

Protease Production by *Bacillus subtilis* Immobilized on Different Matrices

Rakesh Kumar*, Ritika Vats

Biotechnology Department

Dolphin PG College of Life Sciences, Chunni-Kalan-140307, Fatehgarh Sahib, Punjab, INDIA

rakesh_panchal1@yahoo.co.in

Abstract: Protease production by *Bacillus subtilis* with free and immobilized cells was examined in this study. Entrapment method of immobilization was used with gelatin, polyacrylamide, calcium alginate and agar matrices and protease production was studied compared to equivalent weight of free cells. Results showed that the gelatin entrapped cells produce 10.8 U/ml; the maximum enzyme titer followed by polyacrylamide, 9.22 U/ml; calcium alginate, 7.8 U/ml and agar, 6.48 U/ml. Equivalent free cells produce 5.7 U/ml protease enzyme. The cell leakage by gelatin was less, 0.06 mg/ml; by polyacrylamide, 0.22 mg/ml; calcium alginate, 0.32 mg/ml and the maximum, 0.74 mg/ml by agar. On repeated batch culturing it was observed that the gelatin entrapped cells can be reused for 7 cycles, followed by polyacrylamide, 6 cycles; calcium alginate, 5 cycles and agar for 3 cycles. The overall protease production by gelatin entrapped cells was 45.36 U/ml. The study suggests the gelatin, a better matrix than others for protease production by *Bacillus subtilis*. [New York Science Journal 2010;3(7):20-24]. (ISSN: 1554-0200).

Key words: Protease, *Bacillus subtilis*, Immobilization, Cell leakage, Matrices

1. Introduction

Proteases or proteolytic enzymes catalyze the cleavage of peptide bonds in proteins (Godfrey and West, 1996). They conduct highly selective and specific modification of proteins i.e. zymogenic form of enzymes by limited proteolysis, blood clotting and lysis of fibrin clots, processing and transport of secretory proteins across the membrane. They catalyze important proteolytic steps in tumor invasion or in infection cycle of a number of pathogenic microorganisms. Their involvement in the life cycle of disease causing organisms has led to become a potential target for developing therapeutic agents against fatal disease such as cancer and AIDS (Rao *et al.*, 1998). This makes proteases a valuable target for new pharmaceuticals.

Proteases are found in several microorganisms such as protozoa, bacteria, yeast and fungi. The inability of the plant and animal proteases to meet the current world demands (due to extensive use in food, pharmaceutical and detergent industry) has led to an increased interest in microbial proteases (Beg *et al.*, 2002). Bacteria are most important alkaline protease producers with the genus *Bacillus* being the most prominent source (Sen *et al.*, 1993), because of their ability to produce large amount of protease having significant proteolytic activity and stability at high pH and temperature. The microbial proteases are usually produced by either free or immobilized cells. Immobilized whole cell or enzyme is advantageous because such biocatalyst display better operational stability and high efficiency of catalysis (Ramakrishna *et al.*, 1992).

The present study has a goal to immobilize the *Bacillus subtilis* cells by entrapment method using

different matrices and to compare the protease production with free cells.

2. Materials and Methods

2.1 Microorganism and growth conditions

Bacillus subtilis (MTCC 441) was maintained on nutrient broth (NB) medium at 4°C. The cells were transferred to 50 ml sterile inoculum medium [(g/l): glucose, 2.0; casein, 0.5; peptone, 0.5; yeast extract, 0.5 and 50 ml salt solution (g/l) KH₂PO₄, 5.0; MgSO₄·7H₂O, 5.0; and FeSO₄·7H₂O, 0.1) pH 7.0] and incubated at 37°C at 200 rpm. The content of flask was centrifuged at 5000xg for 10 min and supernatant was decanted. The cell pellet was washed thoroughly with sterile 20.0 g/l potassium chloride solution, followed by sodium chloride solution and lastly by distilled water. The cell mass was suspended in sterile saline solution. This cell suspension was used as inoculum for free cell system, immobilization process and for preparation of biomass standard curve.

