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Full Length Research Paper

Evaluation of different matrices for production of alkaline protease from *Bacillus subtilis* –K 30 by entrapment technique

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The present investigation evaluates the suitability of different matrices such as calcium alginate, polyacrylamide, agar-agar and gelatin for production of alkaline protease from *Bacillus subtilis*-K 30 using immobilization approach. Calcium alginate was found to be an effective and suitable matrix for higher alkaline protease productivity compared to other matrices studied. All the matrices were selected for repeated batch fermentation. The average specific volumetric productivities with calcium alginate was 14.83 U/ml/h which is 42.83% higher production over the convention free cell fermentation. Similarly, the specific volumetric productivity by repeated batch fermentation was 12.29 U/ml/h with polyacrylamide, 12.22 U/ml/h with agar-agar and 12.09 U/ml/h with gelatin. From the results, it is concluded that the immobilized cells of *B. subtilis* K-30 in calcium alginate are more efficient for the production of alkaline protease with repeated batch fermentation.

Key words: *Bacillus subtilis* K-30, Alkaline protease, immobilized cells, repeated batch fermentation, matrices.

INTRODUCTION

Proteases constitute one of the most important groups of industrial enzymes that execute a wide variety of functions and have various important biotechnological applications (Mohen et al., 2005). These enzymes occupy a pivotal position due to their applications in detergent industry, leather industry, food industry; pharmaceutical industry, silk industry and bio-remediation processes (Anwar and Saleemuddin, 1998; Gupta et al., 2005; Prakasham et al., 2005; Naidu and Devi, 2010). Although, a variety of proteolytic fungi and bacteria are known; only few microbial strains provide high activities with commercial success (Berla and Suseela, 2002). Moreover, the increasing demand for protease as a detergent supplement justifies a study aiming at a search for proteolytic enzymes from various microbial sources. Commercial proteases are mostly from various sources and it was reported that about 35% of the total microbial enzymes used in detergent industry are the proteases from bacteria (Ferrero et al., 1996). *Bacillus* spp. are extensively exploited for protease production (Karl-Heinz, 2004) due to lack of pathogenicity and ability to grow in simple medium (Joshi, 2010).

At present, the use and applications of alkaline proteases have increased remarkably with large proportions of commercially available alkaline proteases derived from *Bacillus* strains (Sen and Satyanarayana, 1993; Prakash et al., 2005; El Enshasy et al., 2008). Modification of processes using immobilized biocatalysts has recently gained much attention from biotechnologists (Kumari et al., 2009). Use of immobilized or whole cells is advantageous because such biocatalyst offer better operational stability, ability to separate from the bulk liquid for possible reuse, continuous operation and decreasing contamination from the product stream during continuous fermentations without loss in biomass (Helmo et al., 1985, Beshay, 2003; Ahmed and Abdel-Fattah, 2010). Moreover, immobilized cells show modifications in

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Abbreviations: DCW, Dry cell weight; **TEMED,** tetra methyl ethylene diamine.

increased mechanical, thermal or chemical resistance in accordance to the method and materials used for their entrapment. As a result, the same cells can be used multiple times with no apparent decrease of their overall biological activity compared to suspended cells (Lukasz et al., 2011; Adinarayana et al., 2004). In the present study, we report immobilization of *B. subtilis* K-30 cells originally isolated from soil sample for higher alkaline protease production using different entrapment techniques with matrices such as calcium alginate, 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

Microorganism

B. subtilis K-30 was used throughout this study. It was isolated using rice bran as a carbon source in our laboratory (Naidu and Devi, 2005). It was maintained on nutrient agar slants at 4°C and was sub cultured every two weeks.

Inoculum preparation

The inoculum was prepared as previously reported (Adinarayana et al., 2005) with modifications. Briefly, 5 ml of sterile distilled water was added to 24 h old slant of *B. subtilis* K-30. The cells were scraped from the slant into sterile distilled water and the resulted cell suspension at 10% level was aseptically transferred into 250 ml Erlenmeyer flasks containing 45 ml of sterile inoculum medium. The composition of inoculum medium (g/L): 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; $MgSO_4.7H_20$, 5.0, and $FeS0_4.7H_20$, 0.1) and pH was maintained at 7.0. The flask was kept in a shaker at 150 rpm at 37°C.The content of the flask 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 (20.0 g/L) and sterile distilled water. Finally, the cell mass was suspended in sterile sodium chloride solution (9.0 g/L). This cell suspension was used as inoculum source for all immobilization studies.

