Immobilization of Polymethylgalacturonase Producing *Aspergillus niger* on *Luffa* Sponge Material

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The vegetable sponge of *Luffa cylindrica* was studied as a matrix for the immobilization of *Aspergillus niger* 26, producer of polymethylgalacturonase (PMG). Entrapped spores could grow and multiply within the lattice of the sponge. The influence of loofa sponge inoculum content, initial spore inoculum content, and duration of the growth cycle on the enzyme activity and mycelium growth was studied. The best yield of PMG was reached with 1 piece of loofa sponge (approx. 0.10 g dry weight), 10^9 spores per g carrier and 48 h duration of one cycle. Data obtained during long-term semicontinuous cultivation showed that production capacity increased significantly and the production period was extended more than 10 times compared with the free cell culture.

Introduction

Aspergillus niger is well known for its ability to produce extracellular pectinolytic enzymes (Acuña-Arguelles *et al.*, 1995; Minjares-Carranco, *et al.*, 1997; Friedrich *et al.*, 1994; Aguilar and Huitron, 1987). These enzymes constitute a complex enzymatic system, which catalyzes the hydrolysis of one or more bonds in pectin molecule (Fogarty and Kelly, 1983). Pectinolytic enzymes play a key role in fruit juice technology by degrading pectins and thus clarifying the juices (Brumano *et al.*, 1993). They are used also in wine clarification, wood preservation, food and textile industry, etc. (Aguilar *et al.*, 1991; Hours *et al.*, 1994; Spagna *et al.*, 1995).

The possibilities of producing pectin-degrading enzymes by immobilized *Aspergillus cells* have been poorly investigated but improved enzyme yields in comparison with free cells have been reported (Bliyeva, 1982; Bliyeva and Rodionova, 1989; Leuchtenberger *et al.*, 1984; Leuchtenberger *et al.*, 1989). We have previously demonstrated an enhanced polymethylgalacturonase (PMG) biosynthesis from *Aspergillus niger* 26 by Ca-alginate immobilization (Angelova *et al.*, 1998). This enzyme causes cleavage of α-1,4-glucosidic linkages of pectin (Sakai *et al.*, 1993).

In a search for alternatives to the carriers used for immobilization of microbial cells, several authors have found the vegetable sponge of *Luffa cylindrica* to be promising (Iqbal and Zafar, 1993a; Iqbal and Zafar, 1993b; Iqbal and Zafar, 1994; Ogbonna *et al.*, 1994; Ogbonna *et al.*, 1997). Since dried loofa sponges are colorless, odorless and tasteless, the sponges have great potential as a good immobilization carrier for the production of enzymes used in the food industry (Ogbonna *et al.*, 1994). To our knowledge there is no published information on biosynthesis of pectinolytic enzymes by immobilized cells within *Luffa cylindrica* sponge.

The present study reports data on the repeated use of immobilized *A. niger* 26 onto the sponge of *Luffa cylindrica* for the production of PMG.

Materials and Methods

Chemicals

In all experiments reported here, apple pectin with 75% degree of esterification from Pectin PLC (Pernic, Bulgaria) was used. All other chemicals used were of the highest purify available. Vegetable sponge obtained from matured dried fruit of *Luffa cylindrica* was used as a carrier for immobilization. Loofa that grows well in South Bulgaria,

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was bought from the market-place (Plovdiv, Bulgaria).

Microorganism and cultivation

Aspergillus niger 26 from the Mycological Collection of the Institute of Microbiology, Sofia was used throughout and maintained on beer agar pH 6.3, at 4 °C. A spore suspension from a 7-d culture in 0.1% solution of Triton X-100 was used as inoculum (Angelova *et al.*, 1998).

Immobilization procedure and cultivation

Vegetable sponge was cut into pieces (approximately 16 x 18 x 3.5 mm). The sponge pieces were soaked in water with continuous rinsing and after then dried at 105 °C. Either one, two or three dried pieces (each of them approximately 0.10 g) were added to 100 ml water in 500 ml Erlenmeyer flasks and were autoclaved at 121 °C for 30 min. After that the water was removed and 60 ml of the nutrient medium (Angelova *et al.*, 1998) was added into the flasks. A suspension with concentrations 10^5, 10^7 or 10^9 spores per g carrier was used to inoculate each flask. The cultures were precultivated on a rotary shaker (150 rpm) at 30°C. After a 48-h growth period the culture medium was removed and the cells immobilized on the sponge were washed with sterile water, then transferred to the same fresh medium for the next cycle of cultivation. The cultivation cycle was 72 h in the experiments for optimization of the loofa sponge inoculum and initial spore content. Subsequently, to optimize the duration of cycle, the cultivation period was varied from 24 to 96 h. Free cells were cultivated in 500-ml Erlenmeyer flasks as described in Table I.