2.2 Analytical Techniques

2.2.1 Biomass standard curve

Aliquots of pellet in the range of 70 mg/ml to 420 mg/ml were differentially read at absorbance 600 nm. A standard curve was plotted between increasing concentration of biomass and absorbance at 600 nm.

2.2.2 Tyrosine standard curve

A stock solution of tyrosine (100µg/ml) was prepared and aliquots in the range 5 to 100 µg were differentially read at 275 nm. A standard curve was plotted between the increasing concentrations of tyrosine against absorbance at 275 nm.

2.2.3 Enzyme assay

Protease activity was assessed by using casein as a substrate. Ten ml casein substrate was taken in test tubes and placed in a water-bath for 5 min at 37°C. The 2 ml crude enzyme extract was added to this. After 30 min of incubation, Trichloroacetic acid (stopping solution) was added. Content was vortexed and incubated, after 30 min, tubes were removed from water-bath and cooled at room temperature. Unhydrolysed casein was removed by filtration using whatman filter paper and solubilized casein was determined spectrophotometrically at absorbance 275 nm. *One unit of enzyme activity is defined as μ mol of tyrosine released from 1ml crude enzyme in 1 h incubation.*

2.3 Immobilization

2.3.1 Calcium alginate (Johnsen and Flink, 1986)

Sodium alginate suspension (3%) was prepared by suspending 0.9 g sodium alginate in 30 ml boiling water and autoclaved at 121°C for 15 min. The suspension was cooled to room temperature and 47 μ l cell suspensions (equivalent to 0.03 g dry cell weight) was added and mixed for 10 min by stirring with a glass rod. This was taken in a sterile syringe and added drop wise into chilled 0.2 M CaCl_2 solution from 5 cm height with constant stirring. The beads obtained were kept for curing at 4°C for 1 h in refrigerator. The cured beads so formed were washed with sterile distilled water and preserved in 0.9% NaCl solution at 4°C. All the operations were carried out aseptically under a laminar flow hood.

2.3.2 Agar

The 47 μ l of cell suspension (equivalent to 0.03 g dry cell weight) was added to molten agar (2%) maintained at 40°C and poured into sterile flat bottom petriplates and allowed to solidify. The solidified agar blocks were cut into equal size cubes and added to sterile 0.1 M phosphate buffer (pH 7.0) and kept at 4°C for 1 h. After curing, phosphate buffer was decanted and cubes were washed with sterile distilled water 3-4 times and were stored at 4°C.

2.3.3 Gelatin

The 47 μ l of cell suspension was added to gelatin (2%) and poured into sterile flat bottom petriplates and allowed to solidify. The gel was over layered with 5 ml of 5% glutaraldehyde for hardening. The blocks were cut into equal size cubes and washed thoroughly with sterile distilled water for complete removal of excess glutaraldehyde. Cubes were preserved at 4°C.

2.2.4 Polyacrylamide

Acrylamide (9.0 g) and bisacrylamide (0.24 g) were mixed and to this 0.45 g ammonium persulphate was added. After this 3 ml TEMED was mixed and 47 μ l cell suspensions was added to polyacrylamide

solution and poured into flat bottom petriplates for solidification. After polymerization, polyacrylamide gel was cut into equal size cubes. The cubes were transferred to 0.2 M phosphate buffer (pH 7.0) and kept at 4°C for curing for 1 h. The cubes were washed with sterile distilled water and stored at 4°C until use. All operations were carried out aseptically under a laminar flow hood.

2.4 Protease Production

2.4.1 Batch process

The batch experiment was performed in 250 ml capacity of Erlenmeyer flask containing 50 ml medium. The immobilized beads of different matrices were transferred to 50 ml of production medium [g/l: glucose, 5.0; peptone, 7.5 and salt solution, 5% ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0 g/l; KH_2PO_4 , 5.0 g/l and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g/l) pH 9.0]. The flasks were incubated at 37°C for 48 h on shaker incubator at 100 rpm.

