Australian Journal of Basic and Applied Sciences, 5(8): 456-464, 2011
ISSN 1991-8178

Cell Immobilization of *Bacillus Subtilis* DJ5 for Production of Novel Hyperthermostable Extracellular β Amylase

[1]Abhijit Poddar, [2]Ratan Gachhui, [1]Subhas Chandra Jana

[1]Post Graduate Department of Microbiology, Bidhannagar College (Govt. of West Bengal), West Bengal, India.
[2]Department of Life Science & Biotechnology, Jadavpur University, West Bengal, India.

Abstract: A newly isolated mesophilc bacterium was found to produce hyperthermostable β amylase enzyme showing full catalytic activity at 100°C for 15 min. The organism was identified as *Bacillus subtilis* strain DJ5 by morphological, biochemical and 16s rDNA analysis. Strain was improved by exposing it to UV irradiation for 10 min followed by NTG treatment at 0.1 mg/mL concentration. Hyperthermostable β amylase enzyme production with free cells and entrapped cells in different matrices like alginate, polyacrylamide, gelatin, agarose were examined and compared for enzyme production. Result showed maximum enzyme production of 23.86 U/mg in strontium alginate entrapped beads that were more than 2 fold higher than conventional free cell system (10.97 U/mg). Maximum enzyme production by barium alginate 20.57 U/mg, calcium alginate 20.02 U/ mg and polyacrylamide 18.67 U/mg was also higher than free cell system. Enzyme production in agarose entrapped cells (9.59 U/mg) and gelatin entrapped cells (2.19 U/ml/mg) was lower than free cell system. Strontium alginate beads were used ten times in repeated batch fermentation without cell disintegration. From the result it can be concluded that strontium alginate entrapped *Bacillus subtilis* DJ5 cells is a better choice for industrial production of highly thermostable β amylase.

Key words: Immobilization, Strontium alginate, Hyperthermostable β amylase, *Bacillus subtilis* strain DJ5.

INTRODUCTION

With the discovery of starch processing enzyme in 1811 by Kirchoff and later classified as α and β amylases by Ohlsson in 1930, amylases has found great significance in present day biotechnology with application ranging from food, fermentation, textile, detergent to paper industries drawing a revenue of US $ 650 per year comprising 30% of world's enzyme consumption (Van der Maarel *et al.,* 2002). Since gelatinized starches are better substrate for amylases than insoluble solid starches, solid starches are pregelatinized by boiling it at elevated temperature (>70 °C depending on starch type and origin) (Rakshit, 1998). Enzymes that are thermolabile bring an economic burden to the industrial process since thermally gelatinized starches are cooled down before addition of enzyme to maintain stability of the enzyme. This approach increases time and consumes labor that finally reflects in the production cost (Haki *et al.,* 2003). In that respect thermostable amylases has taken a leading edge as thermostable enzymes can be directly added to solid starches and subsequently gelatinized at elevated temperature (90-110°C). This one step approach reduces time and also saves labor by eliminating cooling time and also renders process simplicity (Leveque *et al.,* 2000). In order to draw benefit from thermostable amylases, several thermostable α amylases were isolated, purified and characterized from different mutated strains of *Bacillus* (Declerck *et al.,* 1997; Goyal *et al.,* 2005), thermophilc and hyperthermophilc archaea (Leuschner *et al.,* 1995; Niehaus *et al.,* 1999) that are now used heavily in starch processing industry. β amylase (E.C 3.2.1.2; 1,4-α-D glucan maltohydrolase) cleaves 1,4-α-D glucosidic linkages in an exo fashion from non reducing ends of starch, glycogen and maltooligosaccharides and produce maltose and beta limit dextrin from starch (Hyun *et al.,* 1985). The high maltose syrups have an increasing demand because of their potential use in food and pharmaceutical industries (Ray *et al.,* 1996). In spite of such tremendous importance, very little attention were given to β amylase that was dated back in last century (Obi *et al.,* 1984, Shen *et al.,* 1988).

Moreover, the reported thermostable β amylases from *Clostridium thermosulfurogenes* are neither stable

Corresponding Author: Subhas Chandra Jana, Post Graduate Department of Microbiology, Bidhannagar College (Govt. of West Bengal), EB 2, Salt Lake City, Kolkata 700064, West bengal, India.
E-mail: subhasjana1959@gmail.com

nor active above 80° C (Shen *et al.*, 1988). Gradually whole cell immobilization method is gaining industrial and academic attention (Saville *et al.,* 2004). High water content, biocompatibility, easy controlled diffusion properties, non toxic nature, higher productivity with higher fermentation speed, tolerance to harsh environmental conditions and reusability are the hallmarks of immobilized system (Uludag *et al.,* 2000; Atia *et al.,* 2003) that has made it a better choice over batch fermentation process and is now a versatile tool for food and medicine industry.

