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Biosynthesis of levansucrase by free and immobilized cells of *Bacillus circulans* including continuous fermentation

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Abstract

The optimal conditions for the production of the levansucrase by free and immobilized cells of *Bacillus circulans* were investigated including culture methodology and immobilization conditions with respects to matrix concentration, maximal cell loading and treatment with glutaraldhyde. In batch operation, the activity of the immobilized cells was extended for five repeated cycles, wherein, the activity remained between 15.08-8.66U/ml. Continuous production of levansucrase by immobilized cells of *B. circulans* was also investigated in packed bed bioreactor. Relatively high enzyme activity (14.12 U/ml) together with optimum specific productivity (7.3 U/g wet cells) was attained at dilution rate of 0.4 l/h. Our results revealed the feasibility of the immobilized cell technique.

Keywords: Levansucrase, *Bacillus circulan* , immobilization, continuous fermentation, Levan.

Introduction

Levan, a β 2-6 fructan biopolymer with occasional β 2.1 branching is produced by plants (Ponus and Campillo 1985) and microorganisms (Avigad 1968). It is one of the two main forms of polyfructose which occurs in nature, the other being inulin, which consists primarily of 2.1 linkages and is generally much lower in molecular weight than levan (100 residues average for inulin vs. up to 3 million residues for levan (French 1989). Economic incentives of continuous versus batch fermentation process have stimulated a number of investigators (Keith 1991). Besides, the enhancement of product concentration and productivity, the long term stability of production is decisive for application of continuous culture using immobilized cells.

Moreover, efforts devoted for the application of the cell immobilization for levan production are very scare (Platkova et al. 2005, Gottschalk and Janenick 1991).

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Immobilized cell systems have been applied for many biochemical processes and have been reviewed several times (Klein and Ziehr 1990). The application of the immobilized whole cells for biochemical processes offers many advantages particularly the ability to separate cell mass from the bulk liquid for possible reuse which facilitates and enhances the reactor productivity.

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Immobilized cells are also less subjected to the effects of inhibitory compounds and nutrient depletion. In addition, the immobilized system appears to be less susceptible to microbial contamination and allows the use of dense cell population.

The present paper describes the results of levansucrase production by a local *B. circulans* strain. The study included the immobilization of *B. circulans* cells on different matrices. The cells immobilization conditions of *B. circulans* in agar with respect to matrix concentration, maximal cell loading for maximal production were evaluated. Moreover, the use of agar immobilized cells of *B. circulans* for continuous production of levansucrase with respect to its yield, productivity and long-time operational stability was carried out.

Materials and Methods

Microorganisms

The levansucrase producing strain of *B. circulans* was obtained from the Culture Collection of National Research Center, Dokki, Cairo, Egypt. It was maintained on potato - dextrose agar slants at 4° C.

Methods

Culture medium and growth conditions

The basal medium used for fermentation and enzyme production in both batch and continuous culturing has the following composition (g/l): baker's yeast, 11.5, sucrose, 150, $MgSO_4$, 0.2, K_2HPO_4 5.5, the pH was adjusted to 5.2 using sodium acetate buffer. Cultivation was made in 50 ml of sterile medium portions dispensed in 250 ml Erlenmyer flasks. The inoculum (2% v/v) was transferred to the culture medium and the flasks were incubated at 30 °C for 72 h under shaking condition at 50 rpm. Aliquots were drawn periodically to estimate the growth. The cells obtained from the logarithmic phase of growth of each culture were used for the immobilization experiments.

Immobilization technique

All the immobilization processes were performed under sterile conditions. In separate experiments, the cells obtained from each culture (in logarithmic phase of growth) were collected by centrifugation (5000 rpm, 15 min). The wet cells were then suspended in 0.85 % sterile saline and used for cell immobilization.

Immobilization in Ca-alginate

Unless otherwise stated, the wet cells of *B. circulans*, obtained from 50 ml culture medium were mixed with 10 ml sodium alginate solution. The final sodium alginate concentration was kept at 5 % w/w. The beads were obtained by dropping the tested mixtures in sterile $CaCl_2$ (0.05 M). The beads from 10 ml gel were used for inoculation of 50 ml of the production (basal) medium (Abdel-Naby et al. 2000).