Similarly, one flask containing production medium was inoculated with 47 μ l of cell suspension (equivalent to 0.03 g dry cell weight) and was incubated at 37°C for 48 h. This was treated as free cell system. Samples were withdrawn at regular interval of 6 h from each flasks and enzyme activity was calculated by using casein as a substrate as mentioned above and cell leakage of each sample was determined.

2.4.2 Repeated batch process

The repeated batch culture was established in 250 ml Erlenmeyer flask containing 50 ml medium. The immobilized beads of each matrix were filtered, washed with 30 ml saline and transferred to 50 ml fresh production medium. Samples were withdrawn at regular interval of 6 h from each flasks and enzyme activity was calculated. The process was repeated until the beads get disintegrated.

2.5 Cell leakage

Cell leakage from the gel matrix was determined as cell weight by measuring the optical density at 600 nm. From the standard graph of biomass, the corresponding absorbance was converted to μ g of cell leakage from each matrix.

3. Results and Discussion

Immobilization of protease producing cells has practiced by many authors (Anwar *et al.*, 2009, Kumari *et al.*, 2009, Rao *et al.*, 2008). Among the immobilization methods for microbial cells, entrapment is the most suitable and common method of practice. Immobilization by entrapment is known to be a simple and gentle procedure and keeps the cells from unfavorable conditions (pH, temperature, etc) found in the surrounding media (Fukui and Tanaka, 1982; Kierstan and Ducke 1977). Whole cell immobilization technique is generally being used for higher productivity

by protecting the cells from shear forces, in addition to this the product and cell separation is easy so that the cells can be reused several times (Adinarayana *et al.*, 2005).

3.1 Immobilization of *B. subtilis* in different matrices

Different matrices like agar, gelatin, sodium alginate and polyacrylamide were used for immobilization of a definite amount of logarithm phase, *B. subtilis* cells. A free cell system containing the equivalent cell weight was also run, similarly. Fig 1 and fig 2 represent the comparative protease production by different matrices over a time period of 48 h of incubation and cell leakage, respectively.

The gelatin entrapped cells started the enzyme production at 6 h and gradually it increased to maximum titer (10.8 U/ml), among tested different matrices at 24 h of incubation and after this enzyme production was decreased. Compared to free cell system where at 24 h of incubation the maximum enzyme titer was 5.7 U/ml, gelatin entrapped cells showed 51 % increased enzyme production. Cell leakage from the matrix over the period of incubation was less (0.06 mg/ml) compared to other matrices. Gelatin has used as immobilization matrix for the production of Penicillin G acylase (Norouzian *et al.*, 2002), Glucoamylase, invertase and cellobiase (Kennedy, 1984) and for β - amylase (Ray and Nanda, 1995).

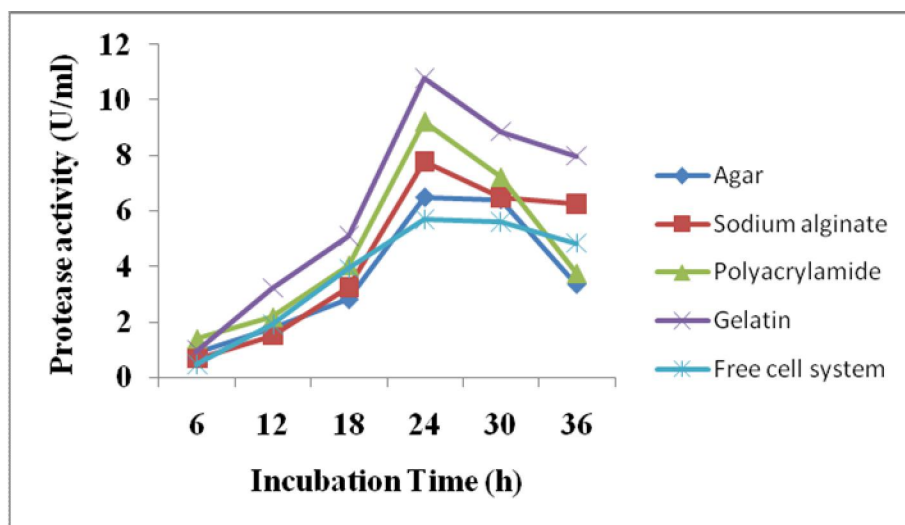


Fig 1. Time course of protease production by Immobilized cells of *Bacillus subtilis* in different matrices

