Alkaline protease production by immobilized cells of *Bacillus pumilis* MTCC 2296 in various matrices

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Abstract

Immobilization techniques provide a special microenvironment in which cells always have different behaviors compared with free cells. In the present work, the effect of *Bacillus pumilis* MTCC 2296 cells immobilized in various matrices; such as calcium alginate, polyacrylamide and agar-agar, for the production of alkaline protease, is studied. Calcium alginate was found to be an effective and suitable matrix for higher alkaline protease productivity (491 U/ml) compared to the other matrices studied. [Life Science Journal. 2009; 6(2): 90 – 92] (ISSN: 1097 – 8135).

Keywords: *Bacillus pumilis*; immobilization; alkaline protease; calcium alginate

1 Introduction

Alkaline proteases constitute one of the most important groups of industrial enzymes. It accounts for at least a quarter of the total global enzyme production (Herbert *et al*, 1992). Proteases are essential constituents of all forms of life on earth including prokaryotes, fungi, plants and animals. Recently, the sue of alkaline protease has increased significantly in various industrial processes such as detergent and feed additives, food, dehairing, pharmaceutical, leather and silk industries. Among these, use as laundry detergent additive is one the most important industrial applications for alkaline proteases (Stevenson *et al*, 1998; Masui *et al*, 1999; Gupta *et al*, 2005). Proteases are also useful and important components in biopharmaceutical products as contact-lens enzyme cleaners and enzymatic debriders (Anwar and Saleemuddin, 2000). The enzyme also could be used in the applications of alkaline environments including aquaculture industry (Fu *et al*, 2005; Shanmughapriya *et al*, 2008). At present, the use of alkaline proteases has increased remarkably with large proportions of commercially available alkaline proteases derived from *Bacillus* strains (Adinarayana *et al*, 2005; El Enshasy *et al*, 2008; Sen and Satyanarayana, 1993).

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 (Kukubu *et al*, 1981; Fortin and Vuillemard, 1990) and higher efficiency of catalysis (Ramakrishna *et al*, 1992; Linko and Haapala, 1996) and they are reusable.

The present study was performed in order to evaluate alkaline protease production by *Bacillus pumilis* MTCC 2296 cells by using different entrapment technique under optimized as well as harsh condition and to check their efficiency with control cells.

2 Materials and Methods

Bacillus pumilis (*B. pumilis*) MTCC 2296 was used through out this study. The culture is routinely maintained on Nutrient agar in 90 mm diameter Petri plates at 37 ºC. *B. pumilis* MTCC 2296 cells were immobilized by using sodium alginate, polyacrylamide and agar-agar. The alginate entrapment of cells was performed according method of Johnsen and Flink (1986). Sodium alginate solution was prepared by dissolving sodium alginate

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in 100 ml boiling water and autoclaved at 121 ºC for 15 minutes.

Immobilization in polyacrylamide was done by adding cells to 2.85 g acrylamide (Sigma-Aldrich), 0.15 g bisacrylamide (Sigma-Aldrich), 10 mg ammonium persulphate (Sigma-Aldrich), and 1 ml TEMED, tetra methyl ethylene diamine (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 (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 (Reyed, 2007).

Encapsulation in agar was done by adding cell suspension into the molten agar-agar. The solidified agar block was cut into equal size cubes (4 mm^3), added to sterile 0.1 M phosphate buffer (pH 7.0), and kept in the refrigerator (overnight) for curing (Veelken and Pape, 1982).

The immobilized beads prepared by using all the three matrices along with control *B. pumilis* MTCC 2296 were transferred into 50 ml of production medium in 250-ml flasks. The composition of production medium was 5 g/L glucose, 7.5 g/L peptone, 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 ºC for 48 hours. Samples were withdrawn at regular intervals of 6 hours and assayed for alkaline protease activity.

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. The culture broth was centrifuged at 8,000 rpm for 10 minutes and supernatant served as the crude enzyme source. One unit of enzyme activity is defined as the amount of enzyme that released 1 µg of tyrosine/ml/minute. Protease activity was also estimated at various pH (8 to 12) using free and immobilized *B. pumilis* MTCC 2296. The buffers used were 20 mM HEPES (pH 8.0 – 9.0) and 200 mM carbonate (pH 10 – 12). All experiments were carried out in triplicate. All the data were analyzed using GraphPad Prism 4.1 software.