Immobilization in agar

The wet cells obtained from 50 ml culture medium were mixed with 10 ml of 5% w/v agar solution at 45° C. The mixture was quickly cooled to 4 °C, cut in 2x2x2 mm fragments and transferred to 50 ml of the production medium (Cheetham et al. 1985).

Immobilization by physical adsorption

In separate experiments, one gram of each carrier (loaf, stone, wool, ceramic) was treated with 10 ml of 0.1% glutaraldehyde overnight. The carriers were then collected by centrifugation, washed with sterile water to remove the excess glutaraldehyde. The cells obtained from 50 ml culture medium were incubated with one gram of each activated carrier suspended in 10 ml saline solution, collected by centrifugation, washed with sterile water and stored at 4 °C. The immobilized cells on one gram carrier were used for inoculation of 50 ml of the production medium.

Production of levansucrase by the free cells in batch culture

Unless otherwise stated, the batch experiments were performed in 250 ml Erlenmeyer flasks each containing 50 ml medium. The flasks were inoculated with 1 ml cell suspension then incubated in a shaking incubator at 30 °C for 72 h at 50rpm. Thereafter, the culture medium was centrifuged in a cooling centrifuge at 4 °C at 5,000 rpm for 15 min. Samples of the clear filtrate were taken for enzyme assay.

Production of levansucrase by the immobilized cells

1. In batch culture

The batch wise technique experiments of the immobilized cells were preformed in 250 ml Erlenmeyer flasks, each containing 50 ml of sterile medium. The flasks were inoculated with calculated amounts of beads. The flasks were incubated in rotary shaker (50 rpm) at 30 °C for 72 h. The culture medium was filtered off, and the clear filtrate was then taken for enzyme assay.

2. In repeated batch culture

This was done in 250 ml Erlenmeyer flasks, each containing 50 ml medium. Each flask was inoculated with calculated amount of beads. Fermentation was conducted at 30 °C for 72 h under shaking condition (50 rpm). At the end of each run, the beads were filtered, washed with 25 ml saline, 0.2 M sodium acetate buffer pH (5.2), distilled water under sterile conditions, and then transferred to 50 ml aliquots of fresh medium.

3. In continuous fermentation

The bioreactor is a glass column of 1.8 cm diameter and 32.0 cm in length. The bioreactor was filled with 7.6 gm wet cells immobilized in 20 ml agar, where bed volume comprised 40 % of the bioreactor volume and the void volume (washing volume) comprises 60 % of its volume. The fresh medium was introduced from the bottom of the reactor. Air was admitted in through a sterile air filter from the bottom of the reactor, the effluent from reactor was collected in a holding tank. The fermentation was conducted at 30° C and carried out in batch operation for the first 24 h, then the continuous operation was started at different dilution rates (0.1-0.5 lh^{-1}). The air flow was optimized at 0.4 v/v/ min. The reactor was considered to be in a steady state after at least 3 residence times.

Enzyme assay

0.5 ml of the enzyme solution in 0.2 M acetate buffer of pH 5.2 was incubated with 1 ml 20% sucrose solution at 30 °C for 15 min (Yanase et al. 1992). The produced reducing sugar was measured by glucose oxidase kits. One unit of the enzyme was defined as the amount of the enzyme that produces 1µmole of glucose per min.

Protein estimation

The protein contents of the enzyme preparations were determined by the method of Lowry et al. (1952). The protein content of the immobilized enzyme was calculated by subtracting the amount of the unbounded protein from the original added protein.

Biomass estimation

The optimal density of the cell growth was measured spectrophotometerically (Spectronic 2000, Bosh&Lomb) at 620nm. Gravimetric determination of the wet weight of the biomass was measured by collecting cell pellets from 50 ml cultivation medium by centrifugation at 5000 rpm at 4 °C for 15 min. The cells were then washed by saline solution, collected by centrifugation and weighed as wet weight cells /ml media. The dry weight was measured after complete drying of the cell pellets at 60 °C to a constant weight.

Results and Discussion

1.Production of levansucrase by free cells

The fermentation course and levansucrase synthesis by the local isolate *Bacillus circulans* at different incubation periods (18-84 h) were tested. Following the fermentation time course of *B.*

circulans adopting the shacked culture technique (Fig.1), the activity of levansucrase was initiated from the early exponential phase and maximal levansucrase activity (18.47U/ml) was reached after 72h, thereafter the enzyme production started to decrease. On the other hand, Park et al. (2001) reported that maximum enzyme production by *B. circulas* was obtained after 24h. Extremely lower yields, which reached (6 U/ml), autolysis of cells and decrease in enzyme stability were recorded over 72 h.

