Whole cell immobilization of thermostable α-amylase from phosphate solubilizing *Bacillus thuringiensis* A5-BRSC

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Abstract

Introduction: Immobilization of the whole cell is a modern approach to fermentative production of different enzymes. The technique has several advantages including easy separation of the fermented product, least chance of contamination and no chance of wash out of cells during product recovery.

Materials and Methods: α-amylase produced from Calcium alginate entrapped cells of *Bacillus thuringiensis* A5-BRSC was purified by ammonium sulphate precipitation, followed by DE-52 ion exchange column chromatography. The molecular weight of the purified enzyme was determined by SDS-PAGE. Study of enzyme kinetics was performed to determine its enzymatic potency.

Results: Immobilization of *Bacillus thuringiensis* A5-BRSC cells in calcium alginate resulted in more than 3.5 times higher α-amylase activity than free cells within 48 hours of batch fermentation. The molecular weight of the purified enzyme was estimated to be 52 kDa by SDS-PAGE. The enzyme exhibited pH optima at 6.9 and temperature optima at 50^0C. K_M and V_{max} of the purified enzyme was determined to be 0.85 mg/ml and 0.98 mg of liberated maltose/ml of enzyme/minute.

Conclusion: α-amylase produced form the immobilized cells found to be more potent than the enzyme produced by the free cells.

Keywords: Cell immobilization, *Bacillus thuringiensis*, α-amylase, Column chromatography.

Introduction

Amylases are most widely distributed enzyme in bacteria, fungi, plants and animals including human. Three major classes of amylases are reported to occur in microorganisms.[1] α-amylase or endo-1, 4- α-D-glucan glucohydrolase (E.C. 3.2.1.1) randomly cleaves α-1,4-glucosidic linkages of starch (amylose or linear chain of amylopectin) to yield glucose, maltose or maltotriose.[2, 3] β-amylase or exoamylase (E.C. 3.2.1.2) were reported to cleave α-1, 4-glucosidic linkages from non-reducing end of starch (amylose or linear chain of amylopectin) to yield maltose units.[4] Another class of amylase is glucoamylase or exo-1, 4- α-D-glucan glucohydrolase (E.C. 3.2.1.3) that hydrolyzes single glucose units from nonreducing ends of amylose or amylopectin in a stepwise manner.[5,6] Among the three classes α-amylase is most common microbial amylase and also widely used in baking, brewing, detergent, textile and paper industries.[7]

Immobilized cells can be defined as cells those are physically confined or localized in a certain defined region of space with the retention of their catalytic activities, which can be used repeatedly and continuously. The central feature of Immobilized cell system is the use of binding structure to confine the cells in some section of the reactor, which is usually achieved by entrapment or attachment to small particles or by involving barriers to cell transport. The remarkable feature of this procedure is that, in spite of this imposed restriction, the cells retain their catalytic activity and facilitate their continuous use and reuse. From an economic standpoint, these Immobilized cell systems provide a reduction in capital investment, helping to achieve a higher yield with a relatively low investment. The use of Immobilized cells offer several advantages over free cells, such as a relative ease of product separation, reuse of biocatalyst, prevention of washout, reduced risk of contamination and operational stability.[8,9,10] Furthermore, using entrapment technique, a dense cell culture can be established leading to improved production. Among different Immobilization techniques, entrapment in calcium alginate gel offers many advantages due to simplicity and non-toxic character.[9,11] The mild and inexpensive method involves the dropwise addition of cells suspended in sodium alginate solution into a solution of $CaCl_2$ whereon the cells are immobilized in precipitated calcium alginate gel in the form of beads.[12]

Phosphate solubilizing property of *Bacillus thuringiensis* A5-BRSC was reported previously. Here whole cell immobilization of same bacterial strain was explored to make a comparative study of the efficacy of α-amylase in free cells and Ca-alginate entrapped cells.

Materials and Methods

Microorganism: *Bacillus thuringiensis* (NCBI gene bank entry: DQ286337) isolated from the agricultural field of West Bengal, India was used as microorganism in the present study.

