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ENTRAPMENT OF *BACILLUS LICHENIFORMIS* CELLS IN ALGINATE GEL FOR PROTEOLYTIC ENZYMES PRODUCTION

ENTRAPAREA CELULELOR DE *BACILLUS LICHENIFORMIS* IN GEL DE ALGINAT PENTRU BIOSINTEZA PROTEAZELOR

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The use of immobilized living cells as biocatalysts represent a new and rapidly growing trend in industrial biotechnology. In this work the immobilization in calcium alginate capsules, viability, and proteolytic activity of a local strain of Bacillus licheniformis was studied. After entrapment end drying, the number of viable cells has stabilized at 1,7 x 10^5 cells/wet capsule of alginate and 3,5 x 10^2 cells/dry capsule of alginate. The protease productivity was 17% higher in first batch in the culture of immobilized cells (683 units/liter) than in free cell culture (580 units/liter). The protease productivity was very low in the case of dry biocatalyst (94% lower than in the culture with wet biocatalyst, or 43 units/liter), due to the death of a large amount of entrapped cells.

Key words: biocatalyst, cell entrapment, Bacillus licheniformis, semi-continuous fermentation

Introduction

Growing need of proteases in several domains, as food industry, pharmaceutics, detergents, agriculture, and medicine lead to continuing research of biotechnologies strain improvement for beater productivity of industrial process. The use of immobilized living cells as biocatalysts represent a new and rapidly growing trend in industrial biotechnology (Scott, 1987). Immobilized cells exhibit many advantages over free cells: maintenance of stable and active biocatalysts, high volumetric production, improvement process control, protection of cells against damage, and reduced susceptibility to contamination (Scott 1987). Among the techniques for immobilizing living cells, gel entrapment in gelled natural biopolymers is favored by numerous researchers for various reasons (Klein 1985, Lacroix 1990): non-toxicity of the matrix (crucial in food-related applications), simplicity of immobilization technique, high viability and productivity of the immobilized cells. These gels are porous and permit leakage of cells into the medium allowing inoculation of the fermentation broth (Lacroix 1990). Recently,

works for optimization of alginate capsule characteristics were reported (Konsoula 2005) and the main parameters to obtain the best capsule characteristics were settled: the gel-to-culture medium volume ratio, the size of pellets, the cell loading in the capsules, the $CaCl_2$ concentration and the amount of sodium alginate to be added in the gel mixture. In the present work we have used the immobilization parameters reported in literature to give the best results. The aim of this work is to study the immobilization in calcium alginate capsules, viability, and proteolytic activity of a local strain of *Bacillus licheniformis*. The operational stability in repeated batch fermentation was evaluated as well.

Materials and Methods

1. Chemicals

Peptone, meat extract, yeast extract, agar were purchased from Difco, NaCl, $CaCl_2$, sodium alginate, were purchased from Merck, $(NH_4)_2SO_4$ from Ingen Laboratory Timisoara, soy meal, corn meal were donated of EuroFuraje Timisoara.

2. Microorganism

The *Bacillus licheniformis Tp4* strain used in this experiment was obtained in our laboratory by genetic transformation of the parental *Bacillus licheniformis B40* strain from the Microbial Biotechnology Center, University of Agricultural Science – Bucharest. The pLC1 plasmid was inserted in this strain, carrying the kanamycin resistance trait, and known as improving the proteolytic activity of the host cells (4, 5).

3. Inoculum preparation

B. licheniformis was cultivated at 37^{o}C on a rotary shaker for 24 h in Nutrient Broth medium (meat extract 0,3%, Peptone 1%, NaCl 0,5%).

4. Testing available fermentation media for protease production

The enzymatic activity of the *B. licheniformis* strain was tested during cultivation in four medium formulas indicated in literature (7): ***fermentation medium M1:*** soluble starch 4%, $(NH_4)_2HPO_4$ 0,5%, yeast extract 0,5%, sodium citrate 0,2%, $MgSO_4x7H_2O$ 0,05%, $CaCl_2x2H_2O$ 0,008%; ***fermentation medium M2:*** soy meal 3,5%, corn meal 2,5%, $CaCl_2$ 0,1%; ***fermentation medium M3:*** glucose 2%, casein 0,5%, peptone 0,5%, yeast extract 0,5%, slats solution 5 ml (KH_2PO_4 5g, $MgSO_4x7H_2O$ 5g, $FeSO_4x7H_2O$ 0,1g / liter); ***fermentation medium M4:*** corn meal 2%, soy meal 1%, $MgSO_4x7H_2O$ 0,05%, $(NH_4)_2HPO_4$ 0,5%, $CaCl_2x2H_2O$ 0,2%. 300 ml Erlenmayer flasks containing 50 ml medium formulas described above were inoculated with *B. licheniformis* strain and incubated at 37^{o}C on a rotary shaker. Periodically samples were taken for proteolytic activity measurement.