Krastanov (1997) selected glutaraldehyde as the best binding agent for immobilization of microbial cells. Glutaraldehyde is introduced as a spacer group which may change the local surface area and consequently decrease the protein crowding of the immobilized enzyme on the carrier which might impair or prevent the proper conformational changes required for catalysis.

Polyacrylamide entrapped cells showed its peak activity (9.22 U/ml) at 24 h of incubation and compared to free cell system it get increased to 35.2% while the cell leakage was 0.22 mg/ml. The results are in line with the finding by Abdel-Naby (1997) who has reported 21.7 U/mg specific activity of protease by *Bacillus mycoides* on polyacrylamide matrix at 2% acrylamide concentration.

Calcium alginate entrapped cells at 24 h of incubation showed 7.8 U/ml enzymes production which was 21% increased than free cell system, but was less than gelatin and polyacrylamide matrix. Our results are in accordance with the study in which 23.2% increased alkaline protease was achieved by *Bacillus*

Licheniformis (Ahmed *et al.*, 2007). The cell leakage was 0.32 mg/ml. 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).

Agar entrapped cells showed least enzyme production (6.48 U/ml) compare to free cells it was only 7.8% increased with maximum cell leakage (0.74 mg/ml). The less protease production was achieved by *Streptomyces avermectinus* at 3% concentration (Ahmed *et al.*, 2008). Nigam *et al.*, (2007) also reported the least enzyme production with agar by *Pseudomonas diminuta* among tested Chitosen, Gelatin and agar matrices.

3.2 Repeated batch process

The reusability of *B. subtilis* was evaluated by transferring the immobilized cells to fresh medium at every 48 h of incubation. Results reveal that the gelatin entrapped cells can be reused for maximum times (7 cycles) with total protease titer of 45.36 U/ml, while agar entrapped cells can be reused for only 3 cycles

(enzyme activity 14.59 U/ml, Table 1). Bajpai and Margaritis (1985) reported 10 batch cycles of inulinase production with gelatin entrapped beads by *Kluyveromyces marxianus*. Adinarayana *et al.*, (2005), Rao *et al.*, (2008) and Anwar *et al.*, (2009) reported 9, 11 and 4 repeated batch cycles with alginate entrapped cells. Beshay (2003) has reported 5 repeat cycles with

calcium alginate obtaining 3.11 fold alkaline protease production. Nigam *et al.*, (2007) reported 6 repeat batch with agar entrapped cells by *Pseudomonas diminuta*.

Table 2 represents the cell leakage from different matrices during repeated batch process. Maximum cell leakage was observed in agar entrapped cells and minimum with gelatin entrapped cells.