Immobilization of whole cell in alginate, polyacrylamide, agar-agar and gelatin

The alginate entrapment of cells was performed according to the method of Johnsen and Flink (1986). Briefly, 3% sodium alginate solution was prepared under sterile conditions. Both alginate slurry and cell suspension [(0.03 g dry cell weight, (DCW)] were mixed and stirred for 10 min. The resultant slurry was added drop wise into 0.2 M calcium chloride solution with the aid of a syringe of 1.0 mm and left for curing at 4°C with mild stirring at 60 rpm for 1 h. The beads were then washed for 3 to 4 times with sterile distilled water. When the beads were not used, they were preserved in 0.9% sodium chloride solution at 4°C. All operations were carried out aseptically in a laminar air flow unit. Immobilization in polyacrylamide was done by the method described by Adinarayana et al. (2005) by adding 0.03 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: 2.85g acrylamide (Sigma-Aldrich), 0.15 g bisacrylamide (Sigma-Aldrich), 10 mg ammonium persulphate (Sigma-Aldrich), and 1 ml tetra methyl ethylene diamine (TEMED), (Sigma-Aldrich). The cell suspension and the above phosphate buffer mixture was mixed well and poured into sterile flat bottom 10 cm-diameter petri plates. After polymerization, 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 h for curing with constant stirring. Whole cell immobilization of *B.subtilis* K-30 was carried out in accordance with the method described by Veelken and Pape (1982). Encapsulation in agar was done by adding cell suspension (2 ml equivalent to 0.03 g DCW) into the molten agar-agar. The solidified agar block was cut into equal size cubes (4 mm^3), added to sterile 0.1M phosphate buffer (pH 7.0), and kept in the refrigerator (overnight) for 1 h for curing. Five milliliters (0.06% DCW) of cell suspension was added to 15 ml of 20% sterile gelatin, maintained at 45°C and poured into sterile petriplate. The gel was over-layered with 10 ml of 5% glutaraldehyde for covalent crosslinking and hardening at 30°C. The resulting block was cut into small cubes (4 mm^3) and the cubes were washed thoroughly with sterile distilled water for complete removal of excess glutaraldehyde.

Production of alkaline protease by batch process with immobilized cells

The immobilized beads, prepared by using all the four matrices along with control were transferred into 50 ml of production medium in 250 ml Erlenmeyer flasks. The composition of production medium was (g/L) glucose, 5; peptone, 7.5; and 5% salt solution ($MgSO_4 \cdot 7H_2O$, 5 g/L; KH_2PO_4, 5 g/L; and $FeSO_4 \cdot 7H_2O$, 0.1 g/L) with a pH of 9.0. The flasks were incubated at 37°C for 48 h. Samples were withdrawn at regular intervals of 6 h and assayed for alkaline protease activity.

Alkaline protease assay

The culture broth was centrifuged at 3000 rpm for 20 min and supernatant served as the crude enzyme source. Protease activity was assessed by the modified procedure based on the method of Tsuchida et al. (1986) using 2.0% casein in 0.2 M carbonate buffer (pH 10.0) as substrate. One unit of enzyme activity is defined as the amount of enzyme that released 1 µg of tyrosine/mL/min. All experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Application of immobilized cells or whole cells is advantageous because such biocatalysts display better operational stability, higher efficiency of catalysis and also their suitability. Immobilization is a strategy for protecting cells from shear forces.

Production of alkaline protease by immobilized cells in calcium alginate

Immobilization of *B.subtilis* K-30 cells using different entrapment techniques with matrices such as calcium alginate, polyacrylamide, agar-agar and gelatin gel was investigated. The enzyme production with immobilized