5. Cell immobilization

Immobilization was carried out under sterile condition, in a laminar flow hood. The following steps were followed:

A. 2 ml of inoculum obtained as described in 2.3 paragraph was centrifuged for 10 minutes at 5000 rpm. The cells were harvested in 2 ml phosphate buffer, pH7.

B. The cell suspension was mixed with 100 ml of 2,5% (w/v) sodium alginate solution.

C. The obtained mixture was dropped through a pipette into 30 ml of 0.2 M $CaCl_2$ sterile solution. The drops were counted. Alginate drops were solidified upon contact with $CaCl_2$, forming capsules and thus entrapping bacterial cells.

D. The capsules were allowed to harden for 24 h at 4^oC and then were washed with sterile saline solution (0.9% NaCl) to remove excess of Ca^{2+} and cells. The obtained biocatalyst was preserved at 4^oC in sterile saline solution.

6. Air drying

The drying of biocatalyst was carried out under sterile condition, in a laminar flow hood. The content of a flask containing 200 alginate capsules containing bacterial cells was poured on sterile filter paper. The saline solution was removed and the capsules are transferred on a second sterile filter paper. The filter paper with capsules in a single layer was transferred in a vacuum dryer and a -0,8 bar vacuum was applied at 42^oC. After the filter paper is saturated with liquid, the capsules were transferred in sterile Petri dishes and kept over night under vacuum. The total drying time (from the filtration to the end of vacuum drying) was 24 hours.

7. Enumeration of viable cells in wet and dry biocatalyst

Samples of 20 wet capsules and 20 dry capsules were put in 10 ml 0.05 M sodium hexametaphosphate sterile solution for 2 hours on an orbital shaker at 200 rpm. If needs, the undissolved fragments of biocatalyst were crushed with a sterile blunt end glass stick. The suspension is then well mixed on a vortex until a homogenous suspension without any residual fragments that may contain entrapped cells is obtained. The suspension is then diluted in sterile saline solution, plated on nutrient agar and the CFU are counted.

8. Fermentation

Wet and dry biocatalysts prepared by the above method (200 capsules) were added to 300 ml flasks containing 50 ml of fermentation medium (M4 formulation). Another flask containing the same medium was inoculated with free cells of *B. licheniformis* prepared as described in 2.3. paragraph. Fermentations with free and immobilized cells were carried out at 37^oC, 180 rpm, on a rotary shaker.

Semi-continuous fermentation

Repeated batch fermentations were conducted by running the fermentation for 72 hours (or 96 h alternatively). At the end of each cycle the production medium was recovered, and the biocatalyst was kept in the flask. The immobilized cells were washed with sterile saline solution, fresh production medium was added and the

fermentation was continued. Obviously, the repeated batch fermentation was applied only for immobilized cells. In the case of free cells (control in this experiment), a simple batch fermentation was carried out.
The operational stability of the immobilized system was determined by the following equation:

$$\text{Operational efficiency (\%)} = 100 \times (C_x/C_1),$$

where C_1 is the protease yield produced in the 1st operation cycle and C_x is the protease yield produced in the xst operation cycle.

9. Protease activity

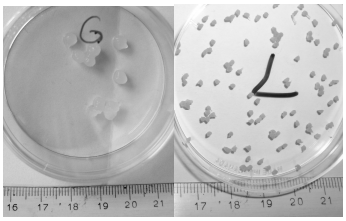
The proteolytic activity of the enzymes produced of free and immobilized cells was determined by the Anson method adapted for casein. One unit is defined as the amount of enzyme which liberates one mM of tyrozin per minute at 37^{o}C.

