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α-Amylase Production by Free and Immobilized *Bacillus subtilis*

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Abstract

The effect of glucose on the α-amylase production by *Bacillus subtilis* ATCC-21556 was studied. Initial glucose concentrations up to 20 g/L were found to be directly proportional to the specific α-amylase production in an immobilized-cell batch system, whereas a free-cell batch system presented an inversely proportional relationship with the initial glucose concentration. This might be owing to the α-amylase repression by the glucose present in the culture medium. Three hundred eighty-five percent of the specific α-amylase production with the free-cell system was produced by the immobilized-cell batch culture.

Index Entries: α-Amylase production; *Bacillus subtilis*; catabolite repression; cells immobilization.

Introduction

The production of α-amylase by free and immobilized *Bacillus* has been reported *(1–12)*. Immobilization on natural or synthetic supports has shown many advantages over conventional fermentation using free cells, such as highest enzyme yield, operational stability, and repeated or prolonged use of cells *(1–3)*.

α-Amylase synthesis by *Bacillus* is frequently controlled by catabolite repression in the presence of glucose or some other rapidly metabolized

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carbon source *(13)*. However, some of the reports dealing with α-amylase production by free and immobilized *Bacillus* using glucose as a carbon source in the production medium did not consider this approach *(6–8)*. Other researchers have worked with glucose catabolite repression-resistant strains to avoid the glucose repression effect *(2–5)*. In this context, the present study focuses on the influence of glucose on α-amylase production by free and immobilized *Bacillus subtilis*. κ-Carrageenan was used as an immobilization support; this gel-forming polysaccharide is considered to be a mild matrix, inexpensive, and easy to work with.

Materials and Methods

Microorganism

B. subtilis ATCC-21556 was maintained in the culture medium containing glycerol 30% (v/v) and stored at –80°C.

Growth Medium

Luria-Bertani (LB) medium *(14)* supplemented with glucose was used. The medium was composed of (w/v): 1.0–20.0 g/L of glucose; 10.0 g/L of casein (Biokar, Beauvais, France), 5.0 g/L of yeast extract (Biokar), and 5.0 g/L of NaCl. In immobilized-cell experiments, KCl was added to a final concentration of 0.1 *M* to ensure mechanical stability of the κ-carrageenan gel beads.

Culturing of the Strain

The microorganism was grown in a 1-L laboratory fermentor with a working volume of 0.3 L (Setric Génie Industriel, Toulouse, France). A flat-bottomed culture vessel was used in order to facilitate recovery of the gel beads *(15)*. For immobilized- and free-cell batch cultures, 30 mL of preinoculum were grown in Erlenmeyer flasks for 12 h at 30°C in a rotary shaker and were used to inoculate 0.27 L of medium in the fermentor.

Immobilization Procedure

Bacterial cells recovered from the 30-mL preinoculum (grown as just described) were mixed with 30 mL of sterile 2% (w/v) κ-carrageenan (E407, Ceca, Paris, France) at 42°C. The bacterial-carrageenan solution was then pumped through a couple of needles and dropped into sterile KCl solution (0.3 *M*) to form gel beads with an average diameter of 3 mm. All immobilization experiments were carried out under aseptic conditions.

Determination of Biomass

Free cells were recovered from the culture by centrifugation, then dried and weighed as described *(16)*. The determination of immobilized biomass was carried out by dissolution of gel beads as described *(16)*. Gel beads were removed from the fermentor at regular intervals and then washed twice in 0.9% NaCl. Beads were weighed and suspended in sterile

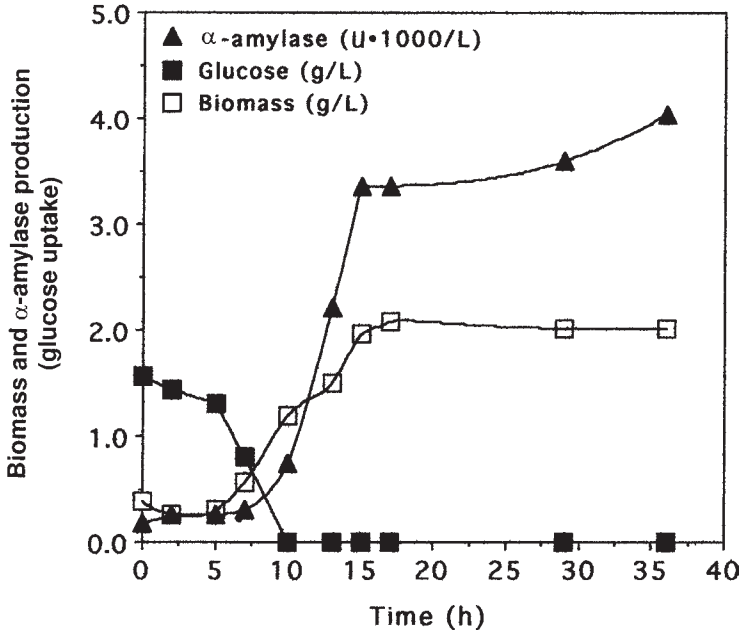

Fig. 1. Monitoring α-amylase production and biomass of free *B. subtilis* ATCC-21556 in batch culture.