Whole cell immobilization: Culture of *Bacillus thuringiensis* A5-BRSC was grown in starch broth medium for overnight at 35 ± 2^0C with gentle shaking in a rotary shaker at 160 rpm. The cells were used as inoculum for both immobilization and free cell

experiments. Cells grown in starch broth medium were centrifuged at 8000 x g for 10 minutes at 4^0C. 20 mg wet weight of cell pellet was suspended in 12.5 ml of 2% sodium alginate solution. The mixture was slowly added drop-wise through 1 ml syringe into 25 ml of 3% $CaCl_2$ solution. The drops became solidified on contact with the $CaCl_2$ solution. The capsules thus formed, entrapping bacterial cells, were allowed to harden for 30 minutes at room temperature. The capsules were then washed with a sterile saline solution (0.9% NaCl, w/v) to remove excess calcium and free cells. Immobilized cells were added in 50 ml of fermentation medium in triplicate sets. Similarly, 20 mg wet weight of *Bacillus thuringiensis* A5-BRSC free cells were added separately in 50 ml of fermentation medium in triplicate sets. All the fermentation flasks were incubated at 35 ± 2ºC for 48 hours on a rotary shaker (160 rpm). For semi-continuous fermentation, after completion of each cycle, the beads of immobilized cells were washed with sterilized phosphate buffer saline (PBS, with 0.9% NaCl, pH- 7.0) and incubated with fresh fermentation medium. Free cells were centrifuged at 10,000 x g for 10 minutes and re-suspended in the fermentation medium.

Recovery of amylase: Amylase from immobilized cells was recovered by dissolving alginate capsules of fermentation medium in 1% sodium citrate solution (w/v). Released cells were centrifuged at 10,000 x g for 10 minutes. The enzyme from the fermentation medium with the free cells was recovered by direct centrifugation of the culture broth at 10,000 x g for 10 minutes. The clear supernatant obtained was used as enzyme source.

For qualitative assay of amylase 50 µl of the enzyme was loaded in the well (5 mm diameter) of starch-agar medium and incubated for 24 hours at 35 ± 2ºC. Addition of Lugol's iodine solution to the starch-agar plate was used to detect amylase activity on the plate. The dextrinizing activity of amylase was estimated by DNS method. [13] Enzyme activity of 1 ml crude amylase in each culture supernatant was expressed as µmols of product formed per minute under specified assay condition. The efficiency of immobilization was calculated by using the formula: [12]

$$\text{Efficiency of immobilization} = C_{imm} / C_{free}$$

[C_{imm} = amylase yield produced by immobilized cells and C_{free} = amylase yield produced by free cells]

Optimization of fermentation parameters in whole cell immobilization: Different concentrations (w/v) of sodium alginate (1%, 2%, 3%, 4% and 5%) were added to the entrapment mixture to study the influence of sodium alginate on the gel bead permeability. In all concentrations of alginate $CaCl_2$ concentration of 3% (w/v) was fixed. Effect of different $CaCl_2$ concentration in immobilization of amylase producing bacterial cells was investigated by maintaining sodium alginate concentration of 2% intact. $CaCl_2$ concentration of 2%, 2.5%, 3%, 3.5%, 4% and 5% was used for the present study.

Fermentation media with free cells as well as immobilized cells were incubated for 24, 48, 72, 96 and 120 hours at 35±2ºC. Each day a small aliquot from each medium was withdrawn and amylase was estimated by DNS method.

Fermentation media with free cells as well as immobilized cells were incubated at different temperatures (35, 48, 60 and 72ºC) to study the effect of temperatures on the rate of amylase production.

To investigate the influence of pH of fermentation media on amylase production from free cells and immobilized cells, a range of pH from 6.0 – 7.5 was set in fermentation media. Effect of pH was studied by incubating the fermentation media for 48 hours at 35±2ºC.

