Production of *Bacillus licheniformis* ATCC 21415 alkaline protease in batch, repeated batch and continuous culture

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ABSTRACT

Bacillus licheniformis ATCC 21415 cells were immobilized on different carriers using different methods of immobilization including physical adsorption, covalent binding, ionic binding and entrapment. The immobilized cells were prepared by covalent binding on wool (as a new carrier) through 1% glutaraldehyde had the highest enzyme activity (9.0 U/mL) with the highest specific productivity (6.17 U/g wet cells/h). Alkaline protease production and the stability of biocatalyst were investigated in both free and immobilized cells. The results showed that the immobilized cells were more efficient for enzyme production by repeated batch fermentation (5 cycles, 480 h) with 57% residual activity whereas the free cells retained 35% after 2 cycles. In continuous production the highest enzyme activity (9.9 U/mL) was obtained at a dilution rate of 0.1/h while the highest enzyme yield (763.6 U/h) and the highest reactor productivity (3.32 U/mL/h) were attained at a dilution rate of 0.4/h. Packed-bed bioreactor was a successful method for continuous production of alkaline protease for a long time (168 h) with 53% relative activity. The bioreactor affected the highest specific productivity (118.2 U/g wet cells/h) which was 12-24 times higher than other systems of enzyme production.

Keywords: B. licheniformis, immobilization, batch, repeated batch and continuous cultures
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INTRODUCTION

Alkaline proteases are one of the most widely studied group of enzymes due to their use in many industrial applications such as in food, pharmaceutical, leather, detergent and in recovering silver from used X-ray films (Singh *et al.*, 2004). Another important application of proteases is to break down protein tissue in order to be able to extract a particular substance, such as heparin (Beshay and Moriera, 2003). Bacilli are well suited for industrial enzymes production due to their ability to secrete large amounts of protein directly into the medium (Harwood, 1992). Thirty to forty of the production cost of industrial enzymes is estimated to be accounted by the cost of the growth media (Joo *et al.*, 2002).

Industrial proteases are normally produced in fed-batch cultivation with prolonged period of intermediate and low specific growth rates. Immobilization of microbial cells for enzyme production offers various advantages, such as the ability to separate cell mass from the bulk liquid for possible reuse, facilitating continuous operation over a prolonged period and decreasing contamination from the product stream during continuous fermentations without loss in biomass (Helmo *et al.*, 1985; Beshay, 2003; Karandikar *et al.*, 2006). Immobilized cells could increase productivity and operational stability or reduce the delays involved in enzyme production (Zhang *et al.*, 1989; Galazzo and Bailey, 1990). Moreover, immobilized cells can show modifications in physiology and biochemical composition when compared with suspended cells (Adinarayana *et al.*, 2004).

The application of immobilized cells for the production of metabolic products by microorganisms has been widely studied and several valuable products such as enzymes, organic acids, amino acids, steroids, alcohol and antibiotics have been successfully obtained (Guo *et al.*, 1990; Adinarayana *et al.*, 2004; Prabakaran and Hoti, 2008). Wool is an attractive and suitable as an immobilization matrix because it is cheap, available and non-toxic. The purpose of the present investigation was to study the immobilization of *B. licheniformis* ATCC 21415 cells for higher alkaline protease production using different methods on different carriers. The reusability of immobilized cells for alkaline protease production under batch, repeated batch and continuous fermentation were also investigated.

MATERIALS AND METHODS

Bacterial strain

B. licheniformis ATCC 21415 was obtained from the American Type Culture Collection, USA. The culture was maintained on nutrient agar medium at 30 °C for 48 h and stored at 4 °C.

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Fermentation media

The optimized medium was used for alkaline protease production and composed of (g/L): $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 0.5; $CaCO_3$, 1.0; dextrin, 35.0; NaCl, 1.0 and peptone, 1.0. The components of the medium were dissolved in 5 % wheat bran extract. The pH was adjusted to 7.0 before autoclaving. Each 250-mL Erlenmeyer flask contained 25 mL of medium and 2% (v/v) 24 h-old inoculum. The preparation of inoculum was done by adding 2 mL of sterile distilled water to 24-h old slants, which were scratched and transferred to each 250-mL Erlenmeyer flask. The flasks were incubated in a rotary shaker regulated at 6000 rev/min and 37 °C for 96 h. The bacterial cells were harvested by centrifugation in a refrigerated centrifuge at 5000 rev/min, 15 min, 4 °C. The clear culture filtrates were assayed for enzyme activity and protein content.

