the strain *Bacillus subtilis* IP 5832 were examined. Based on the amylase productivity level in shake flask cultures after 24 hours of growth, the growth medium containing starch and peptone was selected as the best medium. Amylase production was greatly reduced when glutamate or citrate as sources of carbon were used. Experiments performed at different initial concentrations of starch showed that although the strain grew well with all the starch concentration used, 0.5 % starch was necessary for maximum  $\alpha$ -amylase production, inducing 1.55 IU mL<sup>-1</sup> of amylase to be secreted after 8 h of cultivation in shaking flasks. During the batch fermentation of *B. subtilis* IP 5832 strain in 2 L laboratory fermenter, a 60 % higher activity (2.5 IU mL<sup>-1</sup>) was obtained. The production of the enzyme was directly related to the growth of the strain. Maximum enzyme activity was obtained at the beginning of the stationary growth phase.

**Keywords:** growth;  $\alpha$ -amylase; *Bacillus subtilis*; fermenter; batch cultures.

### INTRODUCTION

The production of economically important alpha-amylases is essential for the conversion of starches into oligosaccharides.<sup>1</sup> This enzyme is extensively used in starch liquefaction, and the paper, food, pharmaceutical and sugar industries.<sup>2–4</sup>  $\alpha$ -Amylase with suitable properties can be very useful in a specific industry, thus it has become essential to characterize all available microbial strains for their

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productivity. Since almost all microorganisms of the *Bacillus* genus synthesise  $\alpha$ -amylase, this genus has the potential to dominate the enzyme industry.

*Bacillus* species are heterogeneous forms of organisms and they are very versatile in their adaptability to the environment. Various factors influence the nature of their metabolic process and the enzyme produced. The composition and concentration of media greatly affect the growth and production of extracellular amylase in bacteria.<sup>5</sup> Optimization of cultural conditions is important for maximum production of microbial strains.<sup>6</sup> In this regard, appropriate media components and suitable conditions must be attained for optimal production of the required products. *Bacillus* species and other forms of microorganisms grow at different rates with specificity to different substrates in the culture medium. The growth conditions also influence their enzymatic activities.<sup>7</sup>

*Bacillus subtilis* IP 5832 has not hitherto been tested for its amylase activity; hence, the present work describes the effects of culture conditions on the growth of the strain and on  $\alpha$ -amylase production in batch experiments in shake flasks and under controlled conditions in a laboratory fermenter.

## EXPERIMENTAL

### Chemicals

All the employed reagents and solvents were of the highest available purity and at least of analytical grade. They were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

### Strain and cultivation conditions

Frozen stock aliquots containing glycerol were prepared from exponential phase *Bacillus subtilis* IP 5832 cultures grown in Luria–Bertani media (LB) and stored at  $-80$  °C. Precultures were routinely grown in Luria–Bertani (LB) broth medium composed of (g L<sup>-1</sup>): peptone, 10.0, yeast extract, 5.0 and NaCl, 10.0. Pre-inoculum cultures were grown from glycerol stocks in 100 mL shake flasks containing 15 mL LB media and incubated overnight at 37 °C in a rotary shaker at 150 rpm. For the shake flasks experiments, 5 mL of pre-inoculum cultures were transferred aseptically to a 500 mL baffled shake flask containing 100 mL of semi-complex growth medium. Unless otherwise stated, the growth medium (BM – basal medium) used for  $\alpha$ -amylase production was composed of (g L<sup>-1</sup>): soluble starch, 5.0; peptone, 20.0; MgSO<sub>4</sub>·H<sub>2</sub>O, 1.0 and K<sub>2</sub>HPO<sub>4</sub>, 3.0. The medium was adjusted to pH 7.0. Cultivations were maintained at 37 °C with agitation at 150 rpm for 24 h.