In the present study one waste isolated mesophilic bacterium, *Bacillus subtilis* strain DJ5 was identified, mutated by exposing it to UV irradiation for 10 minutes followed by treatment with NTG at final concentration of at 0.1 mg/mL and was immobilized for enzyme production. We are reporting first that the mutant strain was found to produce thermostable β amylase enzyme with improved thermostability, being fully active at 121°C for 15 min. In order to maximize enzyme production by optimally exploiting amylase producing machinery of cell and to check reuse efficiency, whole cell of *Bacillus subtilis* DJ5 were immobilized in various matrices such as strontium alginate, calcium alginate, barium alginate, polyacrylamide, agarose and gelatin gel.

MATERIALS AND METHODS

Working Strain:

Highly thermostable beta amylase producing strain, Bacillus subtilis *strain DJ5 was isolated in pure culture from kitchen waste, Darjeeling, West Bengal. It was maintained in sterile Starch-Peptone agar medium at 4° C in our laboratory and was subcultured every three weeks.*

Strain Characterization and Identification:

The strain was identified according to Sneath *et al.* (1986), Thomas *et al.* (1980) and Higashihara *et al.* (1974). Preliminary identification was based on cultural, morphological and biochemical characteristics following Bergey's Manual of Systematic Bacteriology (Garrity *et al.,* 2005). Further it was identified by 16S ribosomal ribonucleic acid (rRNA) gene sequencing. The 16S rRNA gene of the strain*Bacillus subtilis* strain DJ5was amplified by the method described earlier (Das *et al.,* 1996). Primers used for the amplification of 16S rRNA were 5'-GAG TTT GAT CCT GGC TCA G-3' (forward primer) and 5'-AGA AAG GAG GTG ATC CAG CC-3' (reverse primer). DNA amplification was performed with a Thermal Cycler, Model PCT-200 (M.J. Research, Waltham, MA, USA) with the following temperature condition: initial denaturation step at 94°C for 4 min; followed by 30 cycles of 62°C for 1 min, 72°C for 1.5 min and 94ºC for 1 min and final extension at 72°C for 7 min. The PCR product was purified by using the QIAquick PCR purification kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Gel-purified 16S rRNA was sequenced using a CEQ Dye terminator cycle sequencing kit in an automated DNA sequencer Model CEQ 8000 (Beckman Coulter, Fullerton, CA, USA)(Panday and Das, 2010).Nucleotide sequences thus obtained were assembled using the sequence alignment editor program Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Blast searches for homologous sequences of 16S rRNA genes in the public data bases were performed in the NCBI web site (Altschul*et al.,* 1997). Evolutionary distances to other strains of Bacillus were computed by neighbor joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and phylogenetic tree was constructed by software MEGA5 (Tamura *et al.,* 2007)

Inoculum Preparation and Isolation of Crude Enzyme:

Culture was transferred aseptically in 250 mL conical flask containing 100 mL sterile Starch Peptone medium (pH 6.9) containing 0.09% peptone, 0.5% starch (Sigma Aldrich, USA), 0.01% KCl, 0.01% $MgSO_4$, 0.04% $(NH_4)_2SO_4$ and 0.05% Na_2HPO_4. The flask was kept in BOD shaker incubator at 160 rpm at 37°C for 6.5 hour. The culture was centrifuged at 10000 rpm for 10 mins at 4°C in cold centrifuge (Remi, India). The supernatant was collected as source of crude beta amylase enzyme for further assay.

The cell pellet was washed thrice thoroughly in sterile 0.1 M saline phosphate buffer, pH 6.9 and suspended in minimum amount of same buffer. This cell suspension was used as inoculum for immobilization.

Ionotropic Gelation:

Sodium alginate solution (8%) (Loba Chemie, Mumbai, India) was prepared by dissolving sodium alginate in 100 mL boiling water and autoclaved at 121°C for 15 minutes. Equal amount of alginate slurry and cell suspension (cells equivalent to 1ml broth culture in mid log phase i.e. 6.5^{th} hour) were mixed thoroughly and pumped through a sterile syringe into 50 ml (30mM) divalent chloride solutions ($CaCl_2$/ $BaCl_2$/ $SrCl_2$) dropwise from 5-cm height and kept for curing at 4°C for 1 hour.

The cured beads were washed with sterile 0.1 M saline phosphate buffer, pH 6.9 for three to four times. When the beads were not being used, they were preserved in 30 mM solution of respective divalent chloride salt at 4°C. All processes were done aseptically under laminar air flow.