Assay of enzyme activity

Caseinase activity was estimated according to the method of Bergkvist (1963), by determining the amount of casein hydrolysis. For each test, 1 mL of enzyme solution was incubated at 37 °C for 10 min with 1 mL of casein solution (1.5% w/v in 50 mM glycine NaOH buffer at pH 9.5). One unit (U) of enzyme activity was taken as the amount of enzyme that liberated 1 μmol of tyrosine per mL per minute. At least three measurements were made for each experiment and the data given are an average of these results.

Protein determination

Total protein concentrations were determined by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

Carriers for cell immobilization

Sodium alginate, agar, chitosan and *k*-carrageenan were supplied from Sigma Chemical Co., USA. Loofa, sponge and stainless steel were collected from the local market. While wool (Lambs wool) was a personal specimen imported from France.

Cell immobilization procedures

All the immobilization processes were performed under aseptic conditions. The cell pellets of *B. licheniformis* ATCC 21415 obtained in logarithmic phase of growth was collected by centrifugation (5000 rev/min, 15 min) in a refrigerated centrifuge.

Entrapment in Ca-alginate

Bacterial cells obtained from 50 mL culture (0.38 g weight wet) were mixed with 10 mL sodium alginate solution at a concentration of 3% (w/v) through a sterile 5 mL pipette. Gel beads of approximately 2 mm diameter were obtained.

The beads were obtained by dropping mixtures into sterile $CaCl_2$ (0.1 M) through a sterile 5 mL pipette. Gel beads of approximately 2 mm diameter were obtained. The beads from 10 mL gel (150 beads) were used for inoculation of 25 mL of the production medium (Jamuna and Ramakrishna, 1992).

Entrapment in agar and k-carrageenan

The wet cells obtained from 50 mL culture (0.38 g weight wet) were mixed with 10 mL of 3% (w/v) agar or *k*-carrageenan solution at 45 °C. The mixture was quickly cooled at 4 °C, cut into 2x2x2 mm fragments and transferred to 25 mL of the production medium (Cheetham *et al*., 1985).

Covalent binding

One gram of each tested carrier (loofa, wool and stainless steel) approximately 1x1 mm fragments and was treated with 10 mL of 1% glutaraldehyde overnight. The treated carriers were collected by centrifugation and washed with sterile water to remove the excess glutaraldehyde. Then the cells obtained from 50 mL culture medium (0.38 g weight wet) were incubated with each carrier suspended in 10 mL water. The carriers were collected by centrifugation and washed with sterile water and used for inoculating 25 mL production medium (Abdel-Naby, 1993).

Adsorption on chitosan

One gram of chitosan (flakes 1x1 mm) was dissolved in 100 mL of 0.01 M HCl. Then the wet cell pellets obtained from 50 mL culture medium (0.38 g weight wet) were mixed with 100 mL of the previous chitosan solution. The beads were obtained by adjusting the pH value of the mixture to 7.0 with 0.01 M NaOH, washed with distilled water and used for inoculating of 25 mL production medium.

Production of alkaline protease by immobilized cells

Batch experiment

Batch experiment of the immobilized cells was performed in 250-mL Erlenmeyer flasks each containing 25 mL of sterile medium. The flasks were inoculated with the calculated amount of beads. Fermentation was conducted at 37 °C for 96 h in a rotary shaker regulated at 180 rpm. The cultivation medium was filtered off and the clear filtrate was taken for enzyme assay.

Repeated batch experiment

The experimental set up was similar to that of batch cultures. Every 96 h, immobilized cell beads were removed, washed with saline and recultivated into fresh medium. The process was repeated for several batches till the beads started disintegrating.
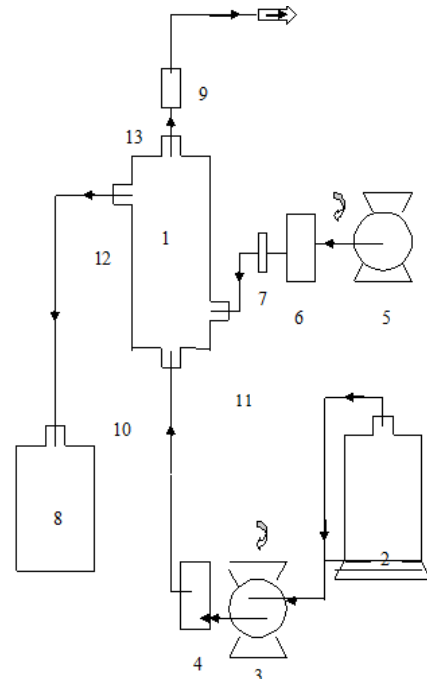
Continuous production using bioreactor

