Int J Pharm Bio Sci 2015 Jan; 6(1): (B) 135 - 144

Research Article **Microbiology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

PRODUCTION OF ALKALINE PROTEASE WITH IMMOBILIZED CELLS OF *BACILLUS CEREUS STRAIN S8* IN VARIOUS MATRICES

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ABSTRACT

The purpose of this investigation was to study the effect of *Bacillus cereus strain S8* cells immobilized in various matrices, such as calcium alginate, k-Carrageenan, polyacrylamide, agar-agar, and gelatin, for the production of alkaline protease. Calcium alginate was found to be an effective and suitable matrix for higher alkaline protease productivity compared to the other matrices studied. All the matrices were selected for repeated batch fermentation. The average specific volumetric productivity with calcium alginate was 210 ± 0.15 U/ml, which was 57% higher production over the conventional free-cell fermentation. The protease yield and operational stability of the immobilized system were increased by tailoring the capsules' characteristics. Capsules prepared from 3% (w/v) sodium alginate and 2.0% (w/v) $CaCl_2$ were the best support for cell immobilization, providing 7.9 fold higher protease productions in comparison to the freely suspended cells. Immobilized biocatalysts sustained 94% of their initial productivity over five sequential batches in a 10-day period, while protease production by free cells declined sharply after further use.

KEYWORDS: Alkaline protease production, B. cereus strain S8, immobilized cells, Alginate, Capsules, repeated batch fermentation.

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INTRODUCTION

The use of immobilized cells offers several advantages over free cells, such as relative ease of product separation, re-use of biocatalysts, prevention of washout, reduced risk of contamination and operational stability. Owing to the continuous production of cells, the increase in biomass can cause problems with regard to the mechanical stability of the matrix, which ultimately may be destroyed. The method of immobilization and the mechanical properties of the matrix are, therefore, significant factors affecting the operational stability of the biocatalyst. Modification of biotechnology and processes, using immobilized biocatalysts, has recently gained the attention of many biotechnologists. Application of immobilized enzymes or whole cells is advantageous, because such biocatalysts display better operational stability [1, 2] and higher efficiency of catalysis [3, 4] and they are reusable. Microbial products are usually produced either by free or immobilized cells. The use of immobilized cells as industrial catalysts can be advantageous compared to batch fermentation process [5, 6]. Whole cell immobilization has been a better choice over enzyme immobilization [7, 8]. Among different immobilization techniques, entrapment in calcium alginate gel offers many advantages due to its simplicity and non-toxic character [9, 10]. Furthermore, using the entrapment technique, a dense cell culture can be established leading to improved productivity [11, 12]. Moreover, by changing the gelation conditions it is possible to control easily some of the capsule characteristics, such as the thickness or permeability to different substrates of the gel membrane [13]. The purpose of the present investigation was to study the immobilization of *Bacillus cereus S8* cells for higher alkaline protease production using different entrapment techniques with matrices such as calcium alginate, k- Carrageenan, Polyacrylamide, Agar-agar and Gelatin gel. The reusability of immobilized cells for alkaline protease production under repeated batch fermentation conditions was also investigated.

MATERIALS AND METHODS

(i) Chemicals

All chemicals (99% purity) used in this study were purchased from Hi-Media Laboratories, Merck (Mumbai, India) and Sigma (U.S.A).

(ii) Microorganism

An alkaline protease–producing strain of *Bacillus cereus S8* (MTCC NO.11901) was isolated [14]. It was maintained on nutrient agar slants at 4°C and was sub cultured for every 2 weeks. It was found to produce protease and other hydrolytic enzymes.

(iii) Inoculum Preparation

Five millilitres of sterile distilled water was added to a 24hr old slant of *B cereus S8*. The cells were scrapped from the slant into sterile distilled water and the resulted cell suspension was transferred, aseptically into 250 ml Erlenmeyer flasks containing 45 ml of sterile inoculum medium. The composition of the inoculum medium is (g/L) (gram/litre): glucose, 2.0; casein, 0.5; peptone, 0.5; yeast extract, 0.5, and salt solution, 50 ml (salt solution containing [g/L]: KH_2PO_4, 5.0; $MgSO_4.7H_2O$, 5.0, and $FeSO_4.7H_2O$, 0.1] with a pH of 7.0. The flask was kept in a shaker incubator at 220 rpm at 37ºC. The content of the flasks was centrifuged at 3000 rpm for 10 min and the supernatant was decanted. The cell pellet was washed thoroughly with sterile 20.0 g/L potassium chloride solution, followed by sodium chloride solution and sterile distilled water subsequently. Finally the cell mass was suspended in sterile sodium chloride solution (9.0 g/L). This cell suspension was used as inoculum for immobilization as well as for free-cell fermentations.