Whole Cell Immobilization in Polyacrylamide:

Bacterial cell suspension (5 mL) was mixed with 15 mL of 12% polyacrylamide solution (SRL, Mumbai, India), 15 mL of distilled water, 3 ml 0.1M phosphate buffer (pH 6.9), 200 µL ammonium persulphate (0.4gm/ml) (SRL, India), and 50 µL TEMED (SRL, India) and allowed to polymerize on a sterile petriplate for 1hour. The polymer sheet was cut into equal size cubes (4 mm^3), rinsed in sterile 0.1 M saline phosphate buffer pH 6.9 and stored at 4° C for curing. The cubes were then washed 3 to 4 times with sterile distilled water and stored in same for further use.

Whole Cell Immobilization in Gelatin:

5 mL of cell suspension was added to 15 mL of 6% sterile gelatin (Hi-media), maintained at 45°C, and poured into a sterile petridish. The gel was overlayered with 10 µL of 5% glutaraldehyde for hardening at 30°C. The resulting block was cut into small-size cubes (4 mm^3) and the cubes were washed thoroughly with sterile 0.1M phosphate buffer (pH 6.9) for complete removal of excess glutaraldehyde. The cubes were then washed 3 to 4 times with sterile distilled water and stored in same for further use.

Whole Cell Immobilization in Agarose:

5 mL cell suspension was added to the molten sterile agarose maintained at 40°C, shaken well for few seconds (without forming foam), poured into sterile petriplate and allowed to solidify. The solidified block was cut into equal size cubes (4 mm^3), added to sterile 0.1 M phosphate buffer (pH 6.9), and kept in the refrigerator (1 hour) for curing. After curing, buffer was decanted and the cubes were then washed 3 to 4 times with sterile distilled water and stored in same for further use.

Production of B Amylase by Batch Process with Immobilized Cell:

The immobilized bead and blocks (cells equivalent to 6% broth culture) were transferred to 100 mL sterile starch peptone production media of previous composition. The flasks were kept in BOD shaker incubator with occasional shaking at 37°C for 24 hours. Samples were withdrawn at 1 hour intervals and assayed for extracellular beta amylase activity.

Production of Beta Amylase by Repeated Batch Process with Immobilized Cell:

Immobilized beads/blocks were used repeatedly in batch fermentation process to determine reuse efficiency of immobilization procedure. After attaining maximum production of beta amylase by the immobilized cells, the spent medium was replaced with same amount of sterile fresh production medium several times until the beads/blocks starts to disintegrate, as determined by measuring bacterial growth at 660 nm in production medium.

Analytical Method:

Assay of β Amylolytic Activity:

β amylolytic activity was measured by the method of Bernfeld (1955). Assay mixture contains 0.5 mL of 0.1M phosphate buffer (pH 6.9), 1 mL soluble starch (0.5% w/v, Sigma Chemicals) and 0.1 mL of crude enzyme. Control was prepared as same without adding substrate. The reaction mixture was incubated at 100°C for 15 min. Enzyme-substrate reaction was then stopped by addition of 1 mL 2M NaOH. Both the assay mixture and control were then allowed to boil in boiling water bath for 10 min after addition of 0.5 mL of 3, 5-dinitrosalisylic acid reagent (Merck, Germany). After cooling the assay mixture at room temperature, absorbance were measured spectrophotometrically (Elico, India) at 540 nm. Amount of maltose released was measured from standard curve of maltose. One unit (U) of β amylolytic activity was defined as the amount of enzyme releasing 1 µmol of maltose equivalent per minute per ml from soluble starch (Sigma) under the standard assay conditions. The specific activity of the enzyme (U/mg of protein) was also determined by measuring protein content of enzyme using bovine serum albumin (BSA) as the standard, according to Lowry *et al.,* (1951). All the experiments were performed in triplicates. The relative β amylase activity was defined as percentage of maximum specific activity measured in assay.

Thermostability Test:

Crude enzyme was boiled in water bath for several hours to check thermostability of the enzyme. Paraffin oil (Merck) bath was used for temperature above 100° C. Residual enzyme activity of heat treated enzyme was measures as described previously. All tests were performed in triplicates.

Cell Growth and Cell Leakage:

Both cell growth in freely suspended cultures and cells leaked from the gel matrix were determined spectrophotometrically (Elico, India) by measuring the optical density at 660 nm and by microscopic observation of matrix inoculated broth medium.