Results and Discussions

1. Cell entrapment

The cell density achieved in the inoculum culture was 3 x 10^8 cells/ml. After 2 ml of inoculum was mixed with 100 ml sodium alginate and knowing that 20 capsules were obtained from 1 ml suspension, will find that the initial cell concentration in one capsule was 3 x 10^5 cells. The cells were viable after entrapment and after drying. After drying, the aspect of gel capsules changed from smooth, translucent, grey-white, elastic, 4 mm diameter; to brownish color, rough-mat, hard, 1-2 mm diameter (Fig.1).
After counting of viable cells in wet and dry biocatalyst we have found the followings (Table1):

- during entrapment, the number of viable cells decreased with 44% (from 3 x 10^5 to 1.8 x 10^5 cells per capsule),
- after three months of storage at +4^{o}C, the number of viable cells decreased only with another 6% comparing to the number of cells counted after immobilization (from 1.8 x 10^5 to 1.7 x 10^5 cells per capsule), which is a negligible decrease,
- As for drying process, the drop of viable number is more dramatic, only few hundreds cells remain viable after drying, and the number doesn't changes much in three months of storage.



(a) (b)

Figure1 Biocatalists consisting of *B. licheniformis* cells entrapped in alginate gel: (a) fresh capsules; (b) dryed.

Table 1

Viability of *B. licheniformis* cells entrapped in alginate gel

Type of capsules / Viability (cells /capsule)	Wet capsules	Dry capsules
Cells added to alginate	3×10^5	3×10^5
After immobilization / drying	$1{,}8 \times 10^5$	1×10^2
1 month	$1{,}7 \times 10^5$	$3{,}5 \times 10^2$
3 months	$1{,}7 \times 10^5$	$3{,}5 \times 10^2$

3.2. Testing available fermentation media for protease production

After 72 hours of incubation in the four medium formulas, the best results were obtained in M4 medium (Fig.2).This medium, which was selected in the next steps as the fermentation medium for batch and semi-continuous fermentation.

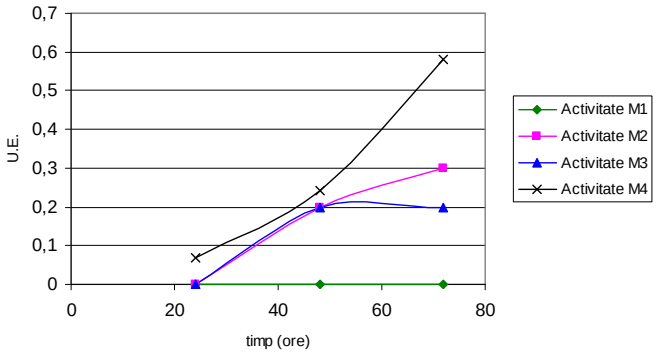


Fig. 2 Proteolytic activity of the strain *B. licheniformis Tp4* in four medium formulas

3. Fermentation

Cells growth and protease production was carried out in a batch system, with *B. licheniformis* free cells (as control) and in semi-continuous system in three cycles (three batches) with immobilized cells. Data presented in figure 3 indicate a high production of proteases in fresh biocatalyst consisting of wet capsules. The protease productivity was 17% higher in first batch in the culture of immobilized cells (683 units/liter) than in free cell culture (580 units/liter). The protease productivity was very low in the case of dry biocatalyst (94% lower than in the culture with wet biocatalyst, or 43 units/liter), due to the death of a large amount of entrapped cells.

In the next cycles of semi-continuous system with immobilized cells, the protease productivity has decreased in both wet and dry biocatalysts systems. In the second cycle the productivity in wet capsules was lower than in free cells, and the

decreasing trend continued in the next cycle. The difference between the two biocatalysts has maintained, with the overwhelming superiority of the wet biocatalyst.