The bioreactor as shown in Figure 1 is a glass column of 1.8 cm diameter and 320 cm in length. The bioreactor was filled with 17.9 g of freshly prepared immobilized beads comprising 7.6 g of wet weight bacterial cells, the working volume of reactor was about 232 mL. The fresh medium was introduced from the bottom of the reactor and control by a peristaltic pump. Air was admitted through a sterile air filter from the bottom of the reactor and the air flow was optimized at 0.3 v/v/min using rotameter. The effluent was collected in a holding tank. The system was considered to be in a steady state only after at least 5 residence times (replacement volumes).

RESULTS AND DISCUSSION

Production of alkaline protease in batch culture

For this purpose the same amount of bacterial cells were immobilized by different methods on different carriers. The specific productivity and the effectiveness factor are the key parameters to select the suitable method and carrier for immobilization process.

The results showed that the activity of the immobilized cells was lower than the free cells and the effectiveness factor of the immobilization was less than one. Similar observations were reported by Jamuna and Ramakrishna (1992). Zaghloul *et al.* (2002) suggested that *B. subtilis* cells appeared to be more active than alginate immobilized cells. This might be due to the fact that viable cells of the free culture were little higher than that of the immobilized culture. This is because they represent a heterogeneous catalysis fermentation, in which the activity or rather synthesis of primary or secondary metabolites is dependent upon the mass transported and adequate oxygen supply. Also, the nutrients are lowering accessible to the immobilized cells. On the other hand, immobilization process leads to

Figure 1: Schematic representation of the continuous fermentation system

1	Bioreactor with the immobilized beads	8	Product collection vessel
2	Medium feed reservoir	9	Outlet air filter
3	Peristaltic pump	10	Medium inlet
4	Flow rat regulator	11	Air inlet
5	Air pump	12	Product outlet
6	Rotameter	13	Air outlet
7	Air filter		

Table 1: Production of alkaline protease in batch culture by free and immobilized *B. licheniformis* ATCC 21415 cells

Carriers	Caseinase activity (U/mL)	Specific productivity (U/g wet cells/h)	Effectiveness factor of immobilization*
Free cells	14.33	9.82	1.00
Immobilized cells			
I. Entrapment			
Agar 3%	6.15	4.21	0.43
Ca-alginate 3%	6.21	4.26	0.43
k-carrageen 3%	5.47	3.75	0.38
II. Covalent binding			
Loofa	8.50	5.83	0.59
Sponge	6.45	4.42	0.45
Stainless steel	1.31	0.89	0.09
Wool	9.00	6.17	0.64
III. Adsorption			
Chitosan	8.13	5.57	0.57

*The activity of immobilized cells/the activity of the same amount of free cells.

changes in the micro environmental conditions, and some metabolic and morphological alteration in the cells may occur. The results in Table 1 showed that the highest specific productivity (6.17 U/g wet cells/h) was reached by covalent binding of *B. licheniformis* ATTC 21415 cells on wool. Immobilization on wool showed the highest effectiveness factor of immobilization (0.64). Wool is considered a new attractive carrier for immobilization and because of its low cost, non-toxic and available.