Results:

Identification of Amylolytic Strain:

Initial tests suggested the strain DJ5 as gram positive, aerobic, spore forming short rod *Bacillus*. The organism was able to grow between 30-40°C and no growth was observed above 42°C exhibiting a true mesophilic character. Several biochemical tests following Bergey's Manual of Systematic Bacteriology indicated the organism as *Bacillus subtilis*. Subsequently PCR amplification and sequencing of its 16s rDNA gene was performed. The sequence obtained was edited to a total length of 1440 bp after direct sequencing. BLAST search showed sequence homology with *Bacillus subtilis*. Phylogenetic tree based on different species of *Bacillus* was constructed using neighbor joining method (Fig. 1) Result indicated strain DJ5 was very closely related with *Bacillus subtilis* strain. Therefore it was named *Bacillus subtilis* strain DJ5.

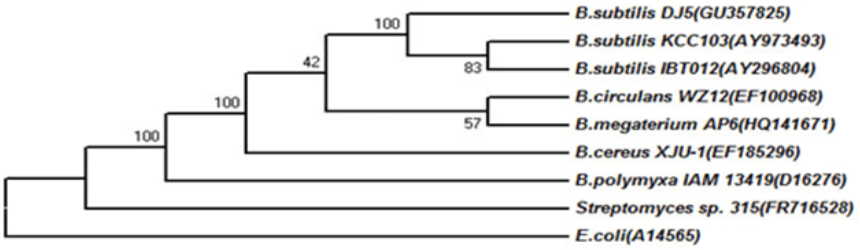

Fig. 1: Phylogenetic relationship of 16s rRNA sequence of *Bacillus subtilis* strain DJ5 with other reference strains of *Bacillus*. Bootstrap values (based on 1000 trials and only values >50%) are shown at nodes. The GenBank accession numbers of strains are given within brackets. *Streptomyces* and *E.coli* are used as outgroup.

Nucleotide Sequence Accession Number:

The neucleotide sequence of 16s rRNA of *Bacillus subtilis* strain DJ5 has been deposited in GenBank database under accession number GU357825.

Thermostability of Crude Beta Amylase Enzyme:

Crude beta amylase isolated from *Bacillus subtilis* DJ5 showed remarkable thermostability that has not been reported earlier by any worker. Though enzyme showed temperature optima of 100°C (data not shown) enzyme showed full thermal stability after boiling at 110°C for 2 hours. Even boiling at 121°C for 15 min it showed 100% stability. Afterwards there was steady decrease of enzymatic activity was recorded (50% activity after 2 hour). Only 20% of original activity was recorded after heating at 130°C for 15 minutes (Fig. 2).

Production of β Amylase by Immobilized Cells in Alginate:

Various concentration of sodium alginate were used to achieve maximum immobilization efficiency as the degree of cross linking directly affects pore size resulting in easy availability of nutrients to cell and cell leakage from entrapped matrix. Among various concentration of alginate used, 8% concentration was found to be suitable for beta amylase production by *Bacillus subtilis* strain DJ5.Out of three types of divalent cations tested, Sr^{+2} proved to be the best both for immobilization of viable cells with subsequent enzyme synthesis and reusability of the cells. A comparative study (Fig. 3) proved that the Sr-alginate beads allow maximum enzyme production of 23.86U/mg at 8^{th} hour that can't be achieved by any beads in the present study. In case of strontium alginate beads enzyme production started at 4 hours with immobilized cells and reached a maximum level (23.86U/mg) by 8 hours. The production is nearly two fold higher in comparison with free cells at 6.5^{th} hour i.e 10.97U/mg.

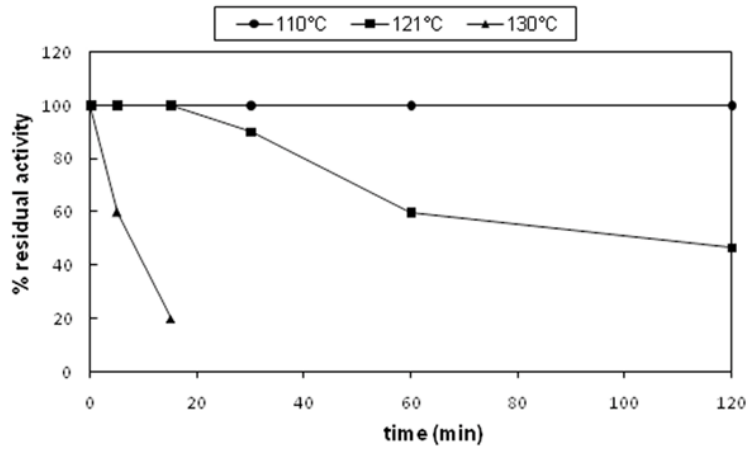

Fig. 2: Temperature stability profile of crude β amylase of *Bacillus subtilis* DJ5.