2.Production of levansucrase by immobilized cells

The bacterial cells at their logarithmic phase of growth were immobilized on different carriers (i.e. agar, calcium alginate, stone, wool and loaf). In another set of experiments the same amounts of the free cells were grown for comparison with the immobilized cells. As shown in table 1 the enzyme activities obtained with the immobilized cells were of lower magnitudes than that of the free cells, whereby the effectiveness factor of the immobilization is less than one. Similarly, Jamuna and Ramakrishna (1992) reported that the effectiveness factor of the immobilized cells would always be less than one. This is because they represent a heterogeneous catalysis fermentation, in which the activity or rather the synthesis of primary or secondary metabolites is dependent on external and internal mass transfer and adequate oxygen supply. In fact, immobilization of the cells in a porous matrix is known to impose a diffusional barrier which presents problems which are not found in the free cells as reduction in nutrient and oxygen availability and /or accumulation of the production inside the beads. This diffusional restriction may exert a negative effect on the activity of the immobilized cells (Abdel-Naby et al. 2000). However, among the tested treatments, the cells of *B. circulans* entrapped in agar showed relatively high specific productivity (9.62 U/gram cells /h) and highest enzyme activity (13.53 U/ml) at 72h incubation (table 1). In addition the highest effectiveness factor of the immobilized *B. circulars* in agar (0.90) justified the application of this treatment in the subsequent experiments.

Entrapment methods are the most common for the preparation of immobilized microbial cells (Fukui and Tanaka, 1982). Immobilization by entrapment is known to be a simple and gentle procedure and keeps the cells from unfavorable conditions (pH, temperature, etc) found in the surrounding media (Kierstan and Ducke 1977). Kim et al. (1998) found that immobilization of levansucrase on calcium phosphate gel strongly increased the polymerase activity. Similarly *B. amyloliquifaciens* cells immobilized on calcium alginate showed the highest enzyme activity (Abdel-Naby et al. 2000).

Effect of matrix concentration

Different concentrations of agar (2-6% w/v) were used for the immobilization process. In all cases, constant amounts of cells (1.12 gm/25 ml agar solution) were used. Inoculation of 50 ml culture medium was performed with the beads resulting from 100 ml agar solution. Results indicated that, the highest enzyme yield (15.68 U/ml) was obtained with 5% agar concentration (Table 2). The increase in agar concentration to 6% resulted in a drop of the enzyme yield. This may be due to the increased gel rigidity which may lead to reduction in the diffusion efficiency of the nutrients and oxygen into the gel matrix. Similar findings were reported by Mamo and Gessesse (1997). It is expected that lower agar concentrations may lead to softness of the gel which may lead to cell leakage and hence uncompleted cell immobilization.

Effect of biomass loading

Evidently, the specific productivity of the entrapped cells proved to depend on the amount of cell biomass loaded in immobilization matrix (Table 3). In this study, the highest specific

productivity (11.05 U/g dry cells /h) together with levansucrase activity of 17.89 U/ml) were obtained with a biomass load of 2.24 g/flask. Gradual increase of the cell load to 4.48 g dry cells decreased both the enzyme activity and specific productivity. Similar results were reported by Jamuna and Ramakrishna (1992) on immobilizing glucosyl transferase enzyme. Jouenne et al. (1993) reported that low levels of entrapped cells led to rapid enzyme biosynthesis while high levels caused diffusion limitation of nutrients.

Effect of binding agent (Glutaraldhyde)

The use of (GA) as a binding agent between *B. circulans* cells and agar exerted an adverse effect on the activity of the produced levansucrase (Table 4). On contrary, Krastanov (1997) selected glutaraldhyde (GA) as the best binding agent for immobilization of microbial cells. Glutaraldhyde is introduced as a spacer group which may change the local surface area and consequently decrease the protein crowding of the immobilized enzyme on the carrier which might impair or prevent the proper conformational changes required for catalysis. This may be also related to the known toxic effect of glutaraldhyde as an organic solvent.