Production of alkaline protease by repeated batch fermentation

Batch culture is a common mode for commercial fermentation in addition to fermentation time; however the production cycle also includes turnaround time (needed for sterilization, inoculation, turnaround etc.). This leads to a reduction in overall productivity and add to production costs. Therefore the possibility of multiple uses (repeated batch) of *B. licheniformis* ATTC 21415 wool immobilized cells was investigated. The results (Figure 2) indicated that wool immobilized cells retained their ability to produce alkaline protease consistently for 5 batches (480 h) with 57% residual productivity. Repeated batches of freely suspended cells had a significant decrease in residual productivity (35%) after 2 cycles. This loss in activity may be due to destruction of cells as well as autolysis during the centrifugation and washing process. The results are in agreement with those obtained by Beshay and Moreira (2003). They also reported on a rapid decrease in protease production by free *Teredinobacter turnirae* cells in the early cycles as compared to porous sintered glass immobilized cells. Adinarayana *et al.* (2005) reported that repeated batch fermentation with *B.subtilis* PE-11 alginate beads were successfully run for 9 batches (9 days).
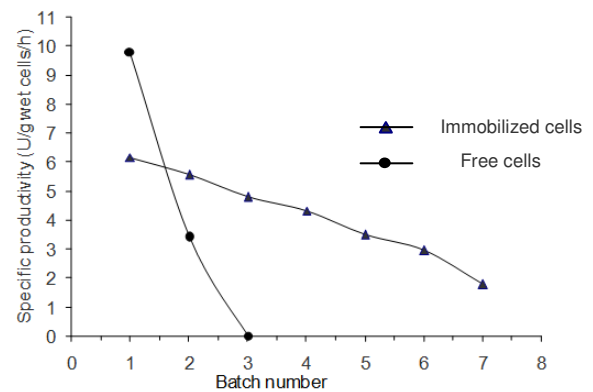
Continuous production of alkaline protease

The fermentation was conducted at 37 °C and carried out in batch operation for 24 h, and then the continuous operation was started at different dilution rates (0.1-0.7/h). The results (Figure 3) indicated that the increase in dilution rate led to a gradual increase in reactor productivity and there was a pronounced decrease at higher dilution rates. Maximal enzyme activity (9.9 U/mL) was attained at a dilution rate of 0.1/h whereby the maximal reactor productivity (3.32 U/mL/h) was obtained at a dilution rate of 0.4/h. Christiansen and Nielsen (2002) found that the maximal production of extracellular *B. clausii* protease was reached at a dilution rate of 0.14/h and it was constant in the range from 0.14 to 0.17/h. The kinetic parameters of the reactor with different dilution rates (data not shown) indicated that the specific productivity increased with the increase in dilution rate up to 0.4/h which gave maximal specific productivity (100.47 U/g wet cells/h). The higher dilution rate led to decrease in specific productivity (88.93 U/g wet cells/h). This trend was expected since the contact time between the medium and the immobilized cells was decreased as the dilution rate increased. Operational stability of the bioreactor was

studied. As shown from the results in Table 2, the reactor was able to keep producing alkaline protease enzyme (8.1 U/mL) for 120 h. After 5 days there was a gradual decrease in the enzyme level to 1.7 U/mL after 216 h (9 days) of the operation.

Table 2: Production of alkaline protease by wool immobilized *B.licheniformis* ATCC 21415 cells in packed-bed bioreactor at dilution rate 0.4/h

Time (h)	Caseinase activity (U/h)	Relative productivity (%)
24	8.3	100.00
48	8.3	100.00
72	8.2	98.80
96	8.1	97.60
120	8.1	97.60
144	6.0	72.3
168	4.4	53.00
192	3.0	36.10
216	1.7	20.50

Figure 2: Repeated batch fermentation for alkaline protease production by free and wool immobilized *B.licheniformis* ATCC 21415 cells
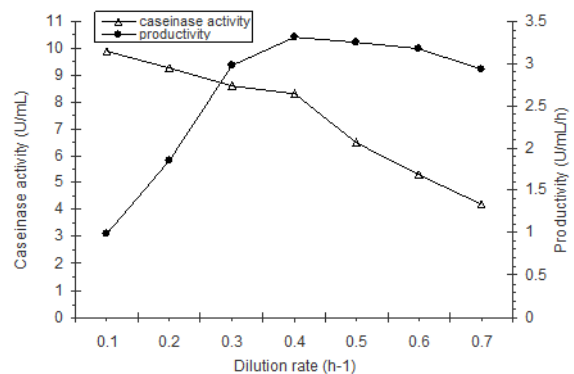
Figure 3: Continuous production of alkaline protease enzyme by wool immobilized *B.licheniformis* ATCC 21415 cells

CONCLUSIONS

Immobilization of *B.licheniformis* ATTC 21415 cells on a new carrier (wool) by covalent binding is a promising method for alkaline protease production. Immobilized cells under continuous operation in the bioreactor resulted in a specific productivity of 118.2 U/g wet cells/h which is 12-24 times higher than the other systems.

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