3 Results and Discussion

Cell immobilization is one of the common techniques for increasing the overall cell concentration and productivity. Immobilization of cells may allow continuous operation of cultivation processes at high dilution rates (Adinarayana *et al*, 2005).

The amount of cell mass entrapped in calcium alginate matrix increased gradually up to 30 hours of incubation after which there was no appreciable change (Figure 1). The enzyme production was started at 6 hours with immobilized cells and reached a maximum level (491 U/ml) by 30 hours. On further incubation, enzyme production was gradually decreased, whereas maximum enzyme titer was observed by 36 hours in the case of free cells. There was negligible change in pH profile of free cells as well as immobilized cells after 30 hours. Maximum alkaline protease production occurred at around pH 10.5 except calcium alginate matrix (pH 11.5) after 30 hours (Table 1). In case of alginate matrix, enzyme activity was continued increasing up to 36 hours at extreme pH 12 as compare to free cells and other matrices. It is evident that the alkaline protease production was higher with calcium alginate immobilized cells (491 U/ml) than that of free cells (399 U/ml). Ramakrishna *et al* (1992) reported the immobilization of *B. cereus* in calcium alginate and employed packed-bed and fluidized-bed reactors to continuously synthesize thermostable α-amylase.

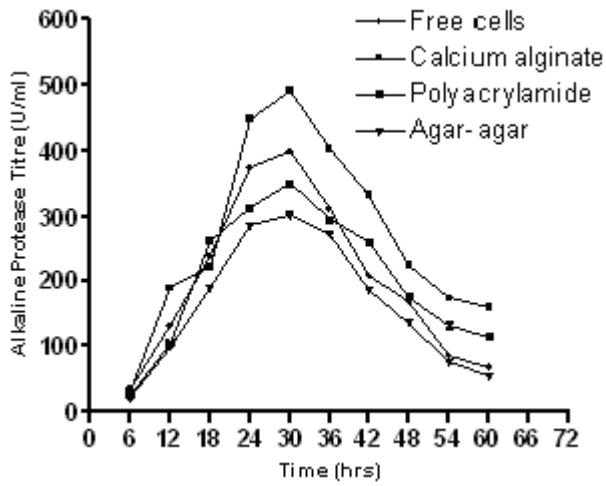

Figure 1. Time course profiles of alkaline protease production by free cell culture and immobilized culture of *B. pumilis* MTCC 2296 in calcium alginate, polyacrylamide and agar-agar.

Polyacrylamide was also found to be one of good matrices for cell immobilization. Using this matrice, gradual increase in alkaline protease production was noticed from 6 hours onwards to 30 hours; on further incubation decline in alkaline protease titer was observed. The maximum alkaline protease titer of 350 U/ml was observed at 30 hours. The alkaline protease titer obtained with this carrier was less than that of free and immobilized cells with the other carrier (calcium alginate).

Alkaline protease production pattern by immobilized

cells in agar-agar was similar to other matrices. The results are similar to Figure 1 and the data indicated that alkaline protease production was started from 6 hours onward and reached a maximum level by 30 hours (303 U/ml). The alkaline protease titer obtained with this carrier was very low compared with the titers of free cells and the immobilized cells of the above-mentioned other carriers. Anna *et al* (2003) reported that the use of agar-entrapped cells of *B. circulans* ATCC 21783 for cyclodextrin glucanotransferase production in a fluidized bed reactor led to enzyme activity (180 U/ml) after 24 hours of cultivation.

Table 1. Comparison of alkaline protease production using *B. pumilis* MTCC 2296 cells entrapped in various gel matrices after 30 hours

Support matrix	Final pH	Alkaline protease titre (U/ml)	Relative percentage of production
Calcium alginate	11.5	49.23[a]	100
Polyacrylamide	10.45	350.03[ab]	71.26
Agar-agar	10.42	302.3[b]	61.54
Free cells	10.48	399.17[a]	81.26

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. In contrast, free cells showed lower enzyme productivity than the calcium alginate-immobilized ones and their activity decreased markedly after 30 hours.

4 Conclusion

In conclusion, calcium alginate is a promising method of *B. pumilis* MTCC 2296 immobilization for alkaline protease production. Alkaline protease production by immobilized cells is better than that of free cells.

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