Na_2CO_3 (0.2 *M*) at 42°C for 10 min. Then they were completely dissolved by the addition of sterile double-distilled water. The biomass was subsequently recovered by centrifugation, dried, and weighed.

α-Amylase Activity

α-amylase activity was assayed to cell-free supernatant following the dinitrosalicylic acid method for determining reducing sugars *(17)*. We have adopted the Bernfield's *(18)* activity unit definition: one unit of α-amylase activity is defined as the amount of enzyme that releases an amount of reducing sugar equivalent to 1.0 mg of maltose from 1% soluble potato starch in 3 min at 20°C and pH 6.9.

Results and Discussion

Batch experiments were carried out with several initial glucose concentrations (1.0–20.0 g/L). Previous studies *(16)* have shown that LB medium supplemented with glucose increased the biomass production. Figure 1 represents a typical batch fermentation with free cells for the initial glucose concentration (1.5 g/L). α-amylase production was repressed by the presence of glucose. When glucose was almost depleted, α-amylase synthesis showed a constant production rate up to the stationary phase, then the production followed by a reduced rate during the stationary phase.

Cell growth presented two growth phases (diauxic-like behavior) separated by the glucose depletion. The first exponential growth phase was

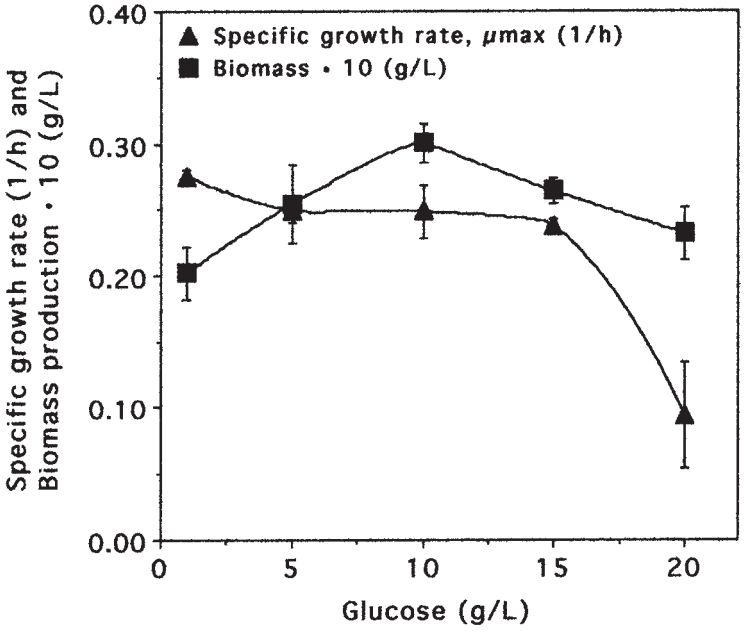

Fig. 2. Maximal specific growth rate and biomass production of free *B. subtilis* ATCC-21556 at different initial glucose concentrations in batch cultures.

supported by glucose consumption in which acetate, lactate, acetoin, and 2,3-butanediol were produced (data not shown) *(16)*.

Figure 2 represents the biomass production and the specific growth rate at different glucose concentrations. The glucose concentration did not have any significant effect over the specific growth rate up to 15 g/L glucose at which the average value was 0.27 h^{-1}. However, at 20 g/L glucose, the specific growth rate fell to 0.09 h^{-1}. The biomass production was increased up to 10 g/L glucose, then fell beyond this point. These results show that initial glucose concentration has a real effect on the biomass production.

Immobilized-cell batch cultures were carried out at the same operational conditions used in free-cell cultures. Figure 3 shows the biomass of immobilized cells and that of released cells from beads at several initial glucose concentrations. The biomass immobilized and that released were 2.5 times higher than that obtained in the free-cell system. The higher biomass production was at 15 g/L glucose, whereas that of free-cell cultures was at 10 g/L (*see* Fig. 2). This difference suggests that, on one hand, there have been some diffusional limitations in the immobilized system related to the immobilization support and to the immobilized biomass distribution. On the other hand, there has been a possible physiological modification altering the behavior of the microorganism.

