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# Screening of a Growing Cell Immobilization Procedure for the Biosynthesis of Thermostable α-Amylases

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## ABSTRACT

Studies were carried out on α-amylase production with immobilized cells of two *Bacillus* strains. High yields of thermostable α-amylases were obtained by *Bacillus licheniformis* 44MB82-G, resistant to glucose catabolite repression and a thermophile *Bacillus brevis* 174, after repeated batch cultivation (270–600 h) of the immobilized biocatalysts. Various cell immobilization techniques were compared, including entrapment in gel matrices (Ca-alginate, κ-carrageenan, agar, and their combinations with polyethylene oxide), adsorption on cut disks of polymerized polyethylene oxide, and fixation on formaldehyde activated acrylonitrile-acrylamide membranes. The optimal immobilization parameters (gel and biocatalyst concentration, initial cell quantity) were determined. Among the gels and supports tested, agar, κ-carrageenan, agar/polyethylene oxide gels, and the membranes were found to be suitable for immobilization and biocatalysts with high operational stabilities were obtained. An enzyme yield of 2750 U/mL culture medium was reached in the fifth repeated batch run with membrane-immobilized *Bacillus licheniformis* cells. This activity represented 176% of the corresponding yield obtained in batch fermentation with free cells. Higher amylase yields than the activity of the control were reached in all experiments and repeated batch runs with immobilized *Bacillus brevis* cells.

**Index Entries:** *Bacillus licheniformis; Bacillus brevis;* immobilization; α-amylase.

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## INTRODUCTION

The immobilization of growing microbial cells is of particular interest becuase of their biotransformational and biosynthetic abilities for production of diverse valuable products. Nevertheless, focus is on the production of low cost and simple products or on biotransformation reactions. α-Amylases are widely used industrial enzymes and they are also good candidates for such studies. The possibility of producing a thermostable (*1,2*) and thermolabile (*3,4*) α-amylase with immobilized *Bacillus* cells was investigated. The most frequently used immobilization method is entrapment in various gel matrices (*2,4–6*). The considerable diffusional limitations, observed in the biosynthesis of this high molecular product (*5*), can be reduced by the application of suitable carriers, microorganisms, and optimal immobilization.

The most extensively studied α-amylase producers are thermophile microorganisms or catabolite repression-resistant and thermotolerant bacterial mutants (*7*). However, to our knowledge there is no report on the immobilization of catabolite derepressed *Bacillus licheniformis* strain that can synthesize a high quantity of thermostable α-amylase on glucose as the carbon source, and on immobilization of a thermophile strain, such as *Bacillus brevis*.

Our aim was to choose suitable carriers, methods, and optimal conditions for immmobilization of growing cells of *Bacillus licheniformis* 44MB82-G and *Bacillus brevis* 174 and to evaluate the immobilized biocataysts in repeated batch fermentations for production of thermostable α-amylases.

## MATERIALS AND METHODS

### Microorganisms, Media, and Growth Conditions

The first bacterial strain used was the thermotolerant and glucose derepressed *Bacillus licheniformis* 44MB82-G (*8,9*). It was grown in a nutrient broth supplemented with 1% (w/v) soluble starch and 2% (w/v) glucose for optimizing biomass yields. Cells for immobilization were harvested in the late exponential phase of growth by centrifugation (3000*g*, 20 min, 4°C). They were washed with saline solution, and, after recentrifugation, the biomass was used for immobilization. The fermentation medium contained (in percentage, w/v): glucose, 6.0; beef extract, 1.5 (Lab-Lemco powder, Oxoid, Basingstoke, Hampshire, UK); peptone, 1.5 (Oxoid); $K_2HPO_4$, 1.04; cornsteep liquor, 0.66; $CaCl_2$, 0.11, pH 6.5.

The second strain used was the thermophile *Bacillus brevis* 174 (*10–12*). It was grown in identical seed and production medium containing (in percentage, w/v): soluble starch, 0.6; peptone, 0.6 (Oxoid); yeast extract, 0.6 (Fluka Chemie AG, Buchs, Switzerland), pH 7.1–7.2. Cells

from the 17th h of cultivation at 50°C were harvested by centrifugation and washed by the same procedure as the cells from *Bacillus licheniformis*.