Purification and kinetic study of the enzyme from immobilized cell

Purification of amylase extracted from immobilized cells of *Bacillus thuringiensis* A5-BRSC was performed by growing the organism in starch broth medium (Hi-media) at 30^0C for 48 hrs. The culture was centrifuged at 8000xg for 30 minutes. The supernatant was collected. Solid ammonium sulphate was slowly added to the filtrate with a continuous stirring by a magnetic stirrer. The solution was kept at 4^0C for overnight. Most of the amylase was precipitated between 65% - 70% saturation. Precipitated protein was centrifuged at 15000 x g for 30 minutes at room temperature. The concentrated protein was re-suspended in 50mM phosphate buffer of pH 6.9 and dialyzed against the same buffer for overnight.

Dialyzed enzyme solution was applied to DEAE-cellulose (DE-52) ion exchange column chromatography (30 cm x 1 cm). The column was pre-equilibrated with 50mM phosphate buffer of pH 6.9 and then dialyzed protein solution was loaded in the column. Unbound protein fraction was collected till the $OD_{280\ nm}$ reached in the negligible reading. Unbound fraction was washed with the same buffer. Proteins were eluted with a concentration gradient of NaCl from 0.1 M to 1 M with a constant flow rate of 0.4 ml/min at 30^0C. Fractions were collected and each fraction was analyzed for protein concentration and amylase activity. Estimation of enzyme protein was carried out according to the method of Lowry.[14]

The Michaelis-Menten constant (K_M) of the purified enzyme was determined for various linear chain and branched chain polysaccharides including amylase, soluble starch and amylopectin.

Determination of molecular weight of amylase by SDS-PAGE: Purified enzyme protein was analyzed in 10% SDS-PAGE, according to the method of Laemmli.[15] 100 µg of protein was mixed with sample buffer and incubated in boiling water bath for 4

minutes. Finally, 50 µg of protein was loaded in 10% SDS-PAGE. After the electrophoresis, protein bands were visualized by silver staining method.[16]

Results and Discussion

Immobilized cells of *B. thuringienis* A5-BRSC exhibited more than 3.5 times amylase yield (immobilization efficiency = 3.63) in 48 hours incubation than free cells at 35 ±2^{0}C (Fig. 1). The cells immobilized with 2% (w/v) Sodium alginate and 3% (w/v) $CaCl_2$ showed maximum amylase production (Fig. 2a & b). The result is quite consistent with previous reports on the similar study.[17] Significant enzyme activity was also noted at the incubation temperature of 35^{0}C, but highest enzyme activity was achieved at 50^{0}C; further, rise of temperature up to 80^{0}C fall the enzyme activity. Fermentation pH of 6.9 was revealed to be the optimum pH for enzyme production from both free and immobilized cells. In case of free cells, soluble starch supplementation showed the best enzyme activity, but in case of immobilized cells, this supplementation drastically reduced enzyme production. This finding is contradictory with previous reports.[18,19] However, it was also reported that soluble starch concentration above 0.5% decreases the rate of amylase production in *B. thuringiensis.*[17] One possible explanation behind this phenomenon might be the large molecular weight of starch. Due to its high molecular weight, it could not penetrate gel beads to reach the entrapped cells and masked the gel beads to reach other nutrients to the immobilized cells.

The stability of immobilized *B. thuringiensis* A5-BRSC cells in repeated batch fermentation was studied in order to access their suitability for long-term use. Figure 3 illustrated the rate of amylase production from immobilized cells for repeated 8 cycles. In 2nd cycle, the activity was highest and up to 6th cycle, amylase activity remained steady, which subsequently fall on 7th cycle onwards. In comparison, free cells showed poor amylase activity from 3rd cycle onwards. Earlier reports on repeated batch fermentation from immobilized cells of *Bacillus* sp. exhibited similar findings.[12,17]

