

Immobilization of *Bacillus circulans* MTCC 7906 for enhanced production of alkaline protease under batch and packed bed fermentation conditions

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Citation

G Kocher, S Mishra. *Immobilization of Bacillus circulans MTCC 7906 for enhanced production of alkaline protease under batch and packed bed fermentation conditions*. The Internet Journal of Microbiology. 2008 Volume 7 Number 2.

Abstract

Alkaline protease production by immobilized cells of *Bacillus circulans* MTCC 7906 was studied using agar-agar and calcium alginate as entrapment materials. Under batch conditions using agar agar, 2% agar concentration, 6.25mm³ bead size and 200 beads/ flask inoculum size were the optimal conditions for a maximum alkaline protease production of 19.9 IU/ml and while for alginate beads, 3% alginate concentration, 5.2mm³ bead size and 200 beads/ flask inoculum size were the optimal conditions for a maximum alkaline protease production of 19.7 IU/ml. The alginate and agar agar beads were reusable for four and nine successive batch cycles respectively. A maximum enzyme production of 28 and 27 IU/ml was obtained with incubation time of 36 h, column length of 30cm and flow rate of 10ml/h with agar-agar and alginate entrapped cells respectively under packed bed fermentation conditions. Scanning electron microscopy (SEM) of the agar agar entrapped cells showed a random distribution of cells on the surface with pockets of high cell density.

INTRODUCTION

Alkaline proteases constitute an important group of industrial enzymes that are now used in a wide range of industrial processes viz; detergent, food, pharmaceutical, leather, and silk industries. Proteases account for 30% of the total worldwide enzyme production and 40% of the total worldwide enzyme sales (Sharma et al 2006) and their demand is expected to reach 22 billion dollars in the year 2009 (Turk 2006). At present, use of alkaline proteases has increased remarkably with their large proportion derived from *Bacillus* strains. *Bacillus* sp. are efficient producers of alkaline protease and are probably the only genera being commercialized for alkaline protease production. However, these producing strains have problems in submerged cultures in that the enzymes have low shelf life and are susceptible to rise in temperature, pH etc (Beshay and Moreira 2001). In this context, alternate technologies like use of immobilized biocatalysts have recently gained attention of many biotechnologists as they offer several advantages in the form of repeated use of enzyme, ease of product separation, improvement of enzyme stability and continuous operations in packed bed reactors. (Adel Naby et al 1998). In literature, cell entrapment has been reported to be the best for immobilization and a number of entrapment materials like calcium alginate, agar-agar, polyacrylamide etc. have been

used (Adinarayana et al, 2005; De' Souza et al, 1997). The choice of entrapment material is an important parameter which involves stability and reusability of beads and of course the cost of material. Although calcium alginate has been used most widely but it faces problem of bead disintegration in the presence of phosphate an important ingredient in enzyme production media (Bajpai and Sharma, 2004). Keeping in view this problem, agar-agar was tried as an alternate immobilization material in this study and compared with alginate entrapped cells for alkaline protease production by our isolate of *Bacillus circulans* MTCC 7906 (enriched from vegetable waste and identified by MTCC lab, IMTECH, Chandigarh) under batch and packed bed fermentation conditions. Earlier, in our labs we have optimized alkaline protease production by this strain and studied its application in detergent powders for stain removal and dehairing of buffalo skin (Jaswal and Kocher, 2008, Jawsal and Kocher, 2008).

MATERIALS AND METHODS

Entrapment of cells in Agar-Agar and calcium alginate: *Bacillus circulans* MTCC 7906 was grown in 50 ml inoculum medium consisting (g l⁻¹) of glucose, 10.0; casein, 5.0; Yeast Extract, 5.0; K₂HPO₄, 1.0; MgSO₄·7H₂O, 0.20 and Na₂CO₃, 10.0 with a pH of 9.5. The inoculated medium was

incubated on a shaker incubator at 120 rpm, 28°C for 72 h. A 2% solution of agar-agar was prepared in 45 ml of 0.9% NaCl solution and sterilized by autoclaving. The 72 h old inoculum (5ml) was added to the molten agar maintained at 40°C, shaken well for few seconds (without forming foam or entrapped bubbles), poured into two sterile flat bottom (100mm) diameter Petri plates and allowed to solidify. The solidified agar blocks were cut into equal size cuboids (6.25, 25 and 56.25 mm³) using fine sterile blades, added to sterile 0.1M phosphate buffer (pH 7.0), and kept in the refrigerator (1 h) for curing. After curing, phosphate buffer was decanted and cuboids were washed with sterile distilled water 3 to 4 times before use (Veelken and Pape, 1982).