IMMOBILIZATION OF CELLS BY DIFFERENT MATRICES

(i) Immobilization of Whole Cells in k-Carrageenan

K-Carrageenan (4%) (Sigma, Mumbai, India) was weighed and added to 18 ml of 0.9% sodium chloride. It was dissolved by gentle heating and sterilized by autoclave. The cell suspension (2 ml equivalent to 0.50 g DCW (Dry cell weight)) was added to the molten k-

Carrageenan solution maintained at 40°C, mixed well, and poured into sterile flat bottom 4-inch diameter petriplates. After solidification, the k-Carrageenan blocks were cut into equal size cubes (4 mm^3) and added to sterile 2% potassium chloride solution and kept in the refrigerator for 1 hour for curing. The cubes were washed 3 to 4 times with sterile distilled water.20

(ii) Immobilization of Whole Cells in Agar-agar

A definite quantity of agar–agar (Hi-media, Mumbai, India) was dissolved in 18 ml of 0.9% sodium chloride solution to get final concentration of 2% and sterilized by autoclaving. The cell suspension (2 ml equivalent to 0.50 g DCW) was added to the molten agar-agar maintained at 40°C, shaken well for few seconds (without forming foam), poured into sterile flat bottom 4-inch-diameter petriplates and allowed to solidify. The solidified agar block was cut into equal size cubes (4 mm3), added to sterile 0.1 M phosphate buffer (pH 7.0), and kept in the refrigerator (1 hour) for curing. After curing, phosphate buffer was decanted and the cubes were washed with sterile distilled water 3 to 4 times.20

(iii) Immobilization of Whole Cells in Gelatin

Five millilitres (0.50% DCW) of cell suspension was added to 15 ml of 20% sterile gelatin (Hi-media), maintained at 45°C, and poured into a sterile petridish. The gel was over layered with 10 ml of 5% glutaraldehyde for hardening at 30°C. The resulting block was cut into small-size cubes (4 mm^3) and the cubes were washed thoroughly with sterile distilled water for complete removal of excess glutaraldehyde.

(iv) Immobilization of Whole Cells in Polyacrylamide

A cell suspension was prepared by adding 0.50 g cells to 10 ml chilled sterile distilled water. To another 10 ml of 0.2 M sterile phosphate buffer (pH 7.0), the following chemicals were added: 2.85 g acrylamide, 0.15 g bisacrylamide, 10 mg ammonium persulphate, and 1 ml TEMED (NNN1N1 tetra methyl ethylene diamine). The cell suspension and the above phosphate buffer mixture was mixed well and poured into sterile flat bottom 10 cm-diameter petriplates. After polymerization (solidification), the acrylamide gel was cut into equal size cubes (4 mm^3), transferred to 0.2 M phosphate buffer (pH 7.0), and kept in the refrigerator for 1 hour for curing. The cubes were washed 3 to 4 times with sterile distilled water and stored in sterile distilled water at 4°C until use.

(v) Whole Cell Immobilization by Entrapment

The alginate entrapment of cells was performed according method of Johnsen and Flink.[15]. Sodium alginate solution (3%) was prepared by dissolving sodium alginate in 100 ml boiling water and autoclaved at 121°C for 15 minutes. Both alginate slurry and cell suspension (equivalent to 0.50 g DCW) were mixed and stirred for 10 minutes to get a uniform mixture. The slurry was taken into a sterile syringe and added drop wise into 0.2 M $CaCl_2$ solution from 5-cm height and kept for curing at 4°C for 1 hour. The cured beads were washed with sterile distilled water 3 to 4 times. When the beads were not being used, they were preserved in 0.9% sodium chloride solution in the refrigerator. All operations were carried out aseptically under laminar flow unit.