Repeated batch operation with the immobilized B. circulans in agar

The efficiency of the immobilized *B. circulans* in agar for the production of levansucrase continuously was evaluated in repeated batch process. The fermentation medium was replaced every 48 h wherein the beads were washed thoroughly with acetate buffer (0.2M pH 5.2) and distilled water at the end of each cycle. The results given in Table 5 represented the enzyme activities during 5 repeated cycles. Under these experimental conditions, the immobilized cells were able to keep producing significant levels of levansucrase. The activity of the immobilized cells was nearly stable during the two first cycles. There after, the enzyme activities were gradually decreased to reach 8.66 U/ml at the end of the fifth cycle. The specific levansucrase productivities of the immobilized *B. circulans* (in term of U/g wet cells/h) were about 9.11–5.23 during the 5 cycles. The enzyme productivities during the tested cycles were reduced from 0.72-0.37(U/ml /h).

Continuous production of levansucrase by the immobilized cells of *B. circulans* was investigated in packed bed reactor. The fermentation process was carried out in batch operation for 24h, and then continuous operation was started. The dilution rate varied between 0.1 to 0.51 h^{-1} (Fig 2). Scanning of the whole range of the dilution rates, revealed that maximal enzyme activity (14.12 U/ml) could be achieved at dilution rate (0.4 h^{-1}), then decreased with the increase of the dilution rate. This may be due to the decrease of the contact time between the medium and the immobilized cells as the dilution rate increased. The enzyme productivity, which represents the efficiency of the system, was increased with the increase of the dilution rate up to the level 0.4 $/h^{-1}$, whereby the maximal reactor productivity was attained (5.64 U/g wet cells/h). This implies that an optimum rate at which maximum enzyme productivity could be attained, adopting the packed bed bioreactor fermentation technique.

The optimal enzyme outputs of the immobilized cells of *B. circulans* in batch and continuous cultures were compared (Table 6). In terms of enzyme activity, the maximum yield was achieved after 48h in batch shaken culture of the free cells (18.47 U/ml). This value was higher than that recorded with of the immobilized cells using continuous or repeated batch techniques respectively. As far as the enzyme productivity was concerned, the immobilized cells in continuous fermentation afforded relatively high values as compared with the other tested experimental treatments. The long term cell viability and continuous metabolic activities have been recorded to be among the most important advantages on using the immobilized cells

particularly with the continuous fermentation system. Rychetra et al. (1987), Jamuna and Ramakrishna (1992) reported that the immobilization process leads to changes in micro environmental conditions, and some metabolic and morphological alteration in the cells may occur (Tonkova et al. 1994). In addition, Vives et al. (1993) reported that immobilization of the cells might alter the mechanism of enzyme biosynthesis. Moreover, Fortin and Vuillemard (1990) reported that the immobilization matrix could reduce the diffusion of the repressors to the immobilized cells. In the present study, the induced stress conditions imposed by the immobilization processes might have affected the enzyme system of the immobilized *Bacillus circulans* to be active for relatively long time (more than 5 cycles).

However, our findings demonstrated the feasibility of the production of levansucrase adopting the immobilized culture technique which allowed the achievement of relatively high enzyme activities in view of the successful repeated utilization of the immobilized cells.

Table 1. Levansucrase production by *Bacillus circulans* immobilized in different carriers.

Carrier	Incubation (h)	protein cone. mg/ml	Levansucrase activity (U/ml)	Specific productivity (U/g dry cells/h)	Effectiveness factor
Free cells	48	0.94	18.47	17.18	1.00
	72	0.82	15.04	9.32	1.00
	96	0.80	11.50	3.95	1.00
Ca-alginate	48	1.05	11.56	10.75	0.62
	72	1.41	13.23	7.16	0.88
	96	0.99	9.66	5.07	0.84
Agar	48	0.96	10.36	9.17	0.55
	72	1.20	13.53	9.62	0.90
	96	1.35	10.23	6.77	0.89
Stone	48	1.02	7.45	6.92	0.40
	72	0.92	10.00	6.20	0.66
	96	0.84	9.20	6.30	0.80
Wool	48	1.05	-		
	72	0.98	8.16	5.50	0.54
	96	0.88	08.08	04.09	0.76
Loaf	48	1.02	-		
	72	0.92	12.45	7.71	0.82
	96	0.82	9.77	05.10	0.85

-Effectiveness factors of immobilization= specific productivity of immobilized cells/ specific productivity of free cells at the same incubation period.
-Specific enzyme productivity= dilution rate × enzyme activity.