In fact, previous studies have shown that polysaccharide gel supports do not represent a homogeneous phase and that the peripheral growth of cells forms a supplementary diffusional limitation *(19)*. Nava-Saucedo et al. *(19)*

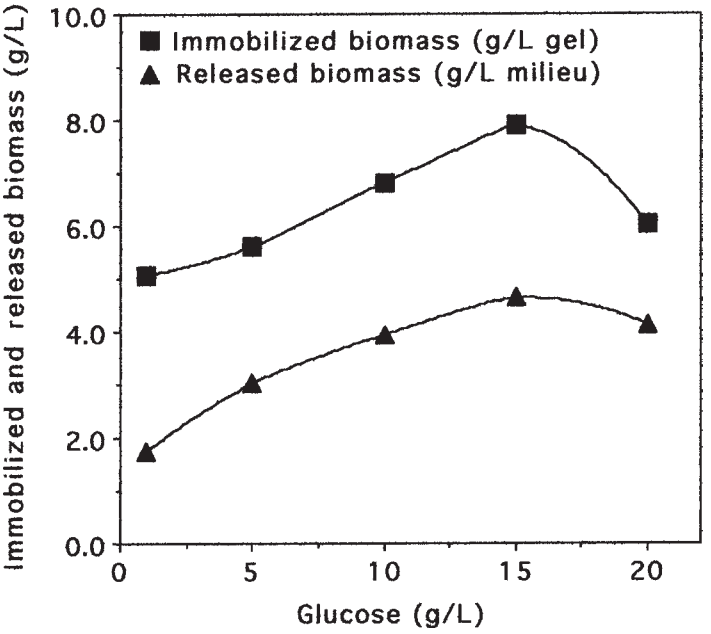

Fig. 3. Immobilized and released biomass of immobilized *B. subtilis* ATCC-21556 at different initial glucose concentrations in batch cultures.

have encountered different types of structural inhomogeneities in polysaccharide gel beads (supercritical crusts, radial shafts and microchannels, discrete cavities and random fractures) that play an important role in the definition of the microenvironment in which the microorganisms grow.

Furthermore, after prolonged incubation, owing to the rapid growth of cells near the gel surface, the gel matrix lost much of its mechanical rigidity, and, consequently, the cavities near the support surface exploded, releasing cells to the solution. Figure 3 also shows the released biomass from the gel beads. We can observe that the quantity of biomass leaked was proportional to the immobilized biomass. Thus, the more biomass grown into the gel beads, the more biomass released to the solution. The gel beads may therefore be considered as a reservoir of cells.

Figure 4 shows the specific α-amylase production by free and immobilized cells at different initial glucose concentrations. A big difference was found between free- and immobilized-cell batch cultures. For the free-cell cultures, the specific α-amylase production was found to decrease with an increase in the initial glucose concentration. At 1 g/L of glucose, 1800 U of α-amylase/g of dry biomass was produced, but at 20 g/L of glucose, only 600 U/g were obtained. This suggests that in the free-cell cultures, higher initial glucose concentrations result in a higher repression of the α-amylase synthesis. These results confirm those reported by Alam et al. *(9)* when working with free cells of *Bacillus* at initial glucose concentrations (0.2–20 g/L). The same trend was also observed by Roychoudhury et al. *(12)* and Yoo et al. *(11)*.

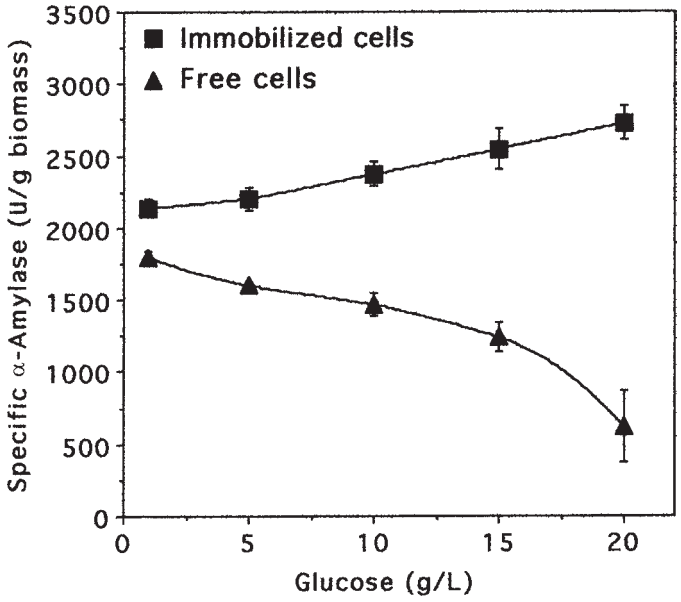

Fig. 4. Specific α-amylase production by free and immobilized *B. subtilis* ATCC-21556 at different initial glucose concentrations in batch cultures.

