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Production of alkaline protease from *Bacillus subtilis* by different entrapment techniques

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Abstract

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* isolated from fermented fish using immobilisation 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 protease production with calcium alginate was 585 U/ml which is 70% higher production over the convention free cell fermentation. Similarly, the protease production by repeated batch fermentation was 380 U/ml with polyacrylamide, 498 U/ml with agar-agar and 438 U/ml with gelatin respectively.

Keywords: Alkaline protease, Immobilized cells, fermentation, Matrices.

Introduction

A protease enzyme hydrolyses the peptide bonds that link amino acids together in the polypeptide chain forming a protein molecule. Proteases are essential constituents of all forms of life on earth, including prokaryotes, fungi, plants and animals. It can be cultured in large quantities in a relatively short time by established methods of fermentation and they also produce an abundant, regular supply of the desired product (Gupta et al. 2002). Alkaline proteases are defined as those proteases that are active in a neutral to alkaline pH range. They are either a serine protease or a metalloprotease.

Alkaline proteases enjoy a big share of the enzyme market with two thirds of share in detergent industry alone (Anwar and Saleemuddin 2000). Besides they are also used in leather, food and textile industries; organic synthesis and waste water treatment (Kumar and Takagi 1999). Microbial alkaline proteases occupy nearly 40% of the total worldwide enzyme sales and use of protease in detergent industry accounts 25% of the total worldwide sales of enzyme (Rao et al. 1998). Micro-organism is excellent source of enzyme than plant and animal due to their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation (Patel et al. 2005). Alkaline proteases can be produced from bacteria, fungi and yeast using fermentation technique (Chandran et al. 2005; Germano et al. 2003; Haki and Rakshit 2003). Although there are several microbial alkaline proteases producers, only a few are considered industrially useful (Gupta et al. 2002). Gram-positive bacteria, especially the genus *Bacillus* are considered an important commercial enzyme producer of proteases. Of them, *Bacillus lichniformis, Bacillus subtilis, Bacillus alcalophilus, Bacillus lentus,* etc are industrial protease producers (Gupta et al. 2002).

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 (Ahmed and Abdel-Fattah 2010; Beshay 2003; Helmo et al. 1985). Moreover, immobilized cells show modifications in 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 (Adinarayana et al. 2004).

In the present study, we report immobilization of *Bacillus subtilis* cells originally isolated from fermented fish 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.

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Materials and Methods

Chemicals

Casein for protease assay was purchased from Himedia. All other chemicals used were of analytical grade, commercially available in India. All experiments have been done in triplicate

Isolation of Organisms and culture

Protease producing *Bacillus subtilis* was isolated from fermented fish, India. The microorganism was grown on nutrient agar slants at 37°C at pH 7.4. It was maintained by sub-culturing on nutrient agar slants kept at pH 7.4. For production experiments, the culture was revived by adding a loop full of pure culture into 50 ml of sterile nutrient broth. For protease production,50 ml complex medium taken in 250 ml Erlenmeyer flask containing (gl^{-1}): starch, 20; soybean meal, 10; K_2HPO_4, 3; KH_2PO_4, 1; at pH-7 (Oberoi et al. 2001) was inoculated with 2% fresh culture ($A_{550\ nm} \approx 0.2$). The inoculated medium was incubated at 37°C and 180 rpm. The culture was centrifuged at 10,000 × *g* for 10 min at 4°C. The cell pellet was discarded and the supernatant was used for assay of protease activity.

Protease enzyme assay

Protease activity was determined by a modified method of Folin and Ciocalteu (Folin and Ciocalteu 1927). 200 µl of the protease broth was added to the reaction mixture, containing 0.65% (wv^{-1}) casein in 800 µl of 50 mM in phosphate buffer (pH 9). The mixture was incubated at 75°C for 10 min. The reaction was stopped by the addition of 1 ml of 5% (wv^{-1}) Trichloroacetic acid (TCA), followed by centrifugation at 10,000×*g* for 15 min. The supernatant were analyzed by the Folin-Ciocalteu reagent. One unit of protease activity was defined as the amount of enzyme that liberated 1µg tyrosine per min per ml of protease broth.