OPTIMIZATION OF THE CAPSULE CHARACTERISTICS

Various amounts of sodium alginate (1–5%, w/v) were added to the gel mixture to study the effect of sodium alginate concentration on the gel capsule permeability. The gelation of the mixture was induced by a 3.5% (w/v) $CaCl_2$ solution. In order to investigate the effect of $CaCl_2$ concentration on the rigidity of the beads the 2% (w/v) sodium alginate solution was extruded drop-wise into varying $CaCl_2$ concentrations from 1 to 5% (w/v).

(i) Fermentation

Immobilized cells prepared by the above method were added to 250 ml flasks containing 50 ml production medium of the following composition: molasses,1%(w/v); potassium nitrate, 0.75%(w/v); salt solution- 5%(v/v) {$MgSo_4.7H_2O$, 0.5%(w/v); KH_2PO_4, 0.5%(w/v)}; $FeSO_4.7H_2O$, 0.01%(w/v) and $CaCO_3$, 0.5%. For the free cell cultures the 50 ml production medium was inoculated with bacterial cells equivalent to those used in immobilized cultures. Batch fermentations with free and

immobilized cells were carried out at 37^0C for24h, 48 h, and 72h on a rotary shaker at 160 rpm. Repeated batch fermentations with optimal characteristics were conducted by running the fermentation for 48 h. At the end of each cycle the production medium was recovered, the immobilized cells were washed with sterile saline, fresh production medium was added and the fermentation was continued. The operational stability of the immobilized system was determined by the following equation:

Operational efficiency (%) = 100 × (Cx/C_1)

Where C_1 is the protease yield produced in the 1st operation cycle and Cx is the protease yield produced in the xth operation cycle. The effectiveness factor of the immobilized biocatalyst was defined as the ratio of the protease activity of the immobilized system to that of the free cells:

Effectiveness factor = C_{imm}/C_{free} Where C_{imm} is the protease yield produced by immobilized cells and C_{free} is the protease yield produced by free cells. Cells leaked from the gel matrix were determined as cell dry weight by measuring the optical density at 600 nm.

(ii) Analytical methods

The results are the mean value of a minimum of three replicas for every fermentation experiment.

(iii) Protease activity

The enzyme activity was determined by using Mc Donald & Chen method [16].

(iv) Protein estimation

Protein estimation was determined according to the method of Lowry [17], using crystalline Bovine serum albumin as standard.

(v) Dry weight estimation

The alginate capsules were dissolved using 1% (w/v) sodium citrate solution. The liberated cells were separated by centrifugation, washed with 0.9% (w/v) NaCl and dried in an hot air oven until it gets constant weight. Biomass was determined from the difference between the total weight of the dissolved alginate gel capsules containing cells and that of alginate capsules without cells (blank) prepared under the same conditions. The dry weight of the bacterial cells was approximately 1.5g.

Statistical Analysis

Data obtained from this study were analyzed using a two-way analysis of variance (ANOVA) and values for $P \leq 0.05$ were considered statistically significant.

RESULTS

(i) Immobilization of cells by different matrices

Cell immobilization is one of the common techniques for increasing the overall cell concentration and productivity. The separation of products from immobilized cells is easier compared with suspended cell systems. Immobilization is a strategy for protecting cells from shear forces. Many different techniques for immobilizing cells have been proposed [18]. Cells entrapped in calcium alginate showed highest production of alkaline protease (210 ± 0.22U/ml) when compared to free cells (131 ± 0.15U/ml). The protease production by immobilized and free cells with respect to different incubation time periods was shown in Fig.1A. Effectiveness factor of different matrices was shown in Fig.1B. Further studies were carried out by using sodium alginate beads which showed highest protease production.