The recovery of extracellular amylase from immobilized cells of was summarized in Table 1. The first step of purification, i.e., ammonium sulphate fractionation, maximum enzyme activity was exhibited between 60 – 70% saturation. This active fraction was further purified by dialysis, followed by DEAE-cellulose (DE-52) column chromatography. The elution profile of the enzyme showed only one sharp peak for amylase (Figure 4a & b). Most of the enzyme protein was eluted at 0.4M NaCl. Maximum purification level achieved was approximately 33 fold and the recovery yield was 12.56%. The fractions that showed amylase activity were pooled together and lyophilized. SDS-PAGE and subsequent silver staining of purified amylase showed a single band of molecular weight 52 kDa (Figure 5). No isozyme of the enzyme was detected, although *Bacillus thuringiensis* CKB 19 was reported to have two isozymes of 59.6 and 44.7 KDa.[17] However, a large number of *Bacillus* sp. was also detected to have no isozyme form of α-amylase.[20,21]

The purified enzyme from immobilized cells showed linear progress in product formation with incubation time up to 8 minutes in 0.1 M phosphate buffer (pH- 6.9) at 37^{0}C and 0.5% soluble starch as substrate. More incubation of the enzyme with the substrate showed zero order kinetics. The enzyme showed maximum activity at 0.25 % concentration (w/v) of soluble starch. Lineweaver-Burk plot revealed K_m and V_{max} for soluble starch. The values of K_m and V_{max} for soluble starch were determined as 0.85 mg/ ml and 0.988 mg of liberated maltose/ ml of enzyme/ minute.

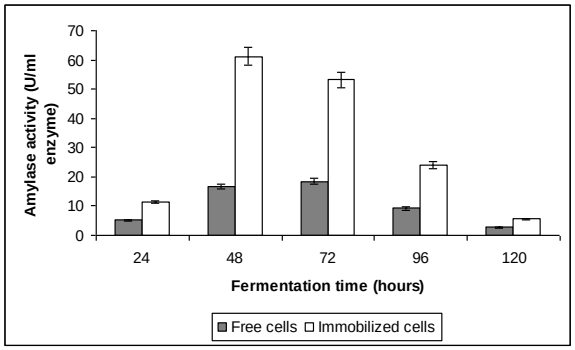

Fig. 1: Comparison of amylase production from free and immobilized cells of *Bacillus thuringiensis* A5-BRSC in fermentation media

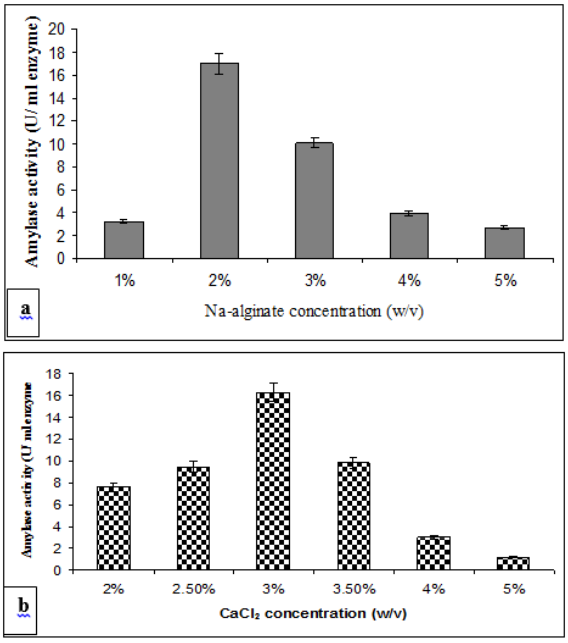

Fig. 2a & 2b: Effect of different concentrations of (a) sodium alginate and (b) calcium chloride on amylase production from immobilized cells of *Bacillus thuringiensis* A5-BRSC

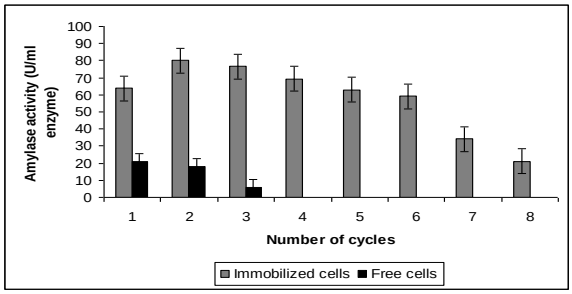

Fig. 3: Re-use efficiency of immobilized cells by repeated batch fermentation in comparison to free cells. Each cycle was carried out for 48 hours at 35^{0}C. After each cycle, the beads were washed with sterilized phosphate buffer saline and incubated with the fresh fermentation medium