Assays

PMG activity was measured by the decrease in viscosity of the substrate solution (Aguilar *et al.*, 1991; Sakai *et al.*, 1993; Chopra and Mehta, 1985): 10 ml 1% (w/v) pectin solution (apple pectin dissolved in McIlvaine buffer, pH 5.5) were mixed with 5 ml enzyme filtrate and incubated at 30 °C for 10 min. Viscosity was measured by viscometer (Reotest 2.1, MLW Leipzig, Germany). One PMG unit was defined as the amount of enzyme in 1 ml culture filtrate that reduced the relative viscosity of the pectin solution by 30%. The relative PMG activity was expresed as percentage of the maximum activity of the free cells after a 72-h cultivation (56 units/ml). The results presented in the figures were evaluated from repeated experiments using three or five parallel samples. Growth of the immobilized cells was determined as dry weight by washing and drying of the sponge pieces with the mycelium to a constant weight at 105 °C and the weight of the sponge piece subtracted.

Results and Discussion

Development of the cell growth within loofa sponge matrix

There were no visible changes in the shape, structure or texture of the sponge after soaking and autoclaving at 121 °C for 30 min. A high cell density was obtained using *Luffa cylindrica* as the immobilization matrix. Cells grew inside and on the surface of loofa pieces and complete coverage of the reticulated sponge was observed immediately after the 1st fermentation cycle. A single compact solid block of fungal mass developed after four cycles. Since the high porosity and high specific pore volume could facilitate mass transfer within the matrix (Ogbonna *et al.*, 1994). Macroscopic examination revealed no release of cells from the immobilization sponge matrix into the medium during 12 cycles of the repeated batch culture studies.

Effect of loofa sponge inoculum

Various numbers of loofa sponge pieces (1, 2, 3) with the initial spore content of $5x10^9$ spores/g carrier were added to 60 ml growth medium. As shown in Fig. 1, the variation of pieces inoculum had no significant effect on both the PMG activity and mycelium growth. Enzyme production with immobilized *A. niger* 26 was identical for all variants and marked a trend of continuous increase during four cycles. The relative PMG activity reached 300% at the 4th cycle. The yield of immobilized mycelium maintained the same trend.

In the variants with 1 or 2 pieces in the culture medium of immobilized system free cells were not present. In case with 3 pieces was established the release of free cells to the medium and their growth outside the sponge (data not shown). The

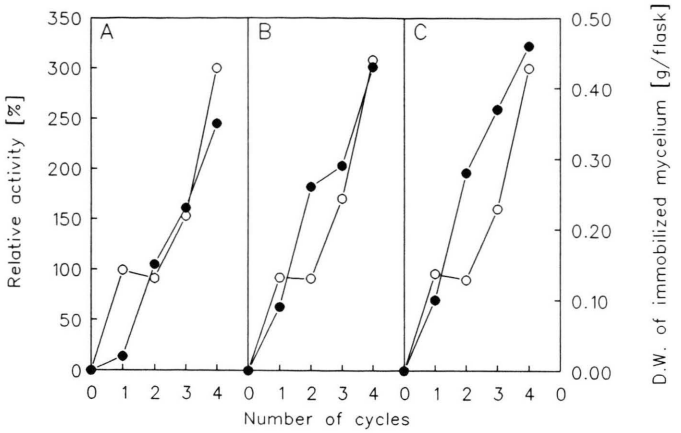

Fig. 1. PMG activity and growth of immobilized *A. niger* 26 depending on the loofa sponge inoculum. (A) 1 piece/flask; (B) 2 pieces/flask; (C) 3 pieces/flask. Open symbols are for relative PMG activity and solid symbols are for dry weight of immobilized mycelium. D. W., dry weight.

dry weight of the free cells was 8–10% of the immobilized biomass dry weight. From these observations it appears that the PMG activity could, therefore, not have been produced by the free cells, but rather by the growing immobilized cells in loofa sponge.

Effect of initial spore content in the Luffa sponge piece

One piece of loofa sponge containing 10^5, 10^7 or 10^9 spores/g carrier was added to 60 ml culture medium and incubated as described above. The initial spore content in piece had an effect on PMG production in the 1st cycle only. The PMG activity increased as the initial spore content increased. After the 1st cycle, the PMG activity did not vary significantly with initial spore content.

With respect to growth of the immobilized mycelium an insignificant increase was observed with the variant with 10^9 spores/g carrier compared to the other variants. The biomass concentration of immobilized mycelium increased in the particular cycles, to a steady-state value of about 12–14 mg/mg carrier for every initial spore content. In all variants in the culture medium of immobilized system free cells were not presented. In this case we may assume that the PMG activity was mainly due to the immobilized cells.