(ii) Protease production by cells of B. cereus strain S8 entrapped in Sodium Alginate

Growth and production of protease were followed up in batch cultures for free and in immobilized cell cultures of *B. cereus S8* (Fig. 1A). The media used for protease production is molasses,1%(w/v); potassium nitrate,0.75%(w/v); salt solution- 5%(v/v) {$MgSo_4.7H_2O$, 0.5%(w/v); KH_2PO_4, 0.5%(w/v)}; $FeSO_4.7H_2O$, 0.01%(w/v) and $CaCO_3$, 0.5%. In free cultures rapid cell growth was observed in the first 12 h of cultivation, and continued up to 72h of incubation with increasing enzyme production (Fig. 2). Immobilized cells, on the other hand, exhibited different kinetics. The increase in biomass entrapped in the gel capsules was continued from 12 h to 48 h, reaching a cell concentration of 1.5g/100ml of production medium in the alginate gel. The time required for the stabilization of the cell content in the gel capsules is approximately 48h of cultivation during which maximum

protease activity was observed, (Fig. 2) suggesting that there is no significant reduction in the total cell biomass production upon immobilization of the bacterium. Finally it should be mentioned that some cells were released from the capsules into the medium. However, this represented only a small part of the entrapped biomass, since the concentration of the released cells in the culture liquid was restricted to 0.01g/100ml (from fourth operational cycle). The data presented in Fig. 1A indicate also that the productivity of protease by the immobilized cells was significantly greater than that of the freely suspended cells. The calcium alginate entrapment technique led to an approximately 3.4 fold increase in the protease yield in comparison to free cell cultures. Cell immobilization resulted in higher production rates, giving total protease yield within the first 48 h of cultivation, while the time required in the case of freely suspended cells exceeded 48 h to 72 h. Since the immobilization led to changes in the micro environmental conditions, some metabolic and morphological alterations in the cells may occur. Similar observations have already been reported for various *Bacillus* species and most researchers attributed the alteration of the enzyme synthesis mechanism to the stress conditions imposed by immobilization [19-21]. To further investigate the effect of changes in the micro environmental conditions on protease production from the immobilized cells, immobilization was carried out by two alternative modes: (1) cells were suspended in the nutrient broth used for the production of protease or (2) washed with 0.9% (w/v) sterile NaCl and resuspended in the sterile saline before mixing with an equal volume of sodium alginate solution. The immobilization of cells suspended in the nutrient broth resulted in higher protease production (250U/ml) in comparison to the immobilization of cells suspended in the sterile saline (210U/ml). The presence of various nutrients and ions in the above mentioned mixture may have contributed to the formation of a more complex gel matrix and concomitantly to the retention of the bacterial cells in the capsules. The limitation of using nutrient broth is cell leakage from the capsules was observed when the cells were suspended in the nutrient broth prior to their mixing with the sodium alginate solution.

(iii) Optimization of parameters for immobilization of B. cereus strain S8 in alginate capsules

Cell immobilization in calcium alginate gel capsules offers the advantage of improving some of the capsule characteristics, such as thickness and percentage of cell leakage by changing certain conditions like sodium alginate and $CaCl_2$ concentration. Figure. 3A shows the effect of the concentration of sodium alginate solution used for the preparation of capsules on protease production and cell leakage. Higher sodium alginate concentrations had an adverse effect on protease production. Although increase in the biopolymer concentration from 2 to 5% (w/v) improved the durability of the capsules and reduced cell leakage but it resulted in 40% lower enzyme yield. This could be ascribed to the reduction in diffusion of nutrients and oxygen into the gel or to limitation of protease release out of it due to its high rigidity. On the other hand, gel capsules prepared from 1% (w/v) biopolymer were fragile and difficult to handle and released significant amount of cells into the culture medium. The highest protease production was obtained with capsules prepared from 3% (w/v) sodium alginate (Fig. 3A). The mechanical strength of alginate capsules appeared to be also highly dependent on the $CaCl_2$ concentration of the gelation solution (Fig. 3B). Increase in the $CaCl_2$ concentration from 1 to 2% (w/v) resulted in limitation of cell leakage from the capsules, which was followed by a 33% increase in protease yield. Use of concentrated $CaCl_2$ solutions decreases the efficiency of the immobilized system.