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 (Johnsen and Flink 1986). Briefly, three percent sodium alginate solution was prepared under sterile conditions. Both alginate slurry and cell suspension [(0.03g dry cell weight, (DCW)] were mixed and stirred for 10 min. The resultant slurry was added drop wise into 0.2M calcium chloride solution with the aid of a syringe and left for curing at 4°C 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. 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. (Adinarayana et al. 2005) by adding cells to 2.85g acrylamide (Sigma-Aldrich), 0.15 g bisacrylamide (Sigma-Aldrich), 10mg 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 0.2M phosphate buffer (pH 7.0), and kept in the refrigerator for 1 h for curing.

Whole cell immobilization of *B.subtilis* was carried out in accordance with the method described by Veelken and Pape (Veelken and Pape 1982). Encapsulation in agar was done by adding cell suspension (2ml equivalent to 0.03g 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 15ml of 20% sterile gelatin, maintained at 45°C and poured into sterile petriplate. The gel was over-layered with 10ml of 5% glutaraldehyde for hardening at 30°C. The resulting block was cut into small cubes (4mm^3) and the cubes were washed thoroughly with sterile distilled water for complete removal of excess glutaraldehyde.

Results and Discussion

Production of alkaline protease by immobilized cells in calcium alginate

Immobilization of *B.subtilis* cells using different entrapment techniques with matrices such as calcium alginate, polyacrylamide, agar-agar and gelatin gel was investigated. The enzyme production with immobilized cells in calcium alginate started at 6 h and reached the maximum level (585 U/ml) by 36 h (Figure 1). On further incubation, enzyme production gradually decreased, while maximum enzyme titre was observed at 48 h in the case of free cells (Figure2).It is evident that the alkaline protease production was higher with immobilized cells (585 U/ml) compared to free cells (344 U/ml). Beshay et al studied the effect of alginate concentration on immobilization of alkaline protease producing bacterial strain,*Teredinobacter turnirae* (Beshay 2003).

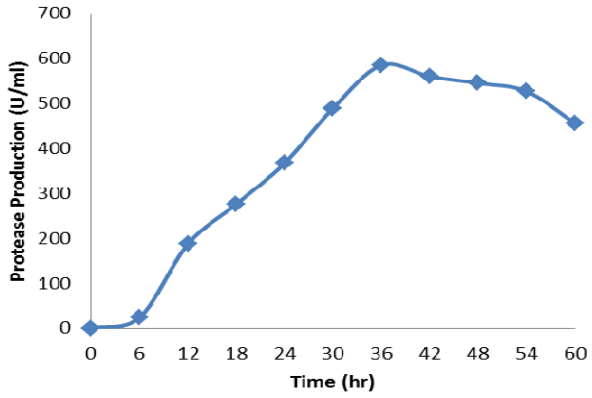

Figure 1: Time course profile of alkaline protease production by immobilized culture of B. subtilis in calcium alginate

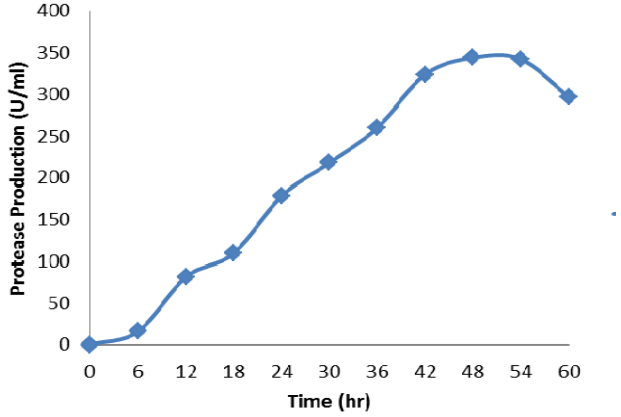

Figure 3: Time course profile of alkaline protease production by free cells of B.subtilis

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 2000; Kim et al. 1994). A gradual increase in alkaline protease production was noticed from 6 h onwards to 36 h (Figure 3). On further incubation, there was a decline in alkaline protease titre with immobilized cells in polyacrylamide. Alkaline protease titre was 380 U/ml at 36 h which was a low titre compared to alginate matrix.