## Repeated-Batch Fermentations

The fermentations with the thermotolerant *Bacillus licheniformis* were carried out at 35°C on a rotary shaker at 240 rpm. The thermophile was grown at 38°C because the thermogels used are unstable at a higher temperature. After each 120 h, when the experiments were carried out with *Bacillus licheniformis*, or 68 h in the case of *Bacillus brevis*, the biocatalysts were washed with sterile water and reintroduced into fresh medium. Parallel experiments were also run with free bacterial cells using the previous fermentation for inoculation of the next run. The experiments with immobilized in Ca-alginate gel cells and the corresponding controls were performed in media without $K_2HPO_4$.

## Immobilization of *Bacillus licheniformis* and *Bacillus brevis* Cells

### *Entrapment in Gel Matrices*

Sodium alginate (LS 20/60, Protan, Drammen, Norway), agar and κ-carrageenan (Fluka) were used in 2.0–6.0% w/v concentration. Wet cells (0.15–0.4 g) were mixed in 50 mL of each gel at ambient temperature or at 50°C (agar and κ-carrageenan). The slurry was made into spherical beads about 2.5–4.0 mm in diameter by dropping into sterile 2.5% (w/v) $CaCl_2$ solution (*13*), sunflower oil (*14*), or cooled 2.5% (w/v) KCl solution. The biocatalysts were solidified 2–4 h in these solutions and were transferred into the fermentation medium after flushing with sterile water.

The composite gels were prepared by mixing 2.0 g of polyethylene oxide (MW 1,200,000, sterilized 6 h by UV irradiation) with 50 mL of each gel, and after that the immobilization was performed as described above.

### *Immobilization by Absorption on Polyethylene Oxide Disks*

The polyethylene oxide disks with a thickness of 3 mm were polymerized by γ-irradiation ($^{60}Co$, 17,000 Ci) to a cumulative dose of 5.0 and 10.0 Mrad and stored in 70% ethanol. The cells (0.2 g) were immobilized by adsorption on the cut disks (3/3/3 mm, 2.0 g) for 18 h at 25°C in saline solution. After that the biocatalysts were washed four to five times with sterile water and then transferred into the fermentation medium.

### *Chemical Coupling on Acrylonitrile-Acrylamide Membranes*

The asymmetric membranes used represented a copolymer of acrylonitrile with acrylamide (10%) on a polyester pad. Their thickness was 0.33 mm, where 0.17 mm was owing both to the selective and support layers. The pore size was 0.012–0.068 μm for the selective layer and 0.071–2.24

μm for the support layer. The membranes (2/2 cm) were activated and sterilized 4 h at 45°C in 50 mL 0.066*M* phosphate buffer, pH 7.5, containing 10% (v/v) of formaldehyde (Fluka) (*15*). Then they were flushed with sterile water and phosphate buffer. Immobilization was performed by: The activated membranes were placed in suspension of washed cells (0.4% wet wt) for 6 h in phosphate buffer at 25°C, or they were transferred into a nutrient broth with growing cells for 18 h at 35°C. After these procedures, the membranes were washed consecutively with sterile water, saline solution, and phosphate buffer for elimination of the non-immobilized cells.

## Enzyme Assay

α-Amylase activity in the medium was measured periodically during the cultivation by starch-iodine method using 1.0% (w/v) soluble potato starch (Lintner Starch, Serva Feinbiochemica, Heidelberg, Germany), dissolved in 0.066*M* phosphate buffer, pH 6.5 as substrate (*16*). One unit of activity was defined as the amount of enzyme that produces one micro-equivalent of anhydrous glucose (0.162 mg) per min from soluble starch at 30°C in the experiments with the *Bacillus licheniformis* and 60°C in the case of *Bacillus brevis.*

## Scanning Electron Microscopy (SEM)

The beads and the membranes were fixed for 2 h in 2.0% v/v glutaraldehyde (Fluka). After washing with saline solution, they were dehydrated in 30–100% water–ethanol series. The air-dried particles were coated with 120–130 Å of gold in argon by the Edwards apparatus S 150 Å. The SEM observations were done on a scanning device to a Zeiss 10C electron microscope at 20 kV accelerating voltage with an electron ray 60 A.