### Effect of different semi-synthetic media on growth and $\alpha$ -amylase production

In addition to the described basal medium (BM) for testing the growth and amylase production, the following media, that have been used by others for testing amylase production, were also used: semi-synthetic medium I (SM I) containing (g L<sup>-1</sup>): tryptone, 2.0; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.05; KH<sub>2</sub>PO<sub>4</sub>, 1; Na<sub>2</sub>HPO<sub>4</sub>, 2.5; NaCl, 1; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 and CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.05; semi-synthetic medium II (SM II) containing (g L<sup>-1</sup>): yeast extract, 20.0; glucose, 5.0; MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.0; FeCl<sub>3</sub>, 0.05; KH<sub>2</sub>PO<sub>4</sub>, 1; Na<sub>2</sub>HPO<sub>4</sub>, 2.5; NaCl, 0.5; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 7.5; CaCl<sub>2</sub>·2H<sub>2</sub>O, 1.0 and thiamine, 0.025, semi-synthetic medium III (SM III) containing (g L<sup>-1</sup>): starch, 5.0; MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.0; FeCl<sub>3</sub>, 0.05; KH<sub>2</sub>PO<sub>4</sub>, 1; Na<sub>2</sub>HPO<sub>4</sub>, 2.5; NaCl, 0.5; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 7.5; CaCl<sub>2</sub>·2H<sub>2</sub>O, 1.0 and thiamine, 0.025, and Luria–Bertani medium (LB).

### *Effect of different carbon sources on the growth and $\alpha$ -amylase production*

The effect of different carbon sources (0.4 % citrate and 0.4 % glutamate instead of 0.5 % starch) and different levels of starch (0.05–1.0 %) in the basal medium (BM) on amylase production were examined.

### *Amylase production in a laboratory fermenter*

For the bioreactor experiments, 100 mL of pre-inoculum culture (grown in LB medium) were transferred to the bioreactor containing 900 mL of BM growth medium. All growths were performed using a Biostat B bioreactor (Sartorius) equipped with a 2 L fermentation vessel under constant temperature, pH and  $pO_2$  values. The pH was maintained at  $7.00 \pm 0.05$  by adding 30 %  $H_3PO_4$  solution to the reactor. The temperature was kept at 37 °C. The  $pO_2$  value was maintained at 50 % of air saturation by adapting the stirrer speed between 450 and 900 rpm and supplying air (enriched with pure oxygen when necessary, *i.e.*, when the  $OD_{600}$  reached 25). The end of the batch phase was identified by a reduction in the oxygen consumption rate and an increase in the acid addition rate. The cultures were centrifuged at 10000 rpm for 20 min at 4 °C using a Beckman J2-21 M/E centrifuge and the cell-free supernatants were used as crude enzyme preparations.

### *Biomass assay*

Growth was followed by optical density measurements at 600 nm ( $OD_{600}$ ). Samples were diluted with deionised water and adjusted to  $OD_{600}$  values between 0.2–0.8.

### *$\alpha$ -Amylase activity assay*

The  $\alpha$ -amylase activity was determined by measuring the formation of reducing sugars released during starch hydrolysis. The reaction mixture containing 0.05 mL of appropriately diluted enzyme and 0.45 mL of 1.0 % (w/v) soluble starch (Merck) in 50 mM phosphate buffer (pH 6.5) was incubated at 50 °C for 15 min. The amount of liberated reducing sugar was determined by the dinitrosalicylic (DNS) acid method.<sup>8</sup> One unit of amylase activity was defined as the amount of enzyme that released 1  $\mu$ mol of reducing end groups per min at 50 °C. D-Glucose was used to construct a standard curve.

## RESULTS AND DISCUSSION

As the nature and amount of carbon and nitrogen sources in culture media are important for the growth and production of extracellular amylase in bacteria,<sup>9,10</sup> optimization of culture conditions is important for maximum enzyme production by microbial strains. The results of the time-course studies on  $\alpha$ -amylase production and cell growth of *B. subtilis* IP 5832 grown in different semi-synthetic media are shown in Fig. 1. The biomass and levels of amylase in the crude culture supernatants varied greatly in response to the carbon and nitrogen source used for the growth of the strain. The maximal growth rate and production of extracellular amylase was obtained when the strain was grown in BM and LB (Fig. 1A). Peptone (BM, LB media) promoted  $\alpha$ -amylase productivity. Moreover, the  $\alpha$ -amylase biosynthesis appeared to be independent of the availability of starch, since enzyme activity was detected in the culture broth containing tryptone as the single carbon source (SM I). This was also the case with amylase from *B. subtilis*.<sup>11</sup> Nevertheless, the addition of starch to the growth medium in-