(iv) Semi-continuous production of protease by immobilized in alginate capsules B. cereus strain S8 cells

The stability of immobilized *B. cereus strain S8* cells in repeated batch fermentations was investigated in order to assess their ability for long-term growth and synthesis of protease. Fig. 4 illustrates the protease production by *B. cereus strain S8* cells, presented as percentage of the initial yield, for 10 batch cultivations. As a control, parallel experiments

with a suspension of free cells were carried out. Free cells lost 50% of their production (data not shown) capacity after the second use. On the contrary the immobilized biocatalysts showed high protease productivity upon re-use. Cells entrapped in calcium alginate gels retained about 94% of their initial efficiency during the first five batches when each cycle continued for 48 h. Under these conditions the immobilized system retained 60% of its initial activity after 10 successive batches. At the end of the 10th cycle the immobilized cells lost only 50% of their initial productivity. The effectiveness factor for the immobilized system is 1.60. The operational efficiency for each operational cycle was shown in Fig.4.

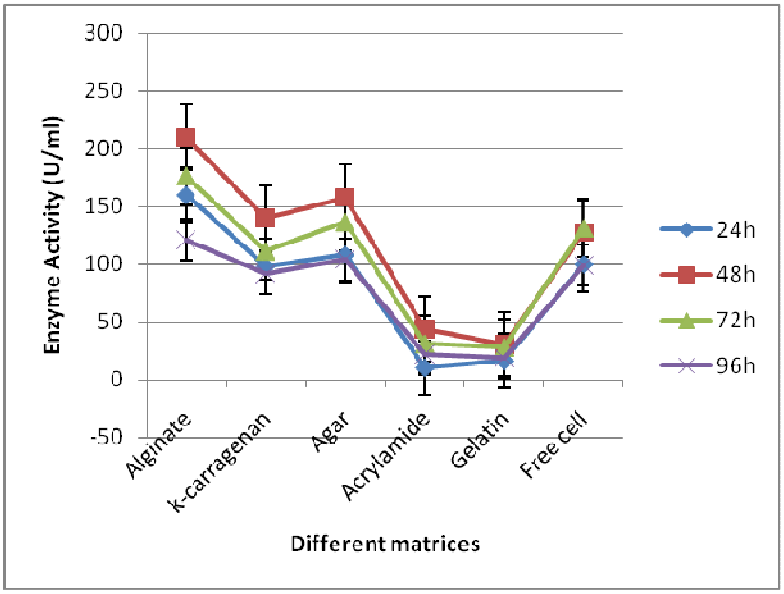

Figure 1A
Protease activity shown by cells in different matrices with respect to different incubation periods.

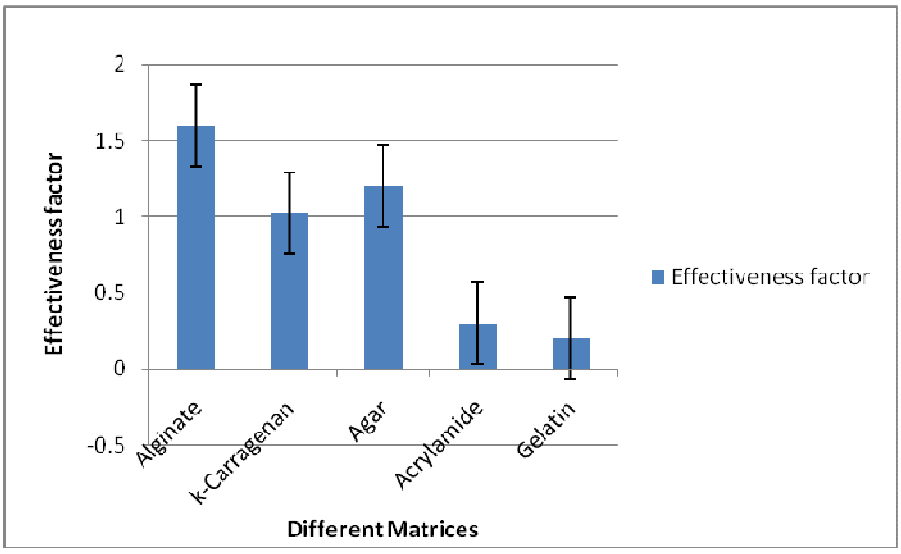

Figure 1B
Effectiveness factor of different matrices.