Effect of the duration of the growth cycle

Entrapped spores of *A. niger* 26 (10^9 spores/g carrier in one piece of loofa sponge) were precultivated for 48 h. Immobilized mycelium thus obtained was subsequently cultivated under different duration: 24, 48, 72 and 96 h respectively (Fig. 2). The lowest PMG activity was obtained with a 24 h cultivation, although in most cycles the enzyme activity was equal or insignificantly lower than that for the free mycelium. In the variants with 72 and 96 h cycles a higher maximum PMG activity was found in compared with the former variant for the first two cycles. In the subsequent cycles the enzyme activity of these variants decreased up to 80–90% of free cells PMG level. The enzyme yield reached its maximum after 48 h operational period and the PMG activity was almost two times that of the free mycelium. Our results demonstrate that the immobilization led to a decrease of the duration of the fermentation cycle in comparison

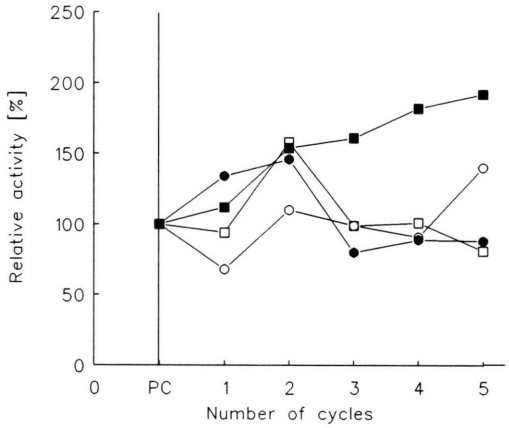

Fig. 2. PMG activity of immobilized *A. niger* 26 by various durations of the cycles. (○) 24 h, (■) 48 h, (○) 72 h and (●) 96 h; PC – precultivation.

with the free cell culture. Moreover, the immobilized mycelium within loofa sponge reached the maximum of PMG activity 24 h early than that immobilized in Ca-alginate gel (Angelova *et al.*, 1998).

Comparison of PMG production by free and immobilized cultures

Figure 3 illustrates the PMG production and the changes in the pH values in semicontinuous replacement fermentations with immobilized and free cells of *A. niger* 26. Table I shows the optimum conditions for operation of free and immobilized cell systems.

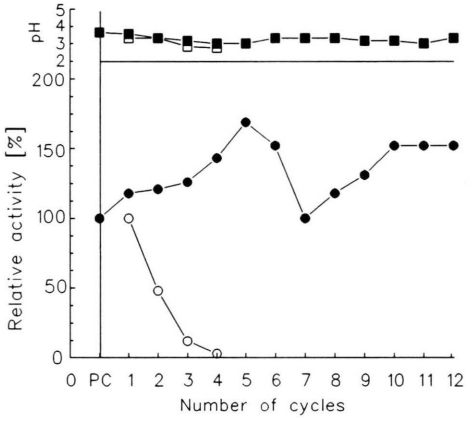

Fig. 3. PMG production (circles) and pH value of culture filtrate (squares) in repeated use of free (open symbols) and immobilized (solid symbols) *A. niger* cells. PC – precultivation.

Table I. Optimum conditions for operation of the free and immobilized growing cell systems.

Operational conditions	Culture of free cells	Culture of immobilized cells
Number of loofa sponge pieces	–	1
Inoculum size	10^8 spores/ml medium	10^9 spores/g carrier
Duration of cycle (h)	72	48
Amount of nutrient medium (ml)	60	60
Temperature (°C)	30	30
Shaker (rpm)	150	150

Maximum of PMG activity by free mycelium was obtained at the 1st cycle. The enzyme level dropped gradually during subsequent cycles, and after the 4th cycle only 3% in comparison with the activity at 1st cycle was obtained. The immobilized system was stable and showed a significant stability of the enzyme biosynthesis. Immediately after the precultivation, biosynthesis of PMG reached a level higher than that of the free mycelium. In spite of the changes in the PMG activity during the semicontinuous cultivation, the reuse efficiency of the loofa sponge pieces was very satisfactory. Even at the 12th cycle PMG activity approximately 150% in comparison with the free culture. Maximum of the relative enzyme activity was obtained at 5 cycle – 169%. Moreover, pH values of culture medium for both the immobilized-cell replacement culture during 12 cycles and free cell culture were comparable. Thus, the higher PMG production by immobilized mycelium does not due to differences in pH. Similar data for immobilized microbial cells within *Luffa cylindrica* sponge concerning both the prolongation and increase of the production for different metabolites were described by Ogbona *et al.* (1994; 1997) and Iqbal and Zafar (1993a; 1994).

In conclusion, the above results show that vegetable sponge of *Luffa cylindrica* is suitable for long-term semicontinuous PMG production.

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