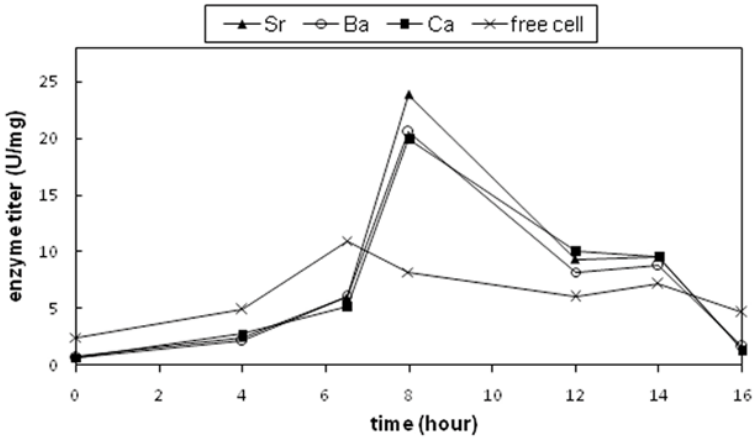

Fig. 3: Comparative study of β amylase production in different divalent cation-alginate beads & free cells.

Production of β Amylase by Immobilized Cells in Other Matrices:

Different entrapment matrices like polyacrylamide, gelatin, and agarose were also used for production of highly thermostable beta amylase. Fig. 4 represents comparative beta amylase production by different matrices over a time period of 16 hour.12% polyacrylamide entrapped cells showed significant production of enzyme at 6th hour that reaches at maximum of 18.67 U/mg at late hour of fermentation i.e. 16th hour. Among various concentration of agar tested, 5% agar entrapped cells started β amylase production at early stage at 2nd hour after incubation onward and reached a maximum level by 12 hours (9.59U/mg). Though there was no disintegration of matrix was recorded yet the enzyme titer is not sufficient as compared with free cell system.Glutaraldehyde, though toxic to cell, has been selected as good immobilizing agent. Glutaraldehyde, as a spacer group change the local surface area and avoid protein crowding surrounding cell and increases catalytic efficiency of cell. The results of this study were not promising. Detectable β amylase reached a maximum production level (2.19U/mg) at 12th hour that is not satisfactory.

Determination of Cell Leakage in Different Matrices:

Successful immobilization results in better productivity without cell disintegration from entrapped beads/blocks. Several different matrices used in this study indicated almost no cell leakage as confirmed spectrophotmetrically (Fig. 5). Only gelatin entrapped blocks showed disintegration after 12th hour. The results of bacterial growth were further supported by microscopic observation of matrix inoculated broth medium.

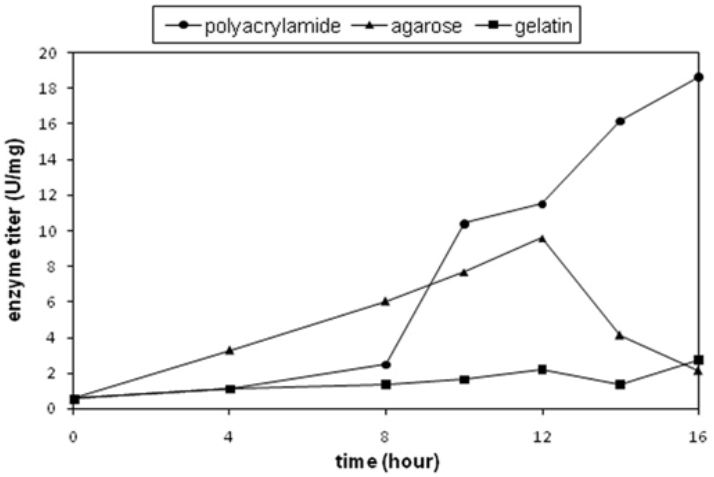

Fig. 4: Study of β amylase production by cells in different entrapment blocks.