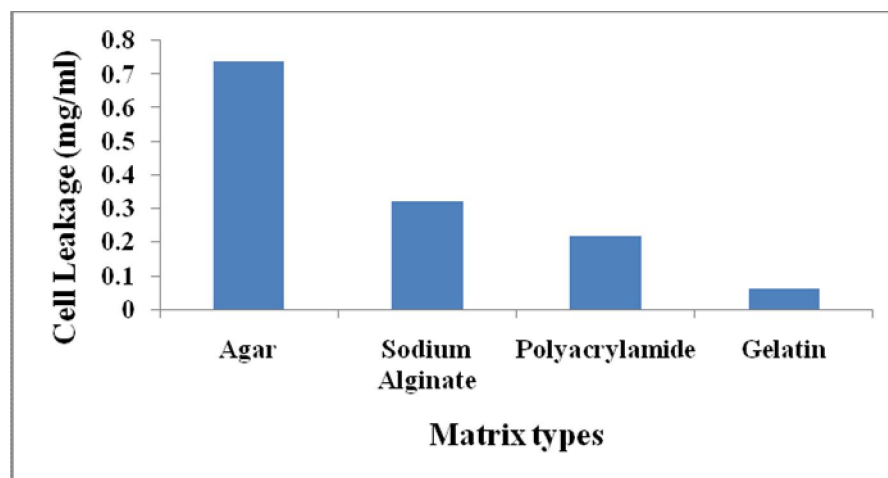


Fig 2. Cell leakage efficiency of different matrices used for immobilization of *B. subtilis*

Table 1 Repeat batches of *B. subtilis* immobilized in different matrices

Matrix Type	Repeat Cycle and Enzyme activity (U/ml)								Relative Efficiency (%)
	1	2	3	4	5	6	7	Total Enzyme Titre (U/ml)	
Gelatin	10.08	9.66	8.23	6.98	5.65	3.20	1.56	45.36	100
Polyacrylamide	8.23	7.45	5.87	3.34	1.45	1.03		27.37	60.33
Calcium alginate	6.20	5.07	4.76	2.0	1.2	-		19.23	42.23
Agar	6.94	4.87	2.78		-	-		14.59	31.08
Free cell system	5.7	-	-	-	-	-		5.7	12.56

Table 2 Cell leakage study by immobilized *B. subtilis* in different matrices during repeat batch process

Matrix Type	Repeat Cycle and Cell Leakage (mg/ml)						
	1	2	3	4	5	6	7
Gelatin	0.08	0.13	0.16	0.25	0.67	9.00	1.34
Polyacrylamide	0.24	0.28	0.58	0.77	0.98	1.86	-
Sodium Alginate	0.34	0.52	0.87	1.06	1.88	-	-
Agar	0.79	1.45	2.22	-	-	-	-

Acknowledgment:

Authors acknowledge the laboratory facilities and financial support provided by the management of Dolphin PG College of Life Sciences, Chunni-Kalan-140307, District- Fatehgarh Sahib, Punjab, INDIA.

Correspondence to:

Rakesh Kumar
Biotechnology Department, Dolphin PG College of Life Sciences, V.P.O., Chunni-Kalan-140307, District- Fatehgarh Sahib, Punjab, INDIA
Cellular phone: +919417941819
Email- rakesh_panchal1@yahoo.co.in