creased the level of secreted enzyme. These results are in agreement with previously reported maximum  $\alpha$ -amylase production when starch was used as the carbon source.<sup>12,13</sup> Based on the amylase productivity level in the shake flask cultures after 24 h of growth (Fig. 1B), the growth medium containing starch and peptone (BM) was selected as the best medium. Amylase activity during growth of the strain in BM was detected from the early stages of growth (results not shown) and it peaked (1.55 IU mL<sup>-1</sup>) at 8 h and was found to decline gradually up to 24 h (0.45 IU mL<sup>-1</sup>). The reason for this might be the simultaneous production of peptidases, which can influence the amylase activity during time. The main advantage of growing *B. subtilis* IP 5832 on BM for amylase production is the very fast production time. Similarly, the production of amylase by *B. thermooleovorans* NP54 peaked at 12 h of growth,<sup>14</sup> while the maximum amylase production by *B. stearotherophilus* AN 002 occurred at 6 h of cultivation.<sup>6</sup> Nevertheless, more often amylase cannot be detected in the culture broth of *Bacillus* sp. before 12 h of incubation.<sup>15–17</sup>

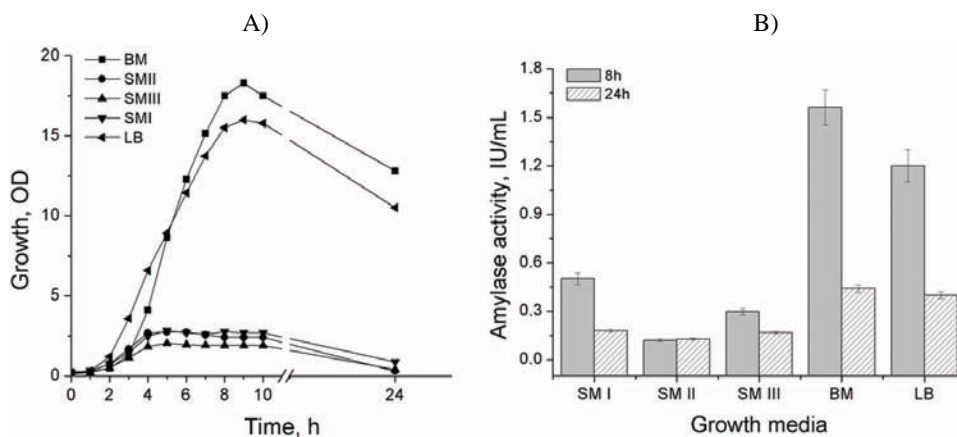


Fig. 1. A) Pattern of growth during 24 h of submerged fermentation of *B. subtilis* IP 5832 at 37 °C in shaking flasks in various growth media. SM I – semi-synthetic medium I; SM II – semi-synthetic medium II; SM III – semi-synthetic medium III; BM – basal medium; LB - Luria–Bertani medium. B) Extracellular  $\alpha$ -amylase activity in the time of maximal production (8 h) and at the end of growth (24 h). Each data point represents the mean of three independent assays (the standard errors were less than 5 % of the means).