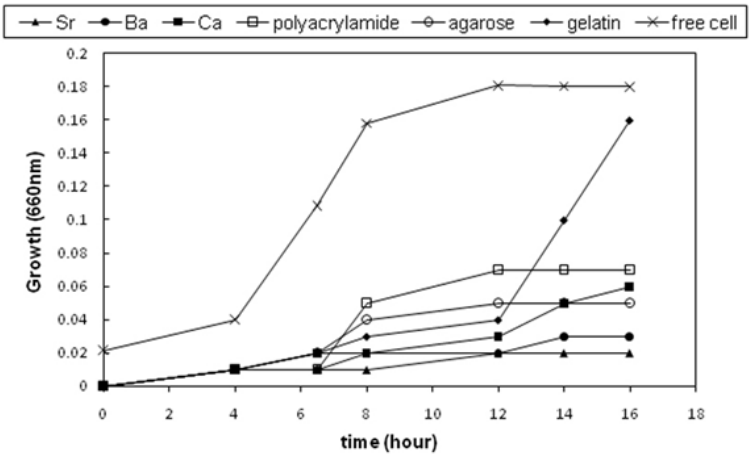

Fig. 5: Comparative study of growth of free & bound cells. Growth of free cells was divided by 10 units to compare the result with other.

Comparison of β Amylase Production by Immobilized Cells in Various Matrices by Entrapment Technique:

The beta amylase titer with immobilized cells in alginate beads was found to higher followed by polyacrylamide (Table 1). Between alginate beads, Sr-alginate beads showed the maximum productivity. Low level of β amylase production was observed in case of gelatin blocks. Probably glutaraldehyde (used for cross-linking with gelatin) was toxic for the cells. A low level to moderate production of β amylase was obtained with agarose that is not promising for Industrial purpose.

Table 1: Comparison of maximum β amylase production by *Bacillus subtilis* strain DJ5.

Sl. No.	Support matrix	Final pH	Specific activity of β amylase (U/mg)	Relative % production	Hour of maximum enzyme production
1	Sr-alginate	7.49	23.86	100	8
2	Ba- alginate	7.30	20.57	86.2	8
3	Ca- alginate	7.46	20.02	83.9	8
4	Polyacrylamide	7.22	18.67	78.2	16
5	Agarose	7.13	9.59	40.2	12
6	Gelatin	7.2	2.19	9.2	12
7	Free cells	6.32	10.97	45.97	6.5

Repeated Batch Fermentation with Strontium Alginate Entrapped Cell:

Due to higher volumetric production and higher physical and chemical stability of strontium alginate entrapped beads, it has been used to evaluate efficiency of repeated batch fermentation. Even after 9 cycles, strontium entrapped beads showed significant amount of β amylase titer (14 U/mg) without disintegration of beads (Fig. 6). After each cycle enzyme titer lowers very slowly. Further repetition after 10 cycles, cell leakage occurs. In order to confirm reproducibility of repeated batch study, this test was repeated three times under same condition.

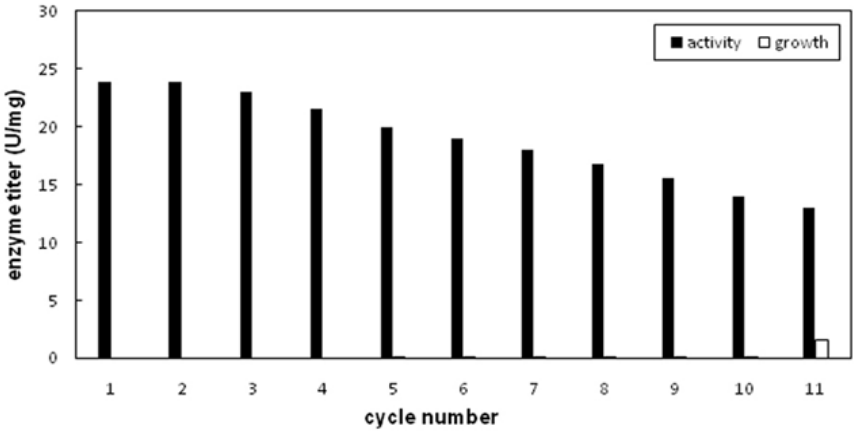

Fig. 6: Repeated batch study of strontium alginate entrapped beads for production of highly thermostable β amylase

Discussion:

With the morphological and biochemical tests followed by 16S rRNA analysis, the strain was identified as *Bacillus subtilis* strain DJ5 that has been found to produce highly thermostable β amylase active over 121°C. Though organism is mesophilic (as growth ceases above 42ºC), hyperthermostable enzyme production by the strain is drawing immense academic interest in this study.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 for several times (Adinarayana *et al.,* 2005). Immobilization study of whole cell for β amylase production has been done by very few scientists (Ray *et al.,* 1995) and they have found alginate as good immobilizing matrix (Kumar *et al.,* 2010). Though several procedures of immobilization used, our results showed that strontium alginate entrapment technique is best method for *Bacillus subtilis* DJ5 immobilization for highly thermostable β amylase production.Higher stability of strontium alginate in comparison to other beads, microenvironment surrounding the immobilized cells in comparison to experienced by their free cell counterparts and immobilization induced cellular or genetic modification helps strontium alginate beads to produce maximum production of enzyme titer in this work (Ramakrishna *et al.,* 2011; Kumar *et al.,* 2010). Lower stability of other alginate beads (Longo *et al.,* 1992), calcium and barium alginate, due to the presence of Na_2HPO_4 in the production media tends to dissolve the beads (Bajpai *et al.,* 2004), showed lesser titer of enzyme in comparison to strontium alginate beads (86.2% in barium alginate and 83.9% in calcium alginate).Moreover Sr-alginate beads permit strong physical and chemical stability avoiding the chance of leakage of cells or contamination in fermentation medium and also increase the reuse possibilities. This also economizes enzyme production by reducing the cost of downstream processing of final product.Polyacrylamide entrapped cells showed its highest activity (18.5 U/mg) at 16 hour of incubation and compared to free cell system, it increased to 32.5% in relative production. Agarose showed highest production at 12 hour of incubation which is actually a decreased production by 5.7% in relative production. The shifting of maximum enzyme production from 8 to 16 hour in polyacrylamide blocks or 8 to 12 hour in case of agarose blocks in comparison to any alginate beads might be explained as delayed permissible exchange of substrate, starch in this work, to the catalytic site because of the degree of cross linking of the gelling agent which varies with the used materials.Delayed exchange of substrate also reflects clearly with this work by the recorded highest enzyme activity at different time incubation for the different beads or matrices as strontium-alginate (at 8 hour of incubation time), polyacrylamide matrix (at 16 hour of incubation) and gelatin matrix (at 12 hour of incubation time).

Hindrances or obstacles for the substrates to stimulate the immobilized bacterial system for the enzyme production is directly proved by the late enzyme production by any type of beads or matrices compare to the free cell system. Lowest production of enzyme (19.2% relative activity) by gelatin matrix cells might be explained as glutaraldehyde prevent proper conformational changes (Krastanov, 1997) of the enzyme required for catalysis, showing less activity as enzyme titer in case of gelatin entrapped cells.

Conclusion:

Mutated strain of *Bacillus subtilis* DJ5 was found to produce novel hyperthermostable β amylase. Production of the hyperthermostable enzyme can be maximized by immobilizing cells of Bacillus subtilis DJ5 in 8% strontium alginate beads for industrial production of β amylase.

ACKNOWLEDGEMENT

This work was supported by *University Grants Commission* (UGC) (F. No. 33-214/2007 (SR)). Authors thank Dr. Subrata K Das for his valuable assistance and Principal, Bidhannagar College for providing all sorts of Infrastructural facility for completion of this work.

REFERENCES

Adinarayana, K., B. Jyothi, P. Ellaiah, 2005. Production of alkaline protease with immobilized cells of *Bacillus subtilis* PE-11 in various matrices by entrapment technique. AAPS Pharma Sci Tech, 6: 391-397.

Altschul, S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res., 25: 3389-3402.

Atia, K.S., S.A. Ismail, M.B. El-Arnaouty, A.M. Dessouki, 2003. Use of co-immobilized beta-amylase and pullulanase in reduction of saccharification time of starch and increase in maltose yield. Biotechnol Prog, 19: 853-857.

Bajpai, S.K., S. Sharma, 2004. Investigation of swelling/degredation behavior of alginate beads crosslinked with Ca^{2+} and Ba^{2+} ions. React Functional Polymers, 59: 129-140.

Bernfeld, P., 1955. Amylase α and β. Methods in Enzymology, 1: 149-150.

Das, S.K., A.K. Mishra, B.J. Tindall, F.A. Rainey, E. Stackebrandt, 1996. Oxidation of thiosulfate by a new bacterium,*Bosea thiooxidans*(strain BI-42) gen. nov., sp. nov.: analysis of phylogeny based on chemotaxonomy and 16S ribosomal DNA sequencing.Int J Syst Bacteriol, 46: 981-987.

Declerck, N., M. Machius, R. Chambert, G. Wiegand, R. Huber, C. Gaillardin, 1997. Hyperthermostable mutants of *Bacillus licheniformis* α-amylase:thermodynamic studies and structural interpretation. Protein Eng, 10: 541-549.

Garrity, G.M., J.A. Bell, T. Lilburn, 2005. The revised road map to the Manual. In Brenner, Krieg, Staley and Garrity (ed.), Bergey's Manual of Systematic Bacteriology, 2, The Proteobacteria, Part A, Introductory Essay. Springer, New York, pp: 159-220.