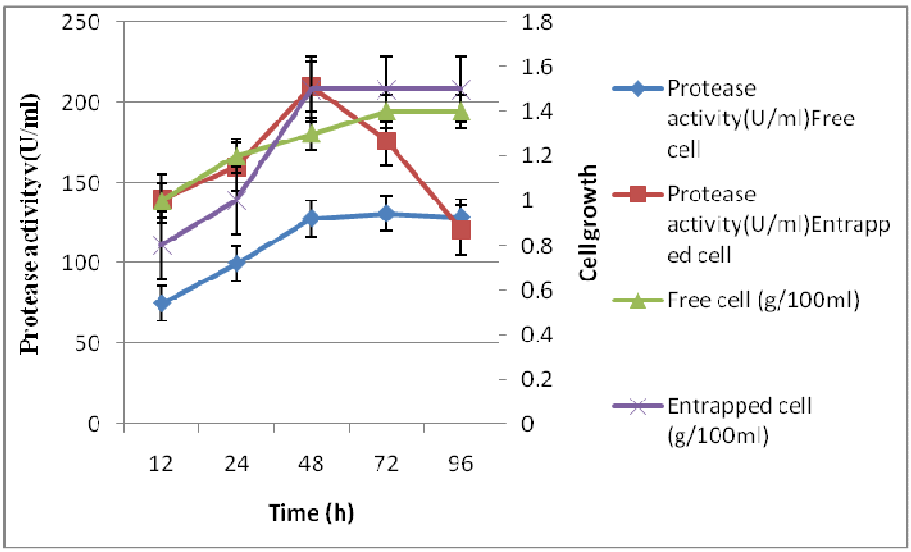

Figure 2
Kinetics of extracellular protease production by free cells and immobilized in calcium alginate gel capsules cells.

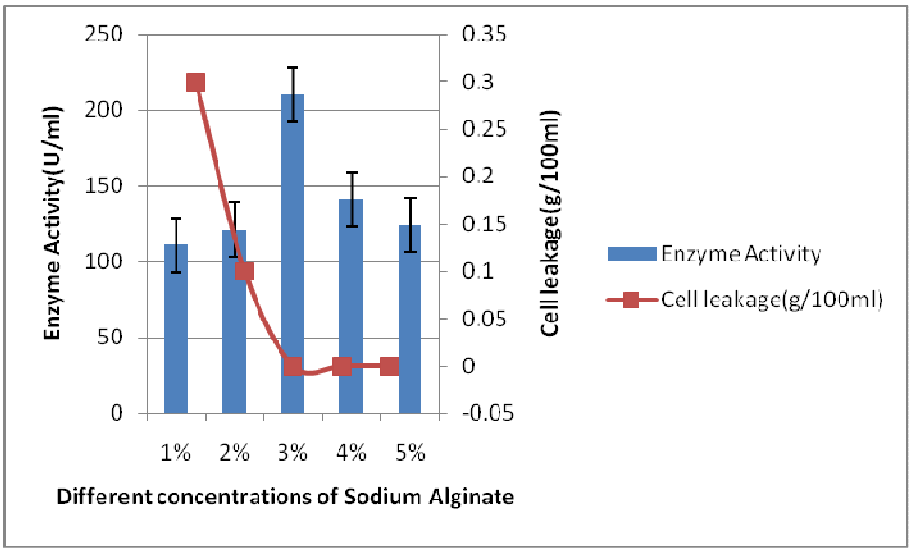

Figure 3A
Effect of sodium alginate concentration used in the preparation of the gel capsules on protease production by immobilized in calcium alginate gel capsules cells.