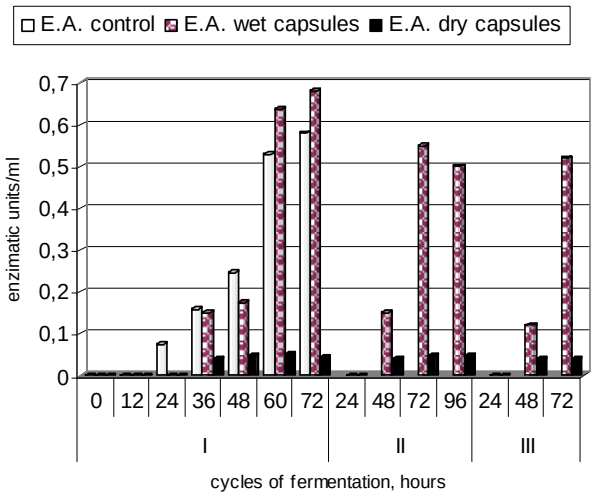


Figure 3 Kinetics of extracellular protease production in free cells culture (E.A. control), wet capsules (E.A. wet capsules) and dry capsules (E.A. dry capsules)

Concerning the operational stability of the biocatalysts, the following results were obtained:

Productivity in Cycle II wet capsules=100 x (683/550)=80,5%
Productivity in Cycle III wet capsules =100 x (683/520)=76%
Productivity in Cycle II dry capsules =100 x (43/46)=93%
Productivity in Cycle III dry capsules =100 x (30/46)=65%

It is obvious the biocatalyst consisting of bacterial cells entrapped in wet capsules has a beater operational stability than biocatalyst consisting of bacterial cells entrapped in dry capsules.

Conclusions

- The enzymatic biosynthesis system for production of proteases with entrapped *B. licheniformis* cells is much more efficient than free cells system. Starting from a single inoculum, in the case of free cells, a single batch can be carried out, as in the case of immobilized cells, continuous or semi-continuous systems can be generated consisting of several cycles of biosynthesis. The real amount of enzyme produced by a biocatalyst in a biosynthesis system consists of the sum of the quantities of enzymes produced in each cycle, or batch. As for the single batch system, with free cells, the bioreactor once inoculated, the fermentation is carried out until the maximum activity is achieved, subsequently the enzyme is extracted and for the next batch a new inoculum will be used. In our

particular case, adding the amounts of enzymes produced in the three cycles of the semi-continuous systems, the following productions are found:

1. 683 + 550 + 520 = 1753 E.U./l obtained with the biocatalyst consisting of bacterial cells entrapped in wet capsules. In the total volume of 150 ml medium 263 proteolytic units were produced.
2. 46 + 43 + 30 = 119 E.U./l obtained with the biocatalyst consisting of bacterial cells entrapped in dry capsules. In the total volume of 150 ml medium 17,85 proteolytic units were produced.
3. 580 E.U./l produced with the free cells in single batch system. In the total volume of 150 ml medium 87 proteolytic units were produced.

- Maximum of proteolytic activity was obtained in 72 hours of fermentation. The second cycle was prolonged for 96 hours, but the proteolytic activity has decreased. This observation can lead to a 24 h shorter cycle, which means more cycles per time unit (2,3 cycles/week at a 72 h cycle, instead of 1,75 cycles/week at a 96 h cycle). At least two cycles / month can be gained, which increases the productivity of the semi-continuous system.

Bibliography

1. **Scott C.D.** (1987) *Immobilized cells: a review of recent literature*. Enzyme Microb Tchnol 9 p. 66-73.
2. **Klein J., Vorlop D**. (1985) *Immobilization technique-cells.* In Moo-Young M Comprehensive biotechnology vol.2 Pergamon Press Biochem 19, p 203-224.
3. **Lacroix C., Pquin C. Arnaud J-P** (1990) *Batch fermentation with entrapped growing cells of L. casei. Optimization of the reological properties of the gel matrix.* Appl. Microbiol Biotechnol 32 p. 403-408.
4. **Ogura M., Tanaka T.,** (1996) - *Transcription of Bacillus subtilis degR Dependent andSuppressed by Multicopy proB* In J. of Bacteriology, vol. 178, p. 216–222
5. **Ogura M., Kawata-Mukai M., M. Itaka K. Takio, Tanaka T.,** (1994) *Multiple Copies of the proB Gene Enhance degS-Dependent Extracellular Protease Production in Bacillus subtilis,* In J. of Bacteriology Vol. 176, (18) p. 5673-5680.
6. **Konsoula Z, Liakopoulou-Kyriakides M.** (2005) – *Thermostable amylase production by Bacillus subtilis entrapped in calcium alginate gel capsules*, **Enz and Microbial Techn. 39, 690-696.**
7. **Vintilă T.**, Dinu L.D., (2004) Tehnologia produselor de biosinteză. Ed. Orizonturi Universitare, Timişoara, p. 26-32.