Goyal, N., J.K. Gupta and S.K. Son, 2005. A novel raw starch digesting thermostable α-amylase from*Bacillus*sp. I-3 and its use in the direct hydrolysis of raw potato starch. Enzyme and Microbial Technology. 37: 723-734.

Haki, G.D., S.K. Rakshit, 2003. Developments in industrially important thermostable enzymes: a review. Bioresource Technology, 89: 17-34.

Higashihara, M., S. Okada, 1974. Studies on β amylase of *Bacillus megaterium* No. 32. Agric Biol Chem, 38: 1023-1029.

Hyun, H.H., J.G. Zeikus, 1985. General biochemical characterization of thermostable extracellular β-Amylase from *Clostridium thermosulfurogenes*. Appl Environ Microbiol, 49: 1162-116.

Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution. 16: 111-120.

Krastanov, A., 1997. Continuous sucrose hydrolysis by yeast cells immobilized to wool. Applied Microbiol and Biotechnol, 47: 476-481.

Kumar, R., R. Vats, 2010. Protease production by *Bacillus subtilis* immobilized on different matrices. New York Science Journal, 3: 20-24.

Leuschner, C., G. Antranikian, 1995. Heat-stable enzymes from extremely thermophilic and hyperthermophilic microorganisms. World J Microb Biotechnol, 11: 95-114.

Leveque, E., S. Janecek, B. Haye, A. Belarbi, 2000. Thermophilic archeal amylolytic enzymes. Enz and Microbial Technol, 26: 3-14.

Longo, M.A., I.S. Novella, L.A. Garcia, M. Diaz, 1992. Diffusion of proteases in calcium alginate beads. Enzyme Microb Technol, 14: 586-590.

Lowry, H.O., N.J. Rosebrough, A.L. Farr, R.J. Randall, 1951. Protein measurement with the Folin phenol reagent. J Biol Chem, 193: 265-275.

Niehaus, F., C. Bertoldo, M. Kahler, G. Antranikian, 1999. Extremophiles as a source of novel enzymes for industrial application. Appl Microbiol Biotechnol, 51: 711-729.

Obi, S.K., J.C. Odibo, 1984. Partial purification and characterization of a thermostable actinomycetes β amylase. Appl Environ Microbiol, 47: 571-575.

Panday, D., S.K. Das, 2010. *Chelatococcus sambhunathii*sp. nov., a moderately thermophilic alphaproteobacterium isolated from hot spring sediment.Int J Syst Evol Microbiol, 60: 861-865.

Rakshit, S.K., 1998. Utilization of starch industry wastes. In: Martin, A (Ed.), Bioconversion of waste materials to industrial products, 2nd Edn, Blackie Academic and professional, London.

Ramakrishna, S.V., R.S. Prakasham, 1999. Microbial fermentation with immobilized cells. Curr Sci, 77: 87-100.

Ray, R.R., G. Nanda, 1996. Microbial β amylases: Biosynthesis, Characteristics, and industrial applications. Crit Rev Microbiol, 22: 181-199.

Ray, R.R., S.C. Jana, G. Nanda, 1995. Beta-amylase production by immobilized cell of *Bacillus megaterium* B6. J Basic Microbiol, 35: 113-116.

Saitou, N., M. Nei, 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4: 406-425.

Saville, B.A., M. Khavkine, G. Seetharam, B. Marandi, Y.L. Zou, 2004. Characterization and performance of immobilized amylase and cellulase. Appl Biochem Biotechnol, 251: 113-116.

Shen, G.J., B.C. Saha, Y.E. Lee, L. Bhatnagar, J.G. Zeikus, 1988. Purification and characterization of a novel thermostable beta amylase from *Clostridium thermosulphurogenes.* Biochem J, 254: 835-840.

Sneath, P.H.A., 1986. Endospore forming gram positive rod and cocci. In: Bergey's Manual of Systematic Bacteriology, Vol 2, Williams & Wilkins, Baltimore pp: 1104-1105.

Tamura, K., J. Dudley, M. Nei, S. Kumar, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution, 24: 1596-1599.

Thomas, M., F.G. Priest, J.R. Stark, 1980. Characterization of an extracellular beta amylase from *Bacillus megaterium sensu stricto*. J Gen Microbiol, 118: 67-72.

Uludag, H., P.D. Vos, P.A. Tresco, 2000. Technology of mammalian cell encapsulation. Advanced Drug Delivery Reviews, 42: 29-64.

Van der Maarel, M., B. Van der Veen, H. Uitdhaag, H. Leemhuis, L. Dijkhuizen, 2002. Properties and application of starch converting enzymes of α amylase family. J Biotechnol, 94: 137-155.