For entrapment of cells in Calcium alginate *Bacillus circulans* MTCC 7906 was grown in 50 ml inoculum medium. The inoculated medium was incubated in a shaker incubator at 120 rpm, 28°C for 72 h. A 3% solution of sodium alginate was prepared in distilled water and sterilized by autoclaving. To the alginate slurry, an equal volume of 72 h old inoculum was mixed and stirred for 10 min to get a uniform mixture. The slurry was filled in a sterile hypodermic syringe and an injection needle was fixed over its end. The bead size was varied by using hypodermic syringe needles of different gauge sizes producing sizes of 41.8, 17.6 and 5.2 mm³ respectively. The alginate solution was dropped into a 0.2M ice cold solution of calcium chloride and beads formed were incubated for overnight for curing. The cured beads were washed with sterile distilled water 4 times and used to inoculate enzyme production media.

PRODUCTION OF ALKALINE PROTEASE WITH IMMOBILIZED CELLS

Batch production: The enzyme production was carried out under batch, repeated batch and packed bed fermentation conditions. The production media for immobilized cells of *Bacillus circulans* consisted (g l⁻¹) of cotton deoiled meal (CDM), 15.0; Yeast Extract, 5.0 and salt solution (g l⁻¹) of K₂HPO₄, 1.0; MgSO₄·7H₂O, 0.20 and Na₂CO₃, 10.0 with a pH of 10 (Jaswal and Kocher, 2008). The effect of different cultural variables (agar agar/ calcium alginate concentration, bead size and inoculum size) was optimized under batch conditions. In case of packed bed experiemts, column length and flow rate were optimized.

The agar-agar and calcium alginate entrapped cells were used as inoculum for alkaline protease production medium (50ml in 250ml capacity erlenmeyer flasks). The flasks were

incubated at 28°C for 120 h. Samples were withdrawn at regular intervals of 24 h and assayed for cell leakage and alkaline protease activity.

Production by repeated batch process: The reusability of *Bacillus circulans* entrapped cells was examined in shake flasks. After attaining the maximum production of alkaline protease, the spent medium was replaced with fresh production medium (50 ml) and the process was repeated for several batches until the enzyme activity remained constant.

Packed bed production: Burettes (50ml capacity) were used as column reactors to which beads were filled upto different lengths (5-30 cm) to optimize the column length. The packed columns were filled with enzyme production medium upto 50 ml mark. The column were activated at room temperature for overnight (16 h), followed by flushing the spent production medium and fresh medium was then poured over the beads. The flow rate was regulated and spent medium was collected every hour and used as crude enzyme extracts. The spent medium was replaced with fresh medium continuously.

STATSTICAL ANALYSIS

All the enzyme production experiments were planned statistically using Random Block Design where atleast three replications of each treatment were taken.

ANALYTICAL METHODS

Extracellular Protease Activity: The protease activity of the periodic samples was determined in a reaction mixture consisting of 0.1 ml of crude enzyme, 2 ml of 0.5% azocasein (in carbonate-bicarbonate buffer, 0.1M, pH 9.5) and 0.9 ml of distilled water and incubated at 60°C for 15 minutes. The proteins were precipitated out thereafter by adding 3 ml of 5% ice-cold trichloroacetic acid (TCA) and free amino acids released by crude protease from casein hydrolysis were estimated by Lowry's method. The protease activity was defined as μMol of tyrosine released per minute per ml of crude enzyme.

Study of cell leakage: The cell leakage from the production media were determined by viable cell plate count method. Periodic samples were serially diluted and plated on inoculum medium and incubated at 28±2°C for 48 h for determining the viable cell count.