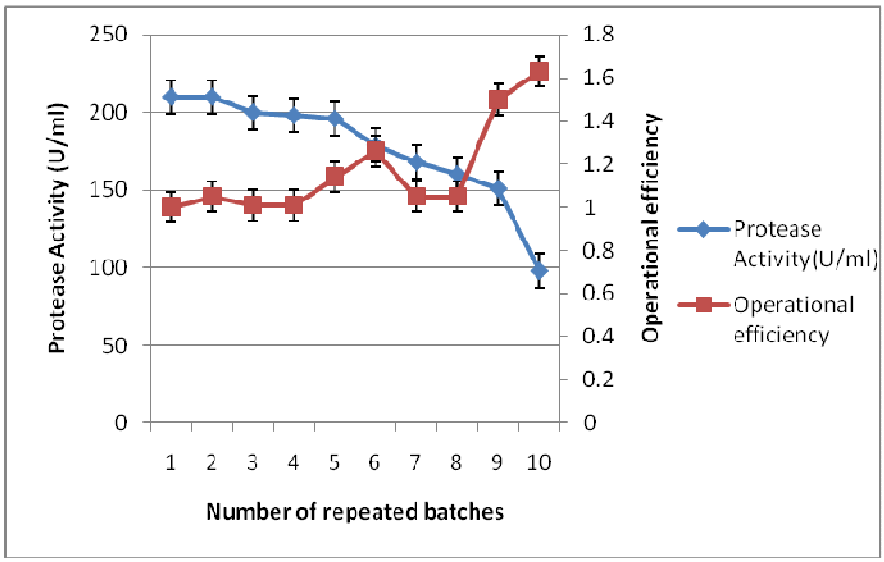

Figure 4
The protease production and operational efficiencies at respective operational cycles.

DISCUSSION

The alkaline protease titre with immobilized cells in alginate matrix was found to be higher followed by Agar. Low level of alkaline protease production was observed with Polyacrylamide and gelatin. Probably both glutaraldehyde (used for cross-linking with gelatin) and Polyacrylamide monomers were toxic for the cells. A low level to moderate titre of alkaline protease was obtained with k-Carrageenan, and also it was proved to be unstable resulting in a considerable amount of cell leakage The productivity of protease by immobilized cells was significantly greater than that by free cells, while the properties of the enzyme, which have been reported in previous work [14], were not affected by immobilization. In contrast to the reports of other researchers, suggesting that immobilized biocatalysts produced lower levels of enzyme in comparison to free cells due to diffusional barriers and reduced oxygen availability to immobilized aerobic cells [21, 22], 7.9 fold higher protease yield was achieved upon immobilization of *B. Cereus strain S8.* The possibility for reuse of the calcium alginate biocatalysts to produce alkaline protease in semi continuous mode the repeated batch fermentation with calcium alginate beads was successfully run for 9 batches (18 days). These findings were in accordance with those obtained previously for the protease production by immobilized *Serratia marcescens* and *Myxococcus xanthus* in calcium alginate beads [23]. It was found that protease production by immobilized *S. marcescens* increased with repeated growth cycles, and reached a maximum after 5 cycles. Bandyopadhyay *et al* [24] studied erythromycin production by *Streptomyces erythreus* entrapped in calcium alginate beads and obtained efficient productivity of erythromycin. They could conduct repeated batch fermentation successfully (each batch 48 hours) for 12 batches (30 days). Similarly, Farid *et al* [25] reported that a good level of oxytetracycline was produced for a period of 28 days (7 batches) using *Streptomyces rimosus* cells immobilized in 4% calcium alginate. The alginate matrix was found to be superior to the other matrices studied in this paper. In addition, the alginate matrix is less expensive, nontoxic, and preparation of biocatalyst involves mild conditions, which is an added advantage. After the immobilized biocatalyst had been in use for about 9 batches, it still possessed alkaline protease production. It has been shown that immobilized cells were able to produce alkaline protease consistently and that they might be used for continuous alkaline protease production [26] Ing-Lung Shih *et al* [27] achieved highest levan production by using

Bacillus subtilis natto cells immobilized on alginate.

CONCLUSION

The results show that calcium alginate is a promising method of *B. cereus strain S8* immobilization for alkaline protease production among different matrices used. Alkaline protease production by immobilized cells is superior to that of free cells because it leads to higher volumetric activities within the same time of fermentation. Specific advantages of this technique such as long life-term stability, reusability, and possibility of regeneration to be adaptable also to scale-up the obtained data. In addition, the experiments with repeated batches of alginate immobilized bacterial growth by introducing fresh nutrients every 24 hours lead to a specific volumetric productivity than free cells.

ACKNOWLEDGEMENT

The authors would like to acknowledge UGC-MRP, New Delhi, India, for providing financial support to carry out this work.

CONFLICT OF INTEREST

Conflict of interest declared none .

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