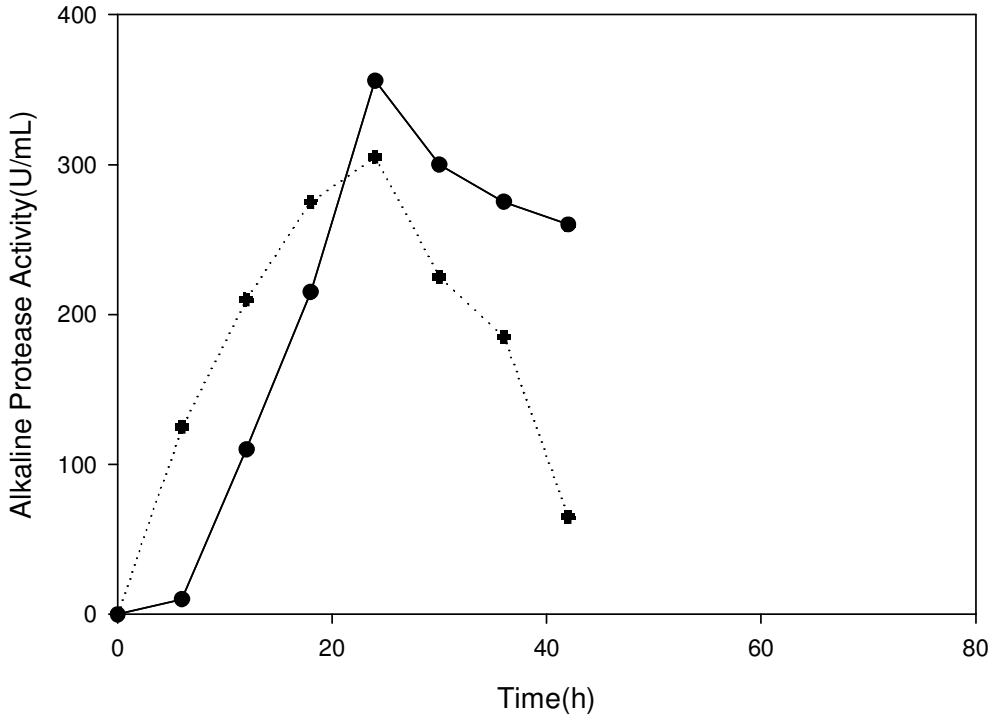

Figure 1. Time course profile of alkaline protease production by free cells and immobilized culture of *B. subtilis* K-30 in calcium alginate.

cells in calcium alginate started at 6 h and reached the maximum level (356 U/ml) by 24 h (Figure 1). On further incubation, enzyme production gradually decreased, while maximum enzyme titer was observed at 48 h in the case of free cells. It is evident that the alkaline protease production was higher with immobilized cells (356 U/ml) compared to free cells (305 U/ml). Beshay (2003) studied the effect of alginate concentration on immobilization of alkaline protease producing bacterial strain, *Teredinobacter turnirae.* The production of alkaline protease improved significantly with increasing alginate concentration and reached a maximum enzyme yield of 8000 U/ml at 25 g/L alginate concentration, which was about 176.8% higher than that obtained by free cells (2890 U/ml). However, at low alginate concentration (20 g/L), the beads are relatively soft and showed rapid leakage of cells from the beads. Another explanation for decrease in protease production with further increase in alginate concentration is low rate of substrate mass transfer and lower porosity of gel beads as judged by earlier reports (Alvaro and Maria, 1996; Fumi et al., 1992). Ramakrishna et al. (1992) reported the immobilization of *Bacillus cereus* in calcium alginate and employed packed bed reactor and fluidized bed-reactors to continuously synthesize thermostable α-amylase. They spun alginate fibres by pulstration technique to reduce the diffusional resistances in the gel matrix, and thereby a 24-fold increase in the productivity compared to batch fermentation with free cells was attained.

Production of alkaline protease by immobilised cells in polyacrylamide

Polyacrylamide was successfully used for immobilization of microbial cells, sub cellular organelles and many enzyme systems (Kierstan and Bucke, 1977; Kim et al., 1994). A gradual increase in alkaline protease production was noticed from 6 h onwards to 24 h (Figure 2). On further incubation, there was a decline in alkaline protease titer with immobilized cells in polyacrylamide. Alkaline protease titer was 295 U/ml at 24 h which was a low titer compared to alginate matrix.

Production of alkaline protease by immobilized cells in agar-agar

Protease production increased gradually from 6 h onwards and reached maximum level by 24 h (294 U/ml). Further, alkaline protease production with immobilized cells in agar-agar was comparable with polyacrylamide, whereas it was less than alginate matrix (Figure 3). The cell leakage from the matrix was gradually increased with increase of fermentation time. The operational stability of *Bacillus circulans* ATCC 21783 immobilized on agar-agar for cyclodextrin glucanotransferase production was studied by Anna et al. (2003) by repeated batch cultivation for 24 h in a fluidized bed reactor and found to be effective. In another report, an acid protease from

