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IMMOBILIZATION OF NITROGEN-FIXING *BACILLUS FLEXUS STRAIN* BLY01 FOR ENHANCED SYNTHESIS OF α-AMYLASE AND ALKALINE PHOSPHATASE

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ABSTRACT

A nitrogen-fixing gram positive spore forming bacteria was isolated from potato waste and produced α-amylase and alkaline phosphatase (APase) when the organism had been fed on starch and insoluble $Ca_2(PO_4)_3$ in nitrogen free medium. The organism was identified as *Bacillus flexus* BLY01 on the basis of morphological as well as phylogenetic analysis using 16S rDNA sequencing. Under optimized conditions in broth culture, fresh cells produced about 56 unit/ml of α-amylase and 16.75 unit/ml of alkaline–phosphatase. The log phase cells of strain BLY01 had been immobilized in Ca-alginate gel capsules and were used for the semi-continuous production of the substrate specific enzymes individually. The immobilized cells showed about 27% and 126% enhancement in activity of α-amylase and APase, respectively. The immobilized system retained 80% - 84% of its initial efficiency after 3rd cycle of fermentation batches.
One unit of α-amylase activity = 10 µg of reducing sugar per ml per min from soluble starch and one unit of APase =10 µg of para-nitrophenol formed per ml per min at 37^0C at pH 8.0.

Keywords: α-Amylase, alkaline phosphatase (APase), *Bacillus flexus*, immobilization, 16rDNA sequencing

INTRODUCTION

The application fields of microbial extracellular malto-oligosaccharide forming α-amylase and alkaline phosphatase (APase) are extremely wide and variable. The α-amylase (EC 3.2.1.1), which is mainly used as a thinning agent in starch hydrolysis, is widely applied in food, paper, textile, and pharmaceutical industries (**Pandey *et al.*, 2000; Konsoula and Liakopoulou-Kyriakides, 2004; Yang *et al.*, 2005**). APase (orthophosphate monoester phosphohydrolases, EC 3.1.3.1.) is the metalloenzyme, nonspecifc, phosphomonoesterases. Phosphatases are the most crucial enzymes for survival of organism that hydrolyze phosphate esters and provide inorganic phosphate (Pi). APase has wide range of applications in the field of disease diagnosis, biotechnology, immunology and molecular biology and serve as biochemical marker for quantitative measurement of disease (**Muginova *et al.*, 2007**). This enzyme has also immense application in agricultural fields for mineralization of insoluble phosphates present in soil and introduced as fertilizers and pesticide (Šarapatka, 2003) Though these enzymes exist in various organisms from bacteria to mammals; however, the microbial enzymes are more appreciable due to their effectiveness, specificity, biocompatibility, biodegradability and also from the economic view point (**Singh Nigam, 2013**).
The technique of cell immobilization has become a common practice for biotechnologists and industrial microbiologists. It has several advantages over fresh cell cultivation from the view points of product separation, re-use of biocatalysts, prevention of washout, reduced risk of contamination and operational stability (**Tanaka and Kawamoto, 2006**). The high density of microbial cells in beads makes them more efficient to show the enzymatic activity and productivity under specific and controlled environment. In comparison to various types of immobilization, the entrapment method using calcium alginate is easier, non-toxic and inexpensive (**Goksungur and Zorlu, 2001**).
The organism *Bacillus flexus* is known to synthesize α-amylase (**Zhao *et al.*, 2008**) and β- amylase (**Amano *et al.*, 2009**) but it has also been recorded as a producer of multiple enzymes like amylase, lipase and protease (**Tambekar and Dhundale, 2013**). Very few reports of APase synthesis by the *B. flexus* has been cited till date (**Patel and Sharma, 2012, 2013**). In the present study, cells of *Bacillus flexus* BLY01 has taken for calcium alginate immobilized bead preparation. The enzymatic efficiency of the fresh cells and immobilized cells to hydrolyze starch and *p*-nitrophenyl phosphate (*p*-NPP) has been investigated. The activities after 1st, 2nd and 3rd cycle of batch fermentation were compared, if any, at the same fermentation condition.