# RESULTS

## Immobilization of *Bacillus licheniformis* Cells

### *Optimization of the Immobilization of* Bacillus licheniformis *Cells*

Different gel concentrations were tested and the higher α-amylase activities were obtained from entrapped in 3.5% κ-carrageenan and 3.0% alginate gels bacterial cells. The pellet durability was improved at gel concentration >4%, but the diffusional limitations, owing to the strong gel consistency, decreased the enzyme yield (Fig. 1A and B). A similar effect of the gel concentration was observed for the agar gel, tested in the same concentration range (data not shown). An optimal gel concentration from 3.0–4.0% w/v was selected from these results for the next experiments.

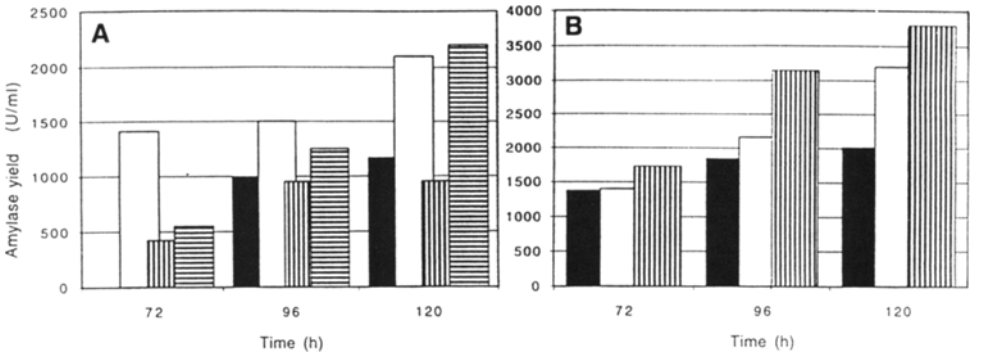


Fig. 1. Effect of the gel concentration on the α-amylase production by entrapped in Ca-alginate (**A**) and κ-carrageenan gels (**B**) *Bacillus licheniformis* cells at initial biomass concentration 0.3% w/v and biocatalyst concentration 40% v/v. (**A**) ■, 2.5%; □, 3.0%; ▥, 6.0% alginate; and ▤, control of free cells; (**B**) ■, 2.5%; □, 3.5% κ-carrageenan; and ▥, control of free cells.

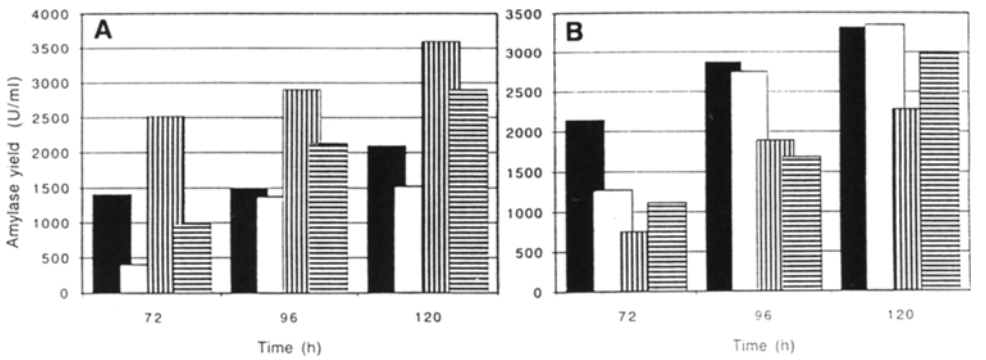


Fig. 2. Influence of the immobilized initial biomass concentration (ICL) on the amylase production by entrapped in pure (**A**) and mixed with PEO gels (**B**) *Bacillus licheniformis* cells at 40% v/v biocatalyst concentration in the medium: A. ■, 0.3% and □, 0.8% ICL in Ca-alginate gel (3.0% w/v); ▥, 0.3% and ▤, 0.8% ICL in agar gel (4.0% w/v). B. ■, 0.4% and □, 0.8% ICL in agar/PEO gel; ▥, 0.6% and ▤, 0.8% ICL in carrageenan/PEO gel.