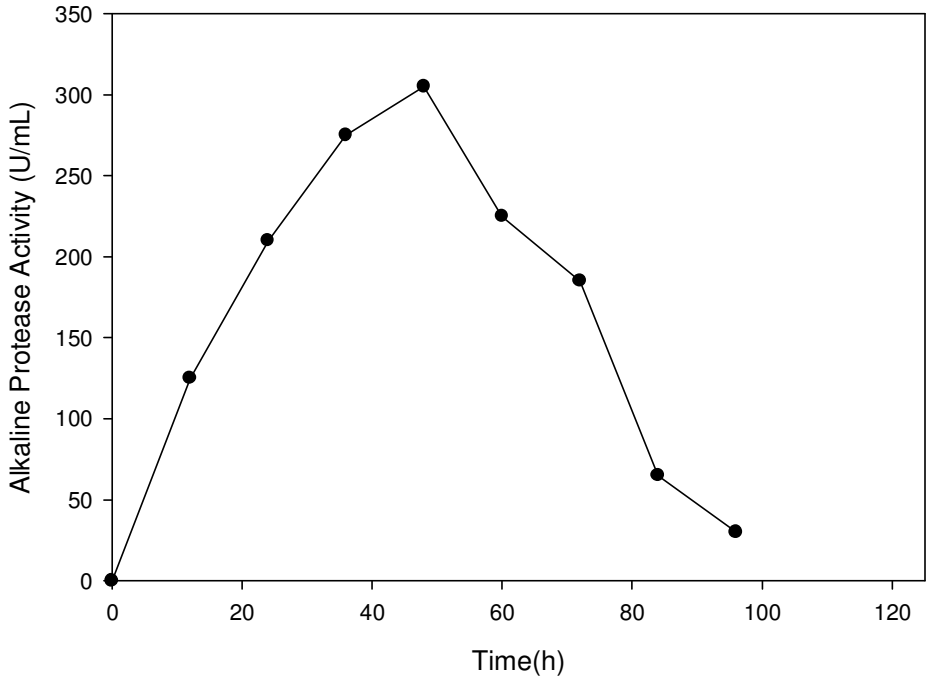

Figure 2. Time course profile of alkaline protease production by free cells of *B.subtilis* K-30.

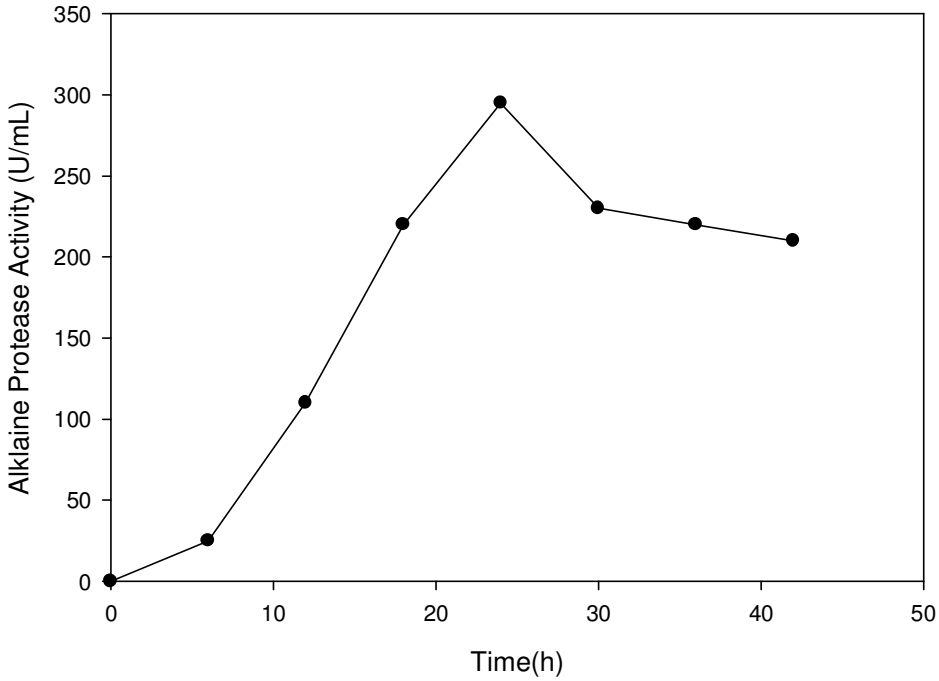

Figure 3. Time course profile of alkaline protease production by *B.subtilis* K-30 in polyacrylamide.