Table 2. Effect of matrix concentration on the production of levansucrase by the immobilized cells of *B. circulans*

Agar conc. (%)	Protein conc. (mg/ml)	Levansucrase activity (U/ml)	Specific productivity (U/g dry cells/h)
2	1.20	14.52	9.62
3	1.25	15.22	1.05
4	1.12	15.25	10.69
5	0.98	15.68	10.99
6	0.95	13.54	8.39

Table 3. Effect of biomass load on the production of levansucrase by the immobilized cells of *B. circulans*

Cell loads (g)	Protein conc. (mg/ml)	Levansucrase activity (U/ml)	Specific productivity (U/g dry cells /h)
1.12	0.98	14.35	8.66
2.24	1.05	17.89	11.05
3.36	1.20	14.25	8.83
4.48	1.25	12.03	7.25

Table 4. Effect of treatment of *B.circulans* cells with glutaraldhyde on the production of levansucrase

Treatment	Protein conc. (mg/ml)	Levansucrase activity (U/ml)	Specific productivity (U/g dry cells /h)
Control(without treatment)	0.99	17.89	11.05
Cells treated with gluteraldhyde	1.05	10.03	6.05

Table 5. Production of levansucrase by immobilized cells of *Bacillus circulans* in repeated batch treatment.

Cycle no	Levansucrase activity (U/ml)	Productivity (U/ml/h)	Specific productivity (U/g wet cells/h)
1	15.08	0.72	9.11
2	14.24	0.66	8.60
3	11.59	0.43	7.00
4	10.07	0.39	6.08
5	8.66	0.37	5.23

Table 6. Comparison of the levansucrase activities and productivities of *B. circulans* in different production systems.

Production techniques	Enzyme activity (U/ml)	Specific productivity (U/g wet cells/h)	Relative production fold
Free cells (at 48h)	18.47	3.84	1
Immobilization treatments: Batch wise	13.23	2.2	0.71
Repeated batch	15.08	2.6	0.81
Continuous production	14.12	7.3	0.76

Figure 1. Effect of different incubation periods on the production of levansucrase by free *B. circulars* cells.

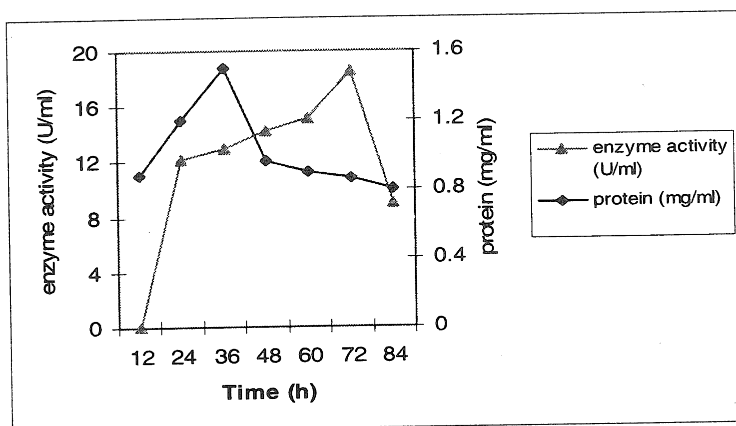
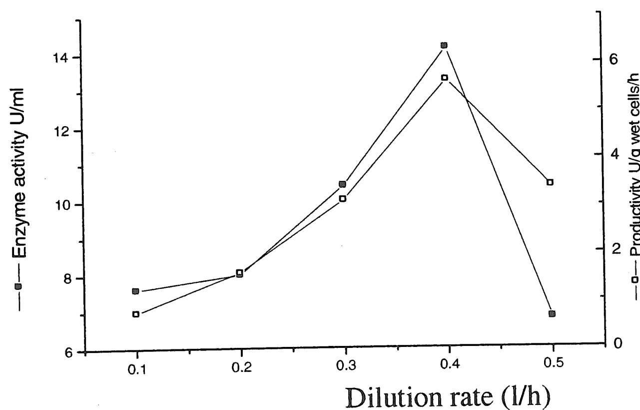


Fig 2. Production of levansucrase produced by continuous cultivation of immobilized cells of *Bacillus circulans* in agar .



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