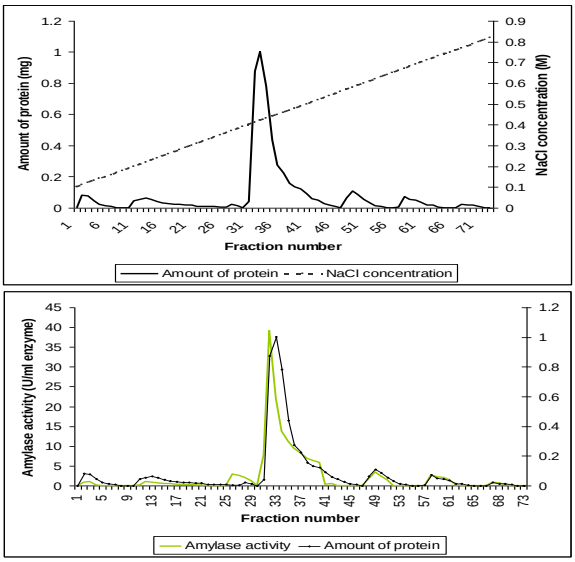

Fig. 4a & 4b: Elution profile of amylase extracted from immobilized cells of *B. thuringiensis* A5-BRSC on DEAE-cellulose (DE-52) column chromatography. (a)The sample was eluted with NaCl gradient of 0.1- 0.9 M (b) Amylase activity in the collected fractions of the eluted protein

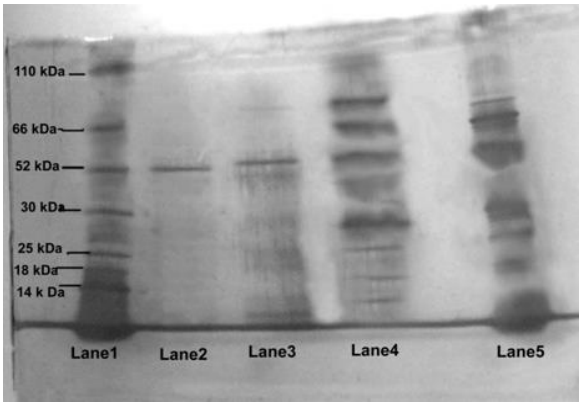

Fig. 5: SDS-PAGE profile of amylase extracted from immobilized cells of *B. thuringiensis* A5-BRSC. [Lane1- Molecular weight marker, lane 2- purified product obtained after DEAE-cellulose column chromatography, lane 3- product obtained after dialysis, lane 4 – commercially available diastase (SRL, India Ltd.) and lane 5- culture filtrate of immobilized cells (crude enzyme source)]

Table 1: Purification and recovery of extracellular amylase from immobilized cells *Bacillus thuringiensis* A5-BRSC

Purification step	Volume (ml)	Protein (mg/ml)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture filtrate	300	1.94	624	1.07	100	1
60–70% ammonium sulphate cut fraction	20	0.52	97.2	9.35	15.6	8.74
Dialyzed product	22	0.32	84.92	12.06	13.6	11.27
DEAE-cellulose chromatography	28	0.08	78.4	35	12.56	32.71

Conclusion

The approach used in this study was to produce a large quantity α-amylase from microencapsulating bacteria as well as from free cells of *Bacillus thuringiensis* A5-BRSC. From the industrial point of view, the amylase is significant due to its high activity at higher temperature and stability at room temperature for more than 48 hours. Such thermostable enzymes have potential to be used in starch liquefying industries, bakeries, medicines and other industrial fields.