Scanning Electron Microscopy: The *B. circulans* cells immobilized in calcium alginate and agar agar were

examined for their distribution pattern on the immobilizing material surface. The samples were fixed with 2% glutaraldehyde and dehydrated in graded series of ethanol. Finally, the samples were dried by critical point drier, coated with gold and observed on a Scanning Electron Microscope (Hitachi S-3400 N, Japan) under different magnifications.

RESULTS AND DISCUSSION

The batch production of alkaline protease by immobilized cells of *B.circulans* 7906 was optimized with respect to 3 parameters viz.; bead size, entrapment material concentration and inoculum size. Fermentation conditions already optimized as pH 10, temp 28°C, agitation 120 rpm with CDM @ 1.5% as C and N source were used (Jaswal and Kocher, 2008).

BATCH PRODUCTION OF ALKALINE PROTEASE

Effect of bead size: The effect of bead size was studied by approximately 150 beads each of agar agar and calcium alginate. And the data was fitted into TWO WAY ANOVA classification. The results presented in Table 1 reveal a better protease yield with low bead size of 6.25, 5.2 mm³ having statistically significant values of 16.6 and 18.4 IU having relative activity of 115.9 and 118.8% over free cells in case of agar agar and calcium alginate entrapped cells respectively. The better performance of entrapped cells over free cells has also been reported elsewhere (Beshay 2003, Adinaryana et al, 2005) and small sized beads have more protease activity due to higher surface area (El-Katany et al, 2003).

Effect of entrapment material concentrations: The concentration of entrapment material is an important parameter and the concentration range of 2-4% studied in this study revealed 2% concentration of agar agar and 3% concentration of calcium alginate as the best for protease yield of 18.2 and 19.5 IU respectively (Table 1). Further increase in concentration of entrapment material decreased activity that may be attributed to diffusion limitation arising out of reduced pore size of beads at increased concentration of entrapment material (Elibol and Moreria ,2003; Rao et al, 2008). It is important to mention that both entrapment materials used behaved similarly as the relative protease activity improved to 127.3 and 125.5 respectively. Rao et al

Figure 1

Table 1. Effect of cultural and fermentation conditions on alkaline protease production by immobilized cells of

Agar agar entrapped cells			Alginate entrapped cells		
Cultural variables	Protease activity (IU)	Relative activity (%)	Cultural variables	Protease activity (IU)	Relative activity (%)
<i>Batch fermentation conditions</i>					
Free cells	14.3	100	Free cells	15.53	100
<i>Bead size (mm³)</i>			<i>Bead size (mm³)</i>		
6.25	16.6		5.2	18.4	
25.0	14.8	115.9	17.6	18.1	118.8
56.25	14.5		41.8	15.5	
C.D. 5%	0.82		C.D. 5%	1.14	
<i>Agar-agar conc. (%)</i>			<i>Calcium alginate conc. (%)</i>		
2	18.2		2	16.7	
3	18.1	127.3	3	19.5	125.5
4	14.6		4	18.8	
C.D. 5%	1.28		C.D. 5%	0.83	
<i>Number of beads¹</i>			<i>Number of beads²</i>		
40	9.5		40	3.7	
80	10.6		80	7.6	
120	12.3	139.2	120	10.2	128.5
160	18.6		160	18.8	
200	19.9		200	19.7	
C.D. 5%	1.08		C.D. 5%	1.17	
<i>Packed bed culture conditions</i>					
<i>Time (h)</i>			<i>Time (h)</i>		
20	11.2		20	14.4	
24	12.4		24	15.1	
28	13.8		28	16.3	
32	18.7	165.7	32	18.5	123.0
36	23.7		36	19.1	
40	22.5		40	18.2	
C.D. 5%	2.08		C.D. 5%	1.43	
<i>Column length (cm)</i>			<i>Column length (cm)</i>		
5	12.7		5	11.8	
10	15.2		10	14.4	
20	19.0	165.7	15	18.2	144.9
30	23.7		30	22.5	
C.D. 5%	1.17		C.D. 5%	1.24	
<i>Flow rate (ml/h)</i>			<i>Flow rate (ml/h)</i>		
5	16.4		5	15.8	
10	28.0		10	27.0	
15	24.4	195.8	15	23.5	151.3
20	23.5		20	22.6	
C.D. 5%	2.71		C.D. 5%	1.84	