The optimum initial bead biomass concentration was investigated by preparing beads from pure gels and mixed with polyethylene oxide gels at 0.3–0.8% (wet wt) cell content. It was proved that the optimal initial cell loading (ICL) of the pure gels (alginate or agar) does not exceed 0.3% w/v. At this condition, the amylase yields (2100 and 3600 U/mL, respectively) were equal or similar to the amylase activity obtained from free bacterial cells. The higher initial cell loading led to a decrease of the enzyme secretion (Fig. 2A).

Polyethylene oxide (PEO) was used to study the possibility of decreasing the gel consistency, enhancing its porosity, and thus decreasing the

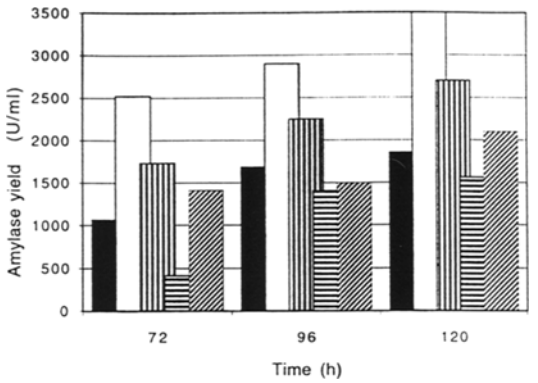


Fig. 3. Effect of the biocatalyst concentration in the medum on the amylase production by immobilized *Bacillus licheniformis* cells at initial biomass concentration 0.3% w/v. ≣, 30% v/v and ▨, 40% v/v Ca-alginate gel (3.0% w/v); ■, 30% v/v; □, 40% v/v and ▥, 50% v/v agar gel (4.0% w/v).

diffusional limitation. At both the low and high initial cell loadings that were applied for entrapment in mixed agar/PEO gel, the amylase production was similar (3350 U/mL) and approximately equal to that obtained from cells entrapped in pure agar at low ICL (Fig. 2B). The enzyme yields, obtained from biocatalysts prepared from carrageenan/polyethylene oxide gel, were not clearly defined (Fig. 2B).

The effect of bead quantity on the fermentation effectiveness was tested at the optimal initial cell loading and optimal gel concentration (Fig. 3). The optimal bead/medium volume ratio was determined to be 1/2.5 (40% v/v) for the two examined gels (alginate and agar).

As mentioned in the Materials and Methods section, the immobilization on acrylonitrile-acrylamide membranes was carried out by two modes: immobilization of washed cells, and immobilization of cells during their growth in a nutrient broth. These immobilized "growing" cells secreted a higher amount of amylase (3630 U/mL) than the immmobilized "washed" cells (2170 U/mL) (Fig. 4). The immobilization of "growing" cells probably led to a better and stronger cell attachment onto the membrane surface. It is also possible that the formation of chemical bonds is more efficient in early stages of cell growth and development.

### *Operational Stability of the Biocatalysts*

We investigated the stability of immobilized cells in repeated batch fermentations to confirm their ability for long-term growth and synthesis of amylase. The results are shown in Fig. 5. At the end of the first batch (120 h of cultivation), the α-amylase yields, obtained from entrapped agar, agar/PEO, and κ-carrageenan gels cells, and after chemical binding on acrylamide-acrylonitrile membranes, reached more than 90% of the activity yield from free bacterial cells. At the end of the fifth cycle, these

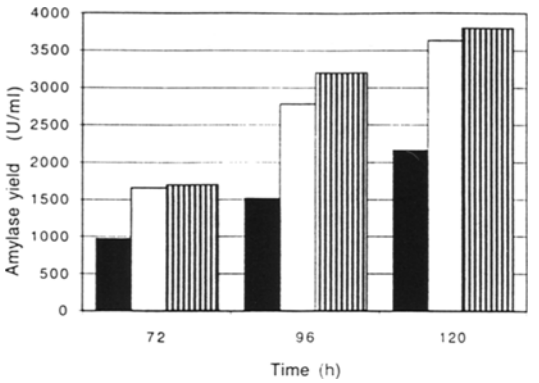


Fig. 4. Effect of the immobilization procedure on the amylase synthesis with chemically bounded on membranes *Bacillus licheniformis* cells. ■, immobilized "washed" cells; □, immobilized "growing" cells; ||||, control of free cells.