The synthesis of amylase was greatly suppressed when the bacterium was grown on glucose (Fig. 1, SM II and SM III) or citrate (Fig. 2B), although in the case of citrate, the growth of the bacterium was comparable to the growth on starch (Fig. 2B). The level of growth resulting from the utilization of various carbon sources (Fig. 2A) did not correlate with the amylase activity (Fig. 2B). Amylase production was greatly reduced when glutamate or citrate were used. These results are in agreement with the reports of others who reported maximum amy-

lase production when starch was used as a carbon source.<sup>12,13,18</sup> On the contrary, *B. subtilis* isolated from Turkish soil produced maximum amylase with citrate as the carbon source.<sup>19</sup>

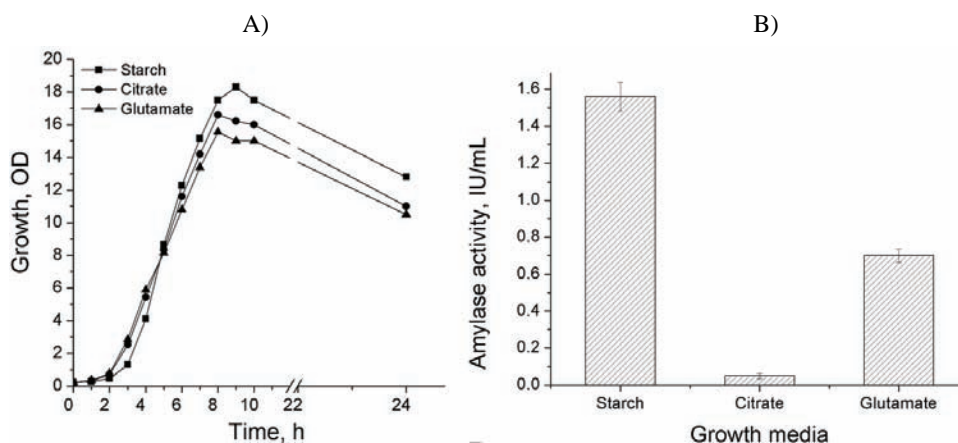


Fig. 2. A) Effect of different carbon sources in BM medium during submerged fermentation of *B. subtilis* IP 5832 at 37 °C in shaking flasks and B) extracellular  $\alpha$ -amylase activity at the time of maximal production (8 h). Each data point represents the mean of three independent assays (the standard errors were less than 5 % of the means).

Experiments performed at different initial concentrations of starch showed that although the strain grew well with all the starch concentration used (Fig. 3A), 0.5 % starch was necessary for the maximum  $\alpha$ -amylase production, inducing the secretion of 1.55 IU mL<sup>-1</sup> of amylase after 8 h of cultivation (Fig. 3B). The best enzyme production by *B. licheniformis* CUMC 305 was also obtained with a starch concentration of less than 1 %.<sup>20</sup> Increasing the starch concentration in the medium beyond 0.5 % did not lead to an increase in the enzyme activity. At higher starch concentrations, the enzyme productivity was comparatively low; such a behaviour was also registered for soil isolates *Bacillus* sp.<sup>15</sup> This can be attributed to the high viscosity of the broth at such concentrations, which interferes with O<sub>2</sub> transfer leading to a limitation of dissolved O<sub>2</sub> for bacteria growth.<sup>21</sup>

The results of the time profiles for both  $\alpha$ -amylase production and biomass of the *B. subtilis* IP 5832 strain grown in the growth medium in the fermenter are shown in Fig. 4. When the strain was cultivated in a laboratory fermenter, the  $\alpha$ -amylase production peaked (2.5 U mL<sup>-1</sup>) at 8 h. When the *B. subtilis* IP 5832 strain was grown in the laboratory fermenter, the amylase activity was 60 % higher than that obtained when the strain was grown in shake flasks. This was probably the result of the higher shaking speed, constant oxygen supply and constant adjustment of pH during the fermenter culture. As in the shake flasks experiments, the maximum  $\alpha$ -amylase production in the fermenter occurred when

optical cell population reached its peak. Amylase production by this strain was found to be growth-associated as the maximum enzyme production was observed during the beginning of stationary phase, when the *OD* reached a plateau. Similar results were obtained for amylase from *Bacillus sp.* IMD 435<sup>22</sup> and amylase from *Bacillus sp.* YX-1.<sup>23</sup>