¹ Number of cells in agar-agar beads¹ was 8 X 10⁶ and alginate beads² was 6X 10⁶
² Mean cell leakage was 3 X 10² in agar-agar beads¹ and 1.5 X 10³ in alginate beads² after 100h of incubation in batch fermentation experiments.

(1986) infact reported a better ethanol production with agar immobilized cells than alginate and l-carrageenan.

Effect of inoculum size: The effect of inoculum size studied by varying the number of beads in production experiments showed increase in protease activities with increase in number of beads/flask at all the 5 levels (40-200 beads/flask) tested. While a significantly higher value of 19.9 IU was obtained with 200 beads/flask of agar agar, a maximum protease activity of 19.7 IU was achieved with similar number of calcium alginate beads. Thus, by batch production the protease activity was improved by 139.2 and 128.5 % over free cells in case of agar agar and calcium alginate beads respectively. Elsewhere, Beshay (2003) and Rao et al (2008) reported 400 and 180 beads/flask of alginate beads for optimum protease production by *T.turnirae* and *B. circulans* respectively.

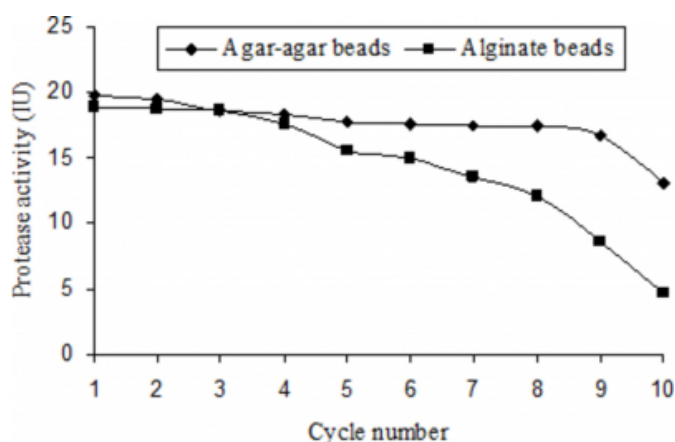
REPEATED BATCH PRODUCTION OF ALKALINE PROTEASE

The reusability of beads was studied to determine their

strength and performance. The results presented in Fig.1 reveal that agar agar beads behaved consistently over 9 consecutive cycles while calcium alginate beads were stable for 4 cycles only before the respective beads started disintegrating. The low stability of calcium alginate beads is attributed to the presence of potassium phosphate in the enzyme production medium that tends to dissolve the beads (Bajpai and Sharma, 2004). On the other hand, agar agar beads had no effect of this salt. Elsewhere, Beshay (2003) and Elibol and Moreira (2003) reported maximum alkaline protease production in 3rd, and 4th cycle while Adinarayana et al (2005) and Rao et al (2008) reported 9 and 11 repeated batch cycles with alginate entrapped cells. Another observation in repeated batches was that maximum enzyme production was decreased from 100h with free cells to 48h in 4th cycle in agar agar and 40h in 3rd cycle in alginate beads (unpublished data). The cell leakage was studied from the periodic samples revealed a mean loss of 289×10^4 cfu/ml at the end of respective cycles.