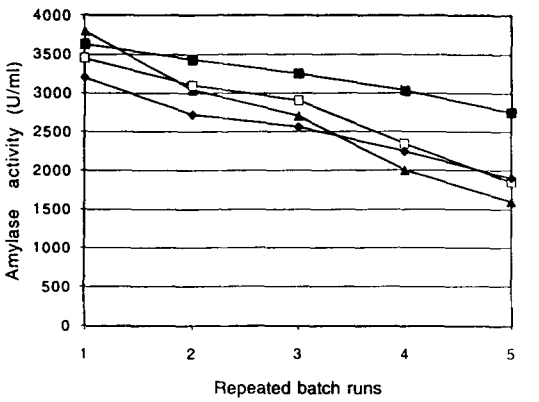


Fig. 5. Repeated batch operations with immobilized ■, on membranes; ◆, in agar and agar/PEO gels; □, in κ-carrageenan gel *Bacillus licheniformis*; ▲, control of free cells.

biocatalysts retained a large part of their initial activities. The membrane-immobilized cells showed 76% higher amylase activity (2750 U/mL) compared with the control of free cells (1700 U/mL).

A sharp decrease in enzyme secretion was observed only when the polyethylene oxide radiation polymer disks were used. In this case, the α-amylase yield decreased with 78% even in the second cycle of cultivation, which was probably because of the easy washing out of the adsorbed cells (data not shown).

### *Scanning Electron Microscopy Studies*

Figure 6, A and B illustrates the surface of a κ-carrageenan gel bead and a κ-carrageenan/PEO gel bead, respectively. The external surface of the κ-carrageenan gel was smooth in comparison with the surface of the