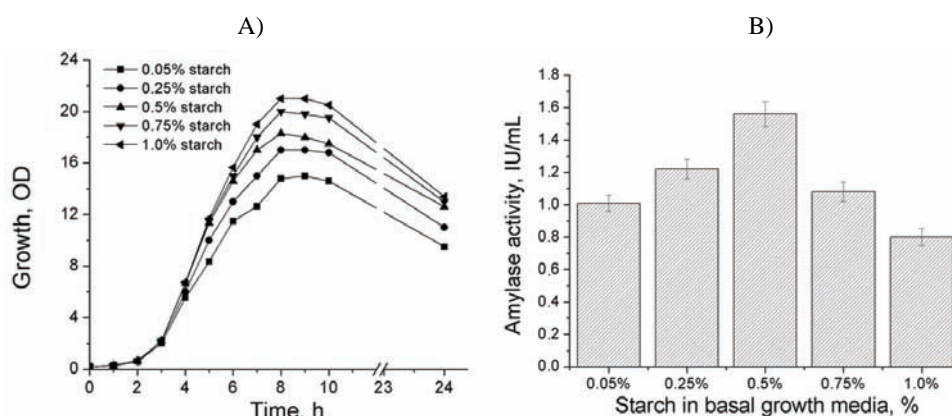


Fig. 3. A) Effect of different starch concentrations in BM medium during submerged fermentation of *B. subtilis* IP 5832 at 37 °C in shaking flasks and B) extracellular  $\alpha$ -amylase activity at the time of maximal production (8 h). Each data point represents the mean of three independent assays (the standard errors were less than 5 % of the means).

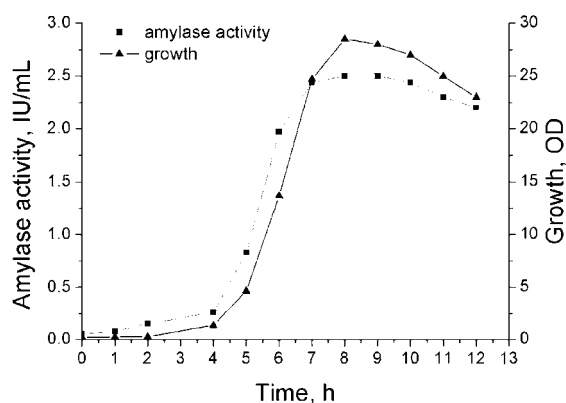


Figure 4. Pattern of growth and extracellular  $\alpha$ -amylase production during submerged fermentation of *B. subtilis* IP 5832 in BM medium at 37 °C in a laboratory fermenter. Each data point represents the mean of three independent assays (the standard errors were less than 5 % of the means).

#### CONCLUSIONS

The optimal N and C sources for growing *B. subtilis* IP 5832 for  $\alpha$ -amylase production were found to be 2 % peptone and 0.5 % starch. The maximum  $\alpha$ -amylase (1.55 IU mL<sup>-1</sup>) was produced during batch fermentation in shaking flask after 8 h of growth, while during batch fermentation in 2 L laboratory fermenter, 60 % higher activity (2.5 IU mL<sup>-1</sup>) was obtained. The production of the enzyme



was directly related to the growth of the strain. Maximum enzyme activity was obtained at the beginning of the stationary growth phase. The proposed process can be considered as a first step in further exploration of possible employment of  $\alpha$ -amylase from *B. subtilis* IP 5832 for starch hydrolysis.

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## ИЗВОД

ОПТИМИЗАЦИЈА РАСТА И ПРОДУКЦИЈЕ  $\alpha$ -АМИЛАЗЕ *Bacillus subtilis* IP 5832 У ШАРЖНИМ КУЛТУРАМА У ЕРЛЕНМАЈЕРУ И ЛАБОРАТОРИЈСКОМ ФЕРМЕНТОРУНАТАША БОЖИЋ<sup>1</sup>, JORDI RUIZ<sup>2</sup>, JOSEP LÓPEZ-SANTÍN<sup>2</sup> и ЗОРАН ВУЈЧИЋ<sup>3</sup>