Aspergillus saitoi was immobilized on agar beads of approximately 100 µm diameters, the hydrolysis of gelatin by immobilized acid protease was found to be 6.8 x 10^{-7} M per second (Gregg and Roger, 1981) Only a detectable level of alkaline protease titer was observed after 6 h of fermentation and reached the maximum level (290 U/ml) by 24 h in gelatin (Figure 4).The alkaline protease titer obtained with this carrier was low compared to that in alginate matrix and comparable to that of free cells, polyacrylamide and agar-agar. *B. subtilis* PE-11

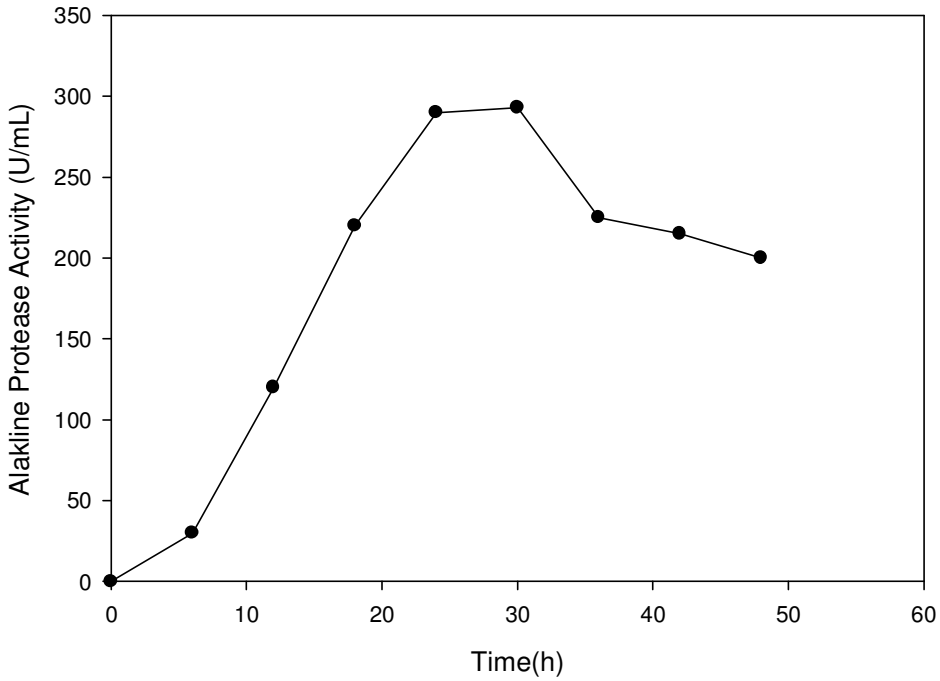

Figure 4. Time course profile of alkaline protease production by *B.subtilis* K-30 in gelatin

Table 1. Comparison of alkaline protease production with cells immobilized in different matrices.

Matrix	Fermentation period for each batch (h)	Number of batches	Total fermentation time (h)	Total alkaline protease titer (U/ml)	Average alkaline protease activity per batch (U/ml)	Specific Volumetric productivity U/ml/h)
Calcium alginate	24	9	216	3204	356	14.83
Polyacrylamide	24	6	144	1170	295	12.29
Agar-agar	24	6	144	1760	294	12.22
Gelatin	24	6	144	1740	290	12.09
Free cells	48	1	48	305	305	6.37

isolated from industrial effluents upon immobilization in gelatin retained 79% of initial activity after 6 cycles of repeated batch fermentation (Adinarayana et al., 2004). Gelatin is employed as a carrier material for the immobilization of *Kluyveromyces fragilis* for β-galactosidase production and Escherichia *coli* for penicillin acylase production (Ramakrishna and Prakasham, 1999).

Comparison of alkaline protease production by immobilized cells in different matrices by entrapment technique

The results show that the average specific volumetric productivity with alginate matrix was 14.83 U/ml/h which is 42.83% higher production over the conventional free cell fermentation (Table 1). Similarly, low level of alkaline protease was observed with polyacrylamide (12.29 U/ml/h), agar (12.22 U/ml/h) and gelatin (12.09 U/ml/h). The maximum enzyme production in immobilized cells required only 24 h where as the free cells required 48 h. From the results, it is concluded that the immobilized cells of *B. subtilis* K-30 in alginate matrix are more efficient for the production of alkaline protease with repeated batch fermentation.

Conclusion

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. The natural polymers such as agar, agarose, pectin and gelatin are employed for cell immobilization (Ramakrishna and Prakasham, 1999). In the present study, we have determined the optimum parameters for maximum production of alkaline protease by the newly

isolated strain by various matrices by entrapment technique using alginate, polyacrylamide, agar-agar and gelatin. Specific advantages of this technique include long life-term stability, reusability, and possibility of regeneration to be adaptable also to scale-up the obtained data. Calcium alginate was found to be a promising matrix to immobilize *B.subtilis* K-30 cells for optimum protease production. In addition, experiments with repeated batch of bacterial growth by introducing fresh nutrients at every 24 h leads to increased enzyme activity which is 1.7 times higher than obtained from free cells.

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