References

1. Bernfield P. Amylases α and β, In: Methods in Enzymology. Vol. 1, Academic Press, New York, USA 1955; 149–58.
2. Anto H., Trivedi U., and Patel K.. Alpha Amylase Production by *Bacillus cereus* MTCC 1305 Using Solid-State Fermentation**.** *Food Technol. Biotechnol*. 2006;44 (2):241–45.
3. Gupta A., Gautam N. and Modi D.R. Optimization of α-amylase production from and immobilized cells of *Aspergillus niger*. *J. Biotechnol. Pharma. Res.* 2010;1(1):1-8.
4. Jonathan D.M. and Preiss J. Purification of a beta amylase that accumulates in Arabidopsis thaliana mutants defective in starch metabolism. *Plant Physiol*. 1990;94(3):1033-39.
5. Anto H., Trivedi U.B., and Patel K.C. Glucoamylase production by solid-state fermentation using rice flake manufacturing waste products as substrate. *Bioresour. Technol*. 2006;97:1161–6.
6. Pandey A., Nigam P., Soccol C.R., Soccol V.T., Singh D., Mohan R. Advances in microbial amylases. *Biotechnol. Appl. Biochem*. 2000;31:135–52.
7. Ramachandran S., Patel A.K., Nampoothiri K.M., Chandran S., Szakacs G., Soccol C.R., Pandey A. Alpha amylase from a fungal culture grown on oil cakes and its properties. *Braz. Arch. Biol. Technol*. 2004;47**:**309-17.
8. Vuellemard, J.C., Goulet, J., Amiot, J. and Terre, S. Continuous Production of Small Peptides from Milk Proteins by Extracellular Proteases of Free and Immobilized *Serratia mercescens* cells. *Enzyme Microbial Tech*. 1988;10:2-8.
9. Goksungur V., Zorlu N. Production of Ethanol from Beet Molasses by Ca-Alginate Immobilized Yeast cell in a Packed-Bed Reactor. *Turk. J. Biol*. 2001;25:265-69.
10. Alva J.C.R. and Rocha-Leao M.H.M. A Strategic Study using Mutant-Strain Entrapment in Ca-Alginate for the Production of *Saccharomyces cerevisiae* cells with High Invertase Activity. *Biotech. App. Biochem*. 2003;38:43-46.
11. Gombotz W.R., Wee S.F. Protein Release from Alginate Matrices. *Adv. Drug Delivery Rev*. 1998;31:267-71.
12. Konsoula Z., Kyriakides L.M. Thermostable α-Amylase Production by *Bacillus subtilis* Entrapped in Calcium Alginate Gel Capsules. *Enzyme Microbial Tech*. 2006;39:690-94.
13. Miller G.L., Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal. Chem*. 1959;31:426–28.
14. Lowry D.H., Rosebrough N. J., Farr A.L., Randall R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. 1951;193:265-75.
15. Laemmli U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature. 1970;277:680-85.
16. Rabilloud T., Mechanisms of protein silver staining in polyacrylamide gels: a 10-year synthesis. Electrophoresis. 1990;11:785-94.
17. Maity C., Samanta S., Halder S K., Das Mohapatra P.K., Pati B.R., Jana M., Mondal K.C. Isozymes of *α*-amylases from Newly Isolated *Bacillus thuringiensis* CKB19: Production from Immobilized cells. Biotechnology and Bioprocess Engineering. 2011;16:312-19.
18. Aiyer P.V., Effect of C:N ratio on alpha amylase production by *Bacillus licheniformis* SPT 27. *Afr. J. Biotechnol*. 2004;3(10):519–22.
19. Banna T.E.E, Ahmed A.A., Mohamed I.A.D., Reham I.I. Production and immobilization of α-amylase from *Bacillus subtilis*. *Pak. J. Biol. Sci.* 2007;10(12):2039-47.
20. Hassan S.A., Ali S.A., Abbasi A., Kamal M. Purification and biochemical characterization of a Ca^{2+}-independent, thermostable and acidophilic α-amylase from *Bacillus* sp. RM16. *Afr. J. Biotechnol*. 2011;10(32):6082-89.
21. Haq Ikram-ul, Ashraf H., Iqbal J., Qadeer M.A. Production of alpha amylase by *Bacillus licheniformis* using an economical medium. *Bioresour. Technol*. 2003;*87*:57–61.