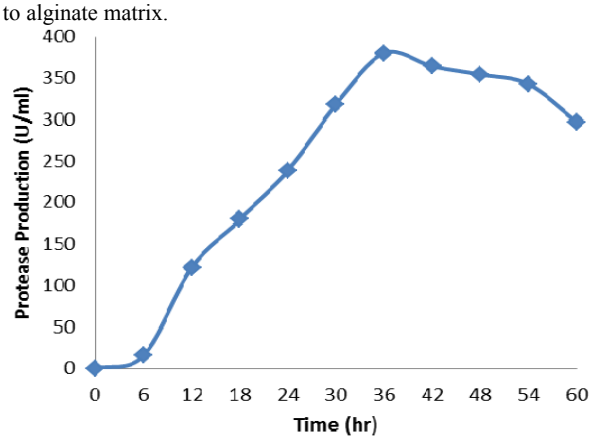

Figure 3: Time course profile of alkaline protease production by B.subtilis in polyacrylamide

Production of alkaline protease by immobilized cells in agar-agar

Protease production increased gradually from 6 h onwards and reached maximum level by 36 h (498 U/ml). Further, alkaline protease production with immobilized cells in agar-agar was comparable with polyacrylamide, whereas it was less than alginate matrix (Figure 4). 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 (Anna et al. 2003). In another report, an acid protease from *Aspergillus saitoi* was immobilized on agar beads of approximately 100 µm diameter, the hydrolysis of gelatin by immobilized acid protease was found to be 6.8x 10^{-7}M per second (Gregg and Robert 1981).

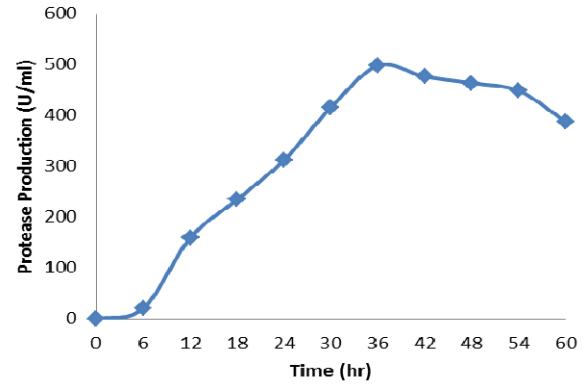

Figure 4: Time course profile of alkaline protease production by B.subtilis of Agar-agar Production of alkaline protease by immobilized cells in Gelatin

Only a detectable level of alkaline proteasetitre was observed after 6 h of fermentation and reached the maximum level (438 U/ml) by 36 h in gelatin (Figure5).The alkaline protease titre 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 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 *E.coli* for pencillin acylase production.

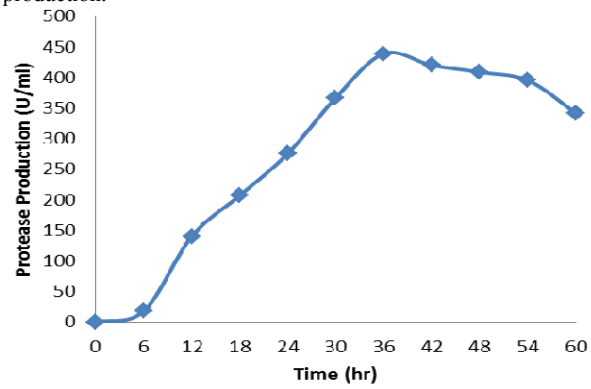

Figure 5: Time course profile of alkaline protease production by B.subtilis in gelatin

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

The results show that the average protease activity with alginate matrix was 585 U/ml which is 70% higher production over the conventional free cell fermentation.Similarly, low level of alkaline protease was observed with polyacrylamide (380 U/ml), agar (498 U/ml) and gelatin (438 U/ml). The maximum enzyme production in immobilized cells required only 36 h where as the free cells required 48 h. From the results, it is concluded that the immobilized cells of *B.subtilis* in alginate matrix are more efficient for the production of alkaline protease with repeated batch fermentation.

Conclusions

The natural polymers such as agar, agarose, pectin and gelatin are also employed for cell immobilization. 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. Calcium alginate was found to be a promising matrix to immobilize *B.subtilis* cells for optimum protease production for long term stability, reusability and possibility of regeneration.

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