On the other hand, immobilized-cell batch cultures presented a direct relationship between the specific α-amylase production and the initial glucose concentration used in the growth medium up to 20 g/L. Immobilized cells seem to present less sensibility to the catabolite repression of the α-amylase synthesis in the presence of high initial glucose concentrations. Thus, the immobilized system allows 385% of specific α-amylase production over the free-cell system at 20 g/L of glucose. These results may be explained first by the diffusional limitations presented by the physical support nature and by the extensive bacterial growth on the surface of the gel beads, and second, by a possible bacterial physiological modification linked to the immobilized microenvironment presented. However, it is hard to dissociate these two phenomena, and further studies need to be conducted to elucidate the mechanisms implicated.

Maximal specific growth rates for the bacterial populations were calculated. Immobilized cells presented a lower maximal specific growth rate compared with that of free cells: 0.18 h^{-1} and 0.27 h^{-1}, respectively. However, the maximal specific growth rate of the released cells from beads was found to be even lower (0.10 h^{-1}) than that of the immobilized cells. These results also suggest a possible physiological modification of the bacteria linked to the immobilization microenvironment.

Conclusion

Under the operational conditions of the batch culture experiments, immobilized-cell cultures of *B. subtilis* ATCC-21556 seem to present less

sensibility to the repression of α-amylase synthesis in the presence of initial glucose concentrations up to 20 g/L. The phenomena apparently implicated in the immobilized living-cell system compartment to behavior (diffusional limitations and physiological modifications related to the immobilization microenvironment) seem to be responsible. At 20 g/L of glucose, 385% of specific α-amylase production with the free-cell system was produced by the immobilized-cell batch culture. An initial glucose concentration of 20 g/L did not repress the α-amylase synthesis in the immobilized-cell system at the same level as it did in the free-cell system. The immobilized-cell system could be advantageous when working with inhibitory or repressor molecules.

Acknowledgments

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References

1. Mamo, G. and Gessesse, A. (1997), *Biotechnol. Tech.* **11,** 447–450.
2. Dobreva, E., Tonkova, A., Ivanova, V., Kabaivanova, L., and Spasova, D. (1998), *J. Ind. Microbiol. Biotechnol.* **20,** 166–170.
3. Dobreva, E., Ivanova, V., Stefanova, M., Tonkova, A., Kabaivanova, L., and Spassova, D. (1998), *Microbiol. Res.* **153,** 157–162.
4. Ivanova, V., Stefanova, M., Tonkova, A., Dobreva, E., and Spassova, D. (1995), *Appl. Biochem. Biotechnol.* **50,** 305–316.
5. Tonkova, A., Ivanova, V., Dobreva, E., Stefanova, M., and Spasova, D. (1994), *Appl. Microbiol. Biotechnol.* **41,** 517–522.
6. Dobreva, E., Ivanova, V., Tonkova, A., and Radulova, E. (1996), *Process Biochem.* **31,** 229–234.
7. Ramakrishna, S. V., Jamuna, R., and Emery, A. N. (1993), *Appl. Biochem. Biotechnol.* **37,** 275–282.
8. Jamuna, R. and Ramakrishna, S. V. (1992), *Enzyme Microb. Technol.* **14,** 36–41.
9. Alam, S., Hong, J., and Weigand, W. A. (1989), *Biotechnol. Bioeng.* **33,** 780–785.
10. Ponzo, J. H. and Weigand, W. A. (1991), *Biotechnol. Bioeng.* **38,** 1065–1081.
11. Yoo, Y. J., Cadman, T. W., Hong, J., and Hatch, R. T. (1988), *Biotechnol. Bioeng.* **31,** 357–365.
12. Roychoudhury, S., Parulekar, S. J., and Weigand, W. A. (1989), *Biotechnol. Bioeng.* **33,** 197–206.
13. Priest, F. G. (1977), *Bacteriol. Rev.* **41,** 711–753.
14. de Taxis du Poët, P., Arcand, Y., Bernier, R., Barbotin, J.-N., and Thomas, D. (1987), *Appl. Environ. Microbiol.* **53,** 1548–1555.
15. Huang, J., Dhulster, P., Barbotin, J.-N., and Thomas, D. (1989), *J. Chem. Technol. Biotechnol.* **45,** 143–147.
16. Durán-Páramo, E. (1997), PhD thesis, Université de Technologie de Compiègne, France.
17. Bailey, M. J. (1988), *Appl. Microbiol. Biotechnol.* **29,** 494–496.
18. Bernfield, P. (1955), *Methods Enzymol.* **1,** 149–158.
19. Nava-Saucedo, J. E., Audras, B., Jan, S., Bazinet, C. E., and Barbotin, J.-N. (1994), *FEMS Microbiol. Rev.* **14,** 93–98.