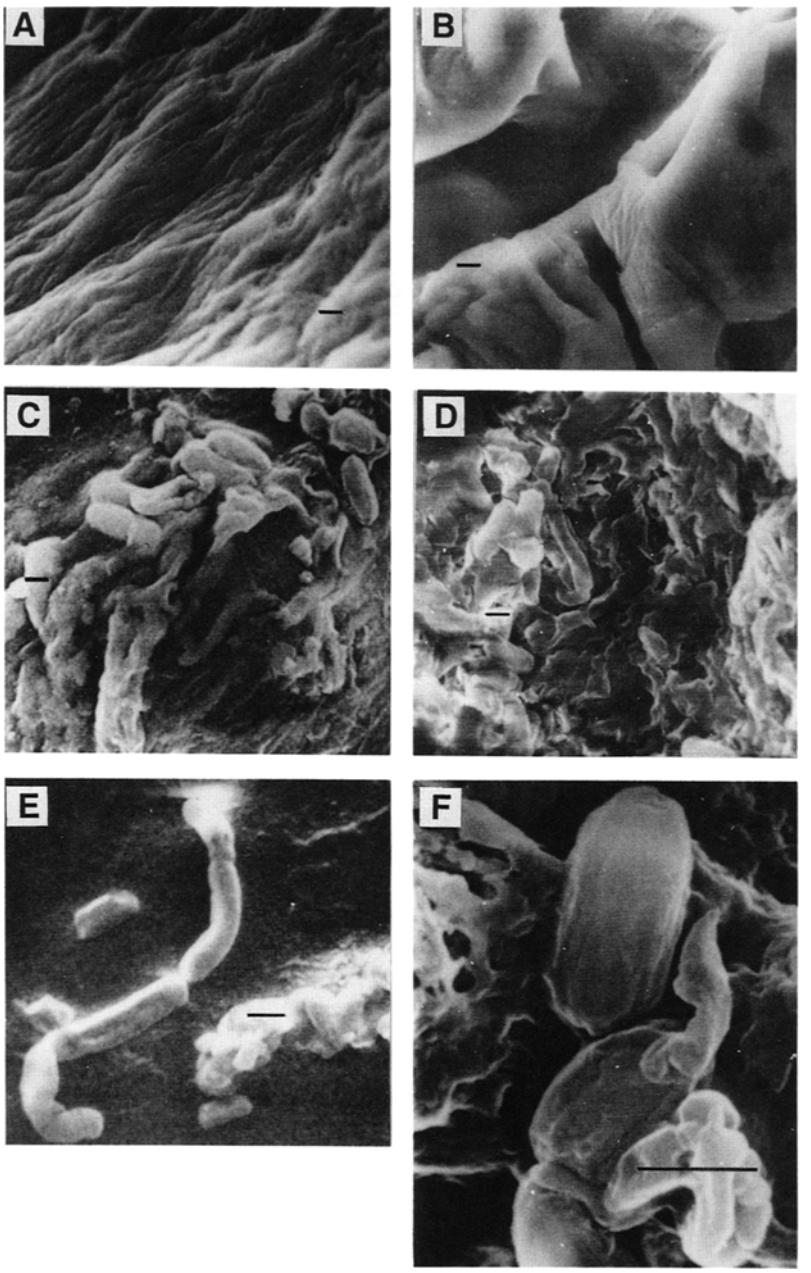


Fig. 6. Scanning electron microphotographs of immobilized *Bacillus licheniformis* cells. Surfaces of beads from pure (**A**) and mixed with PEO carrageenan gel (**B**) (Bar = 2 μm); surfaces of beads from pure (**C**) and mixed with PEO (**D**) carrageenan gel with entrapped cells (Bar = 0.5 μm). Immobilized cells on the selective membrane layer (**E**) and spores on the polyester pad (**F**) (Bar = 0.5 μm).

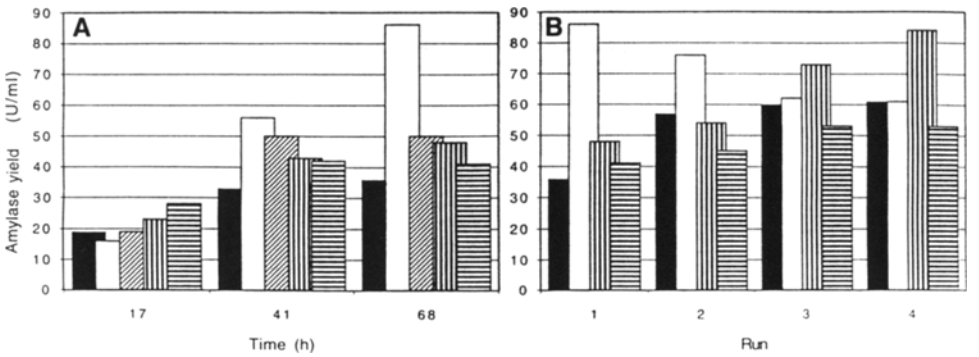


Fig. 7. Comparison between the amylase yields from free and immobilized cells from *Bacillus brevis* 174 (**A**) and operational stability of the biocatalysts (**B**). ■, alginate; □, agar; ▨, carrageenan; ▥, membranes; ≣, free cells.

mixed with polyethylene oxide gel, where considerable folding of its surface was observed. High densities of *Bacillus licheniformis* 44MB82-G cells were observed near the surface of the beads in all cases of gel entrapment (Fig. 6C, and D). After 120 h of cultivation, cells and spores of the strain were found on the membrane selective layer (Fig. 6E) and in the pores of the polyester pad (Fig. 6F).

## Immobilization of *Bacillus brevis* 174 Cells and Operational Stability of the Biocatalysts

In order to analyze the possibilities for immobilization of *Bacillus brevis* 174 cells, using the same gels and supports, the procedures were carried out under optimal conditions for the *Bacillus licheniformis* cells. After 41 h of cultivation (Fig. 7A), the α-amylase yields (except that from alginate) were higher than that obtained with free cells. At 68 h, the α-amylase activities reached from 117 to 210% (equal to 48–86 U/mL) of the activity, obtained from free *Bacillus brevis* 174 cells. The operational stabilities of these biocatalysts (Fig. 7B) were better than the corresponding stabilities of the immobilized *Bacillus licheniformis* 44MB82-G beads. During four repeated batch runs, the enzyme activities remained higher (60–80 U/mL) in comparison with the control. Generally, the *Bacillus brevis* 174 used in these experiments secreted a lower amount of α-amylase than usual, because the fermentations were carried out at a lower temperature than the optimal for cell growth and biosynthesis. A tendency of amylase yield augmentation from the 1st to the 4th run was observed, when membrane-immobilized cells were tested (Fig. 7B).