Figure 2

Fig.1 Alkaline protease production by entrapped in agar-agar and calcium alginate under repeated batch conditions



PACKED BED PRODUCTION OF ALKALINE PROTEASE

Earlier workers have suggested use of immobilized cells for continuous production (Beshay, 2003 and Adinaryana et al, 2005). As the reusability of beads confirmed consistent protease production over successive cycles an effort was made to improve protease production and provide a more handy alkaline protease production by using continuous fermentation. Among the different methods of continuous fermentation, packed bed reactors have become very popular in recent years due to their low manufacturing and operating costs and also due to the ease of process automation in these reactors (Goksungur and Zorlu, 2001). In the present study,

we employed plastic burrettes of 50ml capacity as columns for packing beads as explained earlier in materials and methods. Incubation time, column/bed length and flow rate were the 3 important parameters optimized for packed bed operation. The incubation time was reduced to 36h in both agar agar and calcium alginate beads (Table 1) and the relative protease activity was improved to 165.7 with agar agar beads and 123% with alginate beads compared to free cells. Although, to the best of our knowledge, there are no reports of continuous alkaline protease production by immobilized cells, Adinaryana et al (2007) reported a high CGTase production by *Bacillus* sp. under continuous conditions than batch fermentation.

A column/packed bed length of 30 cm was found to be optimum resulting in an overall increase in relative activity of 165.7 and 144.9% with agar gar and alginate entrapped cells respectively (Table 1). An optimum time of 36h and bed length of 30 cm used to standardize flow rate revealed a recovery of 10ml/h crude enzyme having 28 and 27 IU respectively with agar agar and alginate beads packed beds (Table 1). Thus, by using packed beds we were able to further enhance the protease activity by about 50% and reduce the production time from 100 to 36h. The study also revealed use of agar agar as an alternate equally effective entrapment material in place of calcium alginate the beads of which dissolve over the period of time in phosphate containing media.

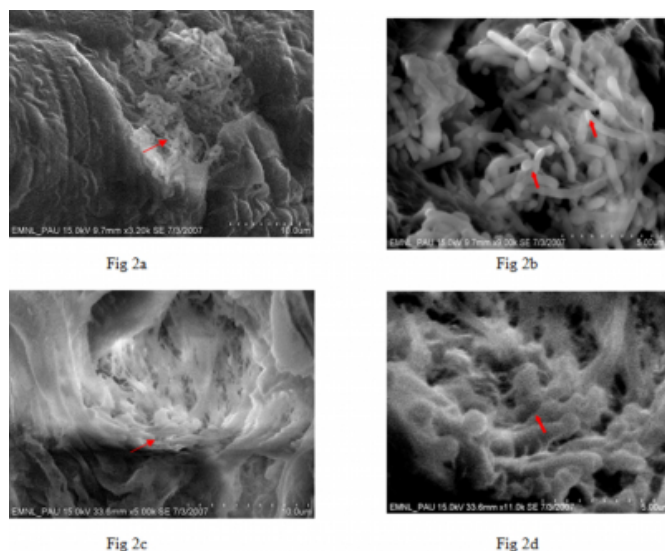
SCANNING ELECTRON MICROSCOPY (SEM) OF IMMOBILIZED CELLS

The surface structure of entrapped *B. circulans* cells were studied using SEM. Both calcium alginate as well as agar-agar beads depicted a random distribution of *B. circulans* rods or vesicular shaped cells on the surface though cells appeared more as vesicles in agar agar beads (Fig. 2b, 2d). There were pockets of high cell density at a scale of 10 μ m in agar agar and alginate beads that were the areas of cell growth, (Fig 2a, 2c) though in case of alginate beads, cells were more relatively uniformly distributed on the surface (Fig 2c). Thus, both in alginate and agar agar beads, the distribution of cells showed almost similar clustering in the areas of growth in the furrows of beads as has been discussed earlier by Rao et al (2008). To the best of our knowledge, we have not come across of any report of SEM of alkaline protease producing agar agar entrapped cells. Though in literature, SEM of alginate entrapped cells is available showing similar observations of random cell

distribution (Beshay 2003, Pai et al 1995, Rao et al, 2008).

Figure 3

Fig 2. SEM observation of calcium alginate (Fig 2a, 2c) and agar-agar entrapped cells of (Figs 2b, 2d). 2a, 2c, 10 μ m and 2b, 2d, 5 μ m.



ACKNOWLEDGEMENTS

The author are thankful to Dr. (Ms.) Rabinder Kaur of Nanoscience Laboratory, PAU, Ludhiana for help during Scanning Electron Microscopy (SEM) studies.

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