References

- [1] Godfrey T and West S. Industrial Enzymology, 2nd ed, p. 3, 1996. Macmillan Publishers inc, New York.
- [2] Rao M.B, Tanksale A.P, Ghatge M.S and Deshpande V.V. Molecular and biotechnological aspects of microbial proteases, microbiology and molecular biology reviews.1998; 62: 597-635.
- [3] Beg QK, Gupta R and Lorenz P. Bacterial Alkaline Proteases. Molecular Approaches and Industrial Applications. *Applied Microbial. Biotechnol.* 2002; 59: 15-32.
- [4] Sen S and Satyanarayana T. Optimization of alkaline protease production by thermophilic *Bacillus licheniformis* S-40. *Indian J Microbiol*, 1993; 33: 43-47.
- [5] Ramakrishna SV, Jamuna R and Emery AN. Production of ethanol by immobilized yeast. *Appl Biochem Biotechnol.*,1992; 37: 275-282.
- [6] Johnsen A, Flink JM. Influence of alginate properties and gel reinforcement on fermentation characteristics of immobilized yeast cells. *Enz. Microb. Technol.* 1986, 8:737-748.
- [7] Anwar A, Qader SAU, Raiz A, Iqbal S and Azhar A. Calcium Alginate: A support material for immobilization of proteases from newly isolated strain of *Bacillus subtilis* KIBGE-HAS. *World Applied Sciences Journal*, 2009, 7(10): 1281-1286.
- [8] Kumari D, Sharma N, Pandove N and Achal V. Alkaline protease production by immobilized cells of *Bacillus pumilis* MTCC 2296 in various matrices. *Life Science Journal*. 2009; 6(2): 8–10.
- [9] Rao CS, Madhavendra SS, Rao RS, Hobbs PJ and Prakasham RS. Studies on improving the immobilized bead reusability and alkaline protease production by isolated immobilized *Bacillus circulans* (MTCC 6811) using overall evaluation criteria. *Applied Biochemistry and Biotechnology*, 2008, 150: 65-83.
- [10] Fukui S and Tanaka A. Immobilized microbial cells. *Ann. Rev. Microbiol.*, 1982, 36: 145-172.
- [11] Kierstan M and Ducke. Immobilization of microbial cells sub-cellular organelles and enzymes in calcium alginate gels. *Biotechnol. Bioeng.*, 1977, 19: 337-397.
- [12] Adinarayana K, Jyothi B and Ellaiah P. Production of Alkaline Protease with Immobilized Cells of *Bacillus subtilis*. *AAPS PharmSciTech* 2005; 6 (3) Article 48 (<http://www.aapspharmscitech.org>).
- [13] Norouzian D, Javadpour S, Moazami N and Akbarzadeh A. Immobilization of whole cell penicillin G acylase in open pore gelatin matrix, PE-11 in Various Matrices by Entrapment Technique. *Enzyme and Microbial Technology*, 2002, 30(1): 26-29
- [14] Kennedy JF, Kalogerakis B, Cabral JMS. Surface immobilization and entrapping of enzymes on glutaraldehyde crosslinked gelatin particles. *Enzyme and Microbial Technology*, 1984, 6(3): 127-13.
- [15] Ray RR and Nanda JG. Immobilization of α -amylase from *Bacillus megaterium* B₆ into gelatin film by cross-linking. *Journal of Applied Microbiology*,1995, 79(2): 157–162.
- [16] Krastanov A. Continuous sucrose hydrolysis by yeast cells immobilized to wool. *Applied Microbiology and Biotechnology*, 1997, 47(5): 476-481.
- [17] Abdel-Naby M.A, Ismail AMS, Ahmed SA, Abdel Fattah AF. Production and immobilization of alkaline protease from *Bacillus mycoides*. *Biores. Technol.*, 1998; 64: 205-210.
- [18] Ahmed SA, Saleh SA and Abdel-Fattah AF. Stabilization of *Bacillus Licheniformis* ATCC 21415 alkaline protease by immobilization and modification. *Australian Journal of Basic and Applied Sciences*, 2007, 1(3): 313-322.
- [19] Bajpai SK, Sharma S. Investigation of swelling/degradation behavior of alginate beads crosslinked with Ca²⁺ and Ba²⁺ ions. *React. Functional Polymers*, 2004; 59:129-140.
- [20] Ahmed SA, Ramadan A, Al-Domany, Nefisa MA, El-Shayeb, Hesham HR and Shireen A. Saleh. Optimization, immobilization of extracellular alkaline protease and characterization of its enzymatic properties, *Research Journal of Agriculture and Biological Sciences*, 2008, 4(5): 434-446.
- [21] Beshay U. Production of alkaline protease by *Teredinobacter turnirae* cells immobilized in Ca-alginate beads. *African Journal of Biotechnology*, 2003, 2(3): 60–65. Available online at <http://www.academicjournals.org/AJB>
- [22] Nigam VK, Kundu S and Ghosh P. Reusability of entrapped cells of *Pseudomonas diminuta* for production of 7-aminoccephalosporanic acid. *Applied Biochemistry and Biotechnology*, 2007, 141(1): 119-125.
- [23] Bajpai P, Margaritis A. Immobilization of *Kluyveromyces marxianus* cells containing inulinase activity in open pore gelatin matrix: Preparation and enzymatic properties, *Enzyme Microb. Technol.* 7 (1985) 373–376.