## DISCUSSION

The substrate transport is a limiting factor of the α-amylase production with immobilized *Bacillus* cells. It was shown (*17*) that starch could not be used effectively in continuous fermentations as a substrate for the production of extracellular α-amylase by gel entrapped cells of *Bacillus amyloliquefaciens*, presumably owing to a decreased rate of substrate mass transfer. In continuous cultivation, the problem was solved (3) by immobilizing cells on ion exchange resin and using an intermittent provision of nutrient and nongrowth media (salts media) for preventing the overgrowth of cells. Nevertheless, in the middle of the fermentation the amylase activity was half of the initial activity, and the immobilized *Bacillus amyloliquefaciens* cells were not able to produce α-amylase in a concentration similar to that obtained in batch cultivation. Other investigations (*6*) showed that after 120 h of fermentation, the biocatalyst activity started to decline gradually. The relative amylase activity of the immobilized cells was 80% of the initial and 66% of the obtained in batch cultivations. The problem was resolved later (2) by the use of fluidized-bed fermentation. Thus an oxygen limitation of the immobilized cells growth and amylase synthesis was proved.

In the present investigation the effect of substrate mass transfer was reduced by replacement of the starch with glucose, using a glucose catabolite derepressed strain of *Bacillus licheniformis*. In the experiments with the thermophile *Bacillus brevis*, the requisite concentration of the soluble starch was low.

The obtained results with immobilized *Bacillus licheniformis* 44MB82-G cells showed that the initial cell concentration has a significant effect on enzyme production and, at a high cell density, the yield decreased. These results are in contradiction to some of the reported observations (*6*) on the effect of initial biomass concentration on fermentation effectiveness, but are in accord with other investigations. It was shown (*5*) that the effective glucose diffusion coefficients in gel beads from κ-carrageenan and alginate decreased when the initial concentration of the microorganism was increased. In addition, these authors (*5*) proved that in alginate gel the glucose mass transfer decreased at high gel concentration, and our results are also in accord with this investigation. At 6% alginate applied, the α-amylase biosynthesis was considerably lower.

Both types of immobilization—gel entrapment in agar, Ca-alginate, κ-carrageenan, mixed with polyethylene oxide agar, and cell coupling on membranes—showed particularly good possibilities. The obtained activities at the 120 h of fermentation were comparable (>90%) to those from free cells. At the end of the fifth repeated batch, the biocatalysts retained from 55 to 78% of the initial amylase activity yield. Thermostable α-amylase was produced from immobilized *Bacillus licheniformis* 44MB82-G cells on glucose as carbon source for at least 600 h (5 batch runs) with a total

yield of $1.5 \times 10^7$ U/L. The relative α-amylase production rate was from 0.875 (alginate) to 1.5 U/h/mL gel (agar) and 0.7 U/h/mg dry cell biomass (free cells). Nevertheless, the biocatalyst's activities declined gradually, and this process was clearly demonstrated in the experiments with entrapped cells. We presume that the oxygen supply was insufficient and when the cells were chemically bound on membranes, the oxygen diffusional problems were partially solved. This biocatalylst showed a better operational stability than all gels tested and could be used successfully in semicontinuous fermentations with immobilized *Bacillus licheniformis* 44MB82-G cells. This presumption is in agreement with the results for the oxygen limitation of the growth and amylase synthesis of immobilized cells obtained previously (*2,3,6*) in batch, repeated-batch, and continuous fermentations.

The immobilized *Bacilus brevis* 174 cells produced higher α-amylase yields for at least 272 h than the free cells in all immobilizing procedures tested. In addition, this strain was able to produce high yields of α-amylase in continuous fermentation (*12*) and thus offered particular possibilities for future investigations into immobilized forms (entrapped in gels or bound on membranes) for continuous production of α-amylase.

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