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Пелијски раст и нивои  $\alpha$ -амилазе након гајења *Bacillus subtilis* IP 5832 испитивани су у зависности од различитих извора угљеника и азота коришћених у подлози. На основу продуктивности амилазе након двадесетчетворочасовног гајења културе у ерленмајерима са мешањем показано је да је најбоља хранљива подлога она која садржи скроб и пептон. Када су glutamate или citrate коришћени као извори угљеника продукција ензима је била значајно редукована. Експериментима у којима је варирана иницијална концентрација скроба показано је да је, иако сој добро расте на свим коришћеним концентрацијама, 0,5 % скроб неопходан за максималну продукцију ензима, индукујући секрецију 1,55 IU mL<sup>-1</sup> амилазе након 8 сати гајења културе у ерленмајерима са мешањем. Током шаржне ферментације *B. subtilis* IP 5832 соја у лабораторијском ферментору од 2 L добијено је 60 % више активности ензима (2,5 IU mL<sup>-1</sup>). Продукција ензима је била директно везана за раст микроорганизама. Максимална ензимска активност добијена је на почетку стационарне фазе раста.

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## REFERENCES

1. H. Pederson, J. Nielsen, *Appl. Microbiol. Biotechnol.* **53** (2000) 278
2. P. Nigam, D. Singh, *Enzyme Microb. Technol.* **17** (1995) 770
3. I. Haq, A. Hamad, I. Javed, M. A. Qadeer, *Bioresour. Technol.* **87** (2003) 57
4. I. Roy, M. N. Gupta, *Enzyme Microb. Technol.* **34** (2004) 26
5. R. A. K. Srivastava, J. N. Baruah, *Appl. Environ. Microbiol.* **52** (1986) 179
6. R. L. Bezbaruah, B. K. Gogoi, K. R. Pilla, *J. Basic Microbiol.* **34** (1994) 139
7. A. O. Ajayi, O. E. Fagade, *Afr. J. Biotechnol.* **5** (2006) 440
8. P. Bernfeld, in *Methods in Enzymology*, I. P. De Murray, Ed, Academic Press INC Deutcher, San Diego, CA, USA, 1955, p. 149

9. A. Pandey, P. Nigam, C. R. Soccol, V. T. Soccol, D. Singh, R. Mohan, *Biotechnol. Appl. Biochem.* **31** (2000) 135
10. P. V. Dharani Aiyer, *Afr. J. Biotechnol.* **3** (2004) 519
11. Z. Konsula, M. Liakopoulou-Kyriakides, *Process Biochem.* **39** (2004) 1745
12. L. L. Lin, M. R. Tsau, W. S. Chu, *Appl. Microbiol. Biotechnol.* **42** (1994) 51
13. R. K. Saxena, K. Dutt, L. Agarwal, P. Nayyar, *Bioresour. Technol.* **98** (2007) 260
14. R. Malhotra, S. M. Noorwez, T. Satayanarayana, *Lett. Appl. Microbiol.* **31** (2000) 378
15. E. de O. Santos, M. L. L. Martins, *Braz. Arch. Biol. Technol.* **48** (2003) 129
16. M. Asgher, M. Javaid Asad, S. U. Rahman, R. L. Legge, *J. Food Eng.* **79** (2007) 950
17. N. Goyal, J. K. Gupta, S. K. Soni, *Enzyme Microb. Technol.* **37** (2005) 723
18. T. E. El-Banna, A. A. Abd-Aziz, M. I. Abou-Dobara, R. I. Ibrahim, *Pak. J. Biol. Sci.* **10** (2007) 2039
19. E. Sarikaya, V. Gürgün, *Turk. J. Biol.* **24** (2000) 299
20. T. Krishnan, A. K. Chandra, *Appl. Environ. Microbiol.* **48** (1983) 430
21. R. Rukhaiyar, S. K. Srivastava, *World J. Microbiol. Biotechnol.* **10** (1995) 76
22. L. M. Hamilton, C. T. Kelly, W. M. Fogarty, *Process Biochem.* **35** (1999) 27
23. X. D. Liu, Y. Xu, *Bioresour. Technol.* **99** (2008) 4315.