MATERIAL AND METHODS

Microorganisms, cultivation and storage

The organism *Bacillus flexus* BLY01 has been isolated from potato cold storage waste material collected from Bally, district Howrah, West Bengal, India. Enrichment culture following serial dilution and spread plate techniques the bacterial strain has been isolated on modified Stockdale nitrogen free medium having pH 7.7 (**Stockdale *et al.*, 1968**) containing starch, 1% (w/v) and calcium phosphate 0.2% (w/v) instead of glucose and dipotassium hydrogen phosphate, respectively. The organism was grown at 37^0C under shake flask condition (180 rpm) for 24h in the said broth medium and was stored on the agar slant at 4^0C.

Sequencing of 16S rDNA and identification of strain

Genomic DNA was extracted from the 24h grown culture using Genei Ultrapure TM Bacterial Genomic DNA Purification kit KT162 (Cat # 612116200021730). The universal primer sequence used for the amplification of 16s rDNA were: the Forward primer -- 5'-AGAGTTTGATCMTGGCTCAG -3' and the Reverse primer - 5' -TACGGYTACCTTGTTACGACTT-3'. Using primers, the ~1.5 kb 16S rDNA fragment was amplified using *Taq* DNA polymerase (3U). The final PCR mix was composed of genomic DNA: ~20ng; dNTP mix (2.5mM each): 1.0µl; Forward Primer: 100ng; Reverse Primer: 100ng; Taq Buffer A (10X): 1X; Taq Polymerase enzyme: 3U; $MgCl_2$: 25 mM; Glass distilled water: to make up the volume 50µl. The program for PCR was as follows: 94^0C for 5 min, 35 cycles of 94^0C for 30 Sec., 55^0C for 30 Sec, and 72^0C for 1.30 min, and extension at 72^0C for 10 min. Amplification was done using Perkin Elmer Gene Amp PCR system 2400. The PCR product was bi-directionally sequenced using the forward, reverse and an internal primer. Amplicons were visualized by electrophoresis on 1% agarose gel with StepUpTM 500bp DNA ladder (Cat# 612651970501730) and after staining with ethidium bromide. The Sequence data was aligned and analyzed for finding the closest homologous microbes using combination of the database of National Center for Biotechnology Information (NCBI) GenBank and ribosomal database project (RDP) website.

Nucleotide sequence accession number

The 16S rDNA partial sequence of the strain *B. flexus* BLY01 was deposited in the NCBI Gene Bankit nucleotide sequence database under accession number KF000098.

Determination of growth and cell number in bead

The growth of the organism was determined following the total count method using a haemocytometer and the cell number present in bead was confirmed after bursting of beads following the viable cell count method on fresh agar plate. The starch phosphate broth was inoculated with growing BLY01 cells of 12h at 4% (v/v) level and was incubated up to 35 h at 37^0C under shake flask condition (140 rpm) for fermentation.

Immobilization of cells

The fresh cells were harvested aseptically by centrifugation (10,000 rpm) at 4 ^{0}C and taken for immobilization following the method of **Kierstan and Bucke (1977**) with some modification. About 100 mg of fresh biomass was well mixed to the 50 ml of sodium alginate solution (4%, w/v). The mixture obtained was extruded drop wise through 0.1 ml fine tip pipettes into a 100 ml sterile $CaCl_2$ solution of 0.1M, used as cross linking agent. Alginate drops (approx. 2mm-3mm) were solidified in contact with $CaCl_2$ and thus entrapping the bacterial cells. Repeated washing and incubation in the same solution at 10^0C made the beads more hard and stable. Finally, the beads were washed with sterile saline to remove excess calcium ions. After determination of beads volume (approx. 0.01 - 0.16 cc) it was found that average bead contained about 0.02 - 0.03 mg of biomass.

Fermentation with immobilized cells

Fresh immobilized beads prepared following the above method contained approximately 0.02 mg of biomass each (calculated from the total volume of gel mixture and the amount of cells added to it) were suspended in starch-buffer solution (pH 8.0) for amylase activity determination. The phosphatase enzyme activity of immobilized cells was determined in glycine- NaOH buffer of pH 8.0 (pH optima for the enzyme). Beads were incubated for different time period and for 1-3 time cycles at 37 ^{0}C.

Analytical methods

Determination of extracellular amylase activity

The amylase enzyme activity was detected following chemical estimation of reducing sugar. The sugar produced in culture filtrate was assayed using di-nitrosalicylic acid reagent (**Miller, 1959**). Activity of the enzyme produced by the strain, fresh cells and immobilized cells was measured in units (U). One unit of α-amylase activity is defined as the amount of enzyme that releases 10 µg of reducing sugar per ml per min from soluble starch at pH 8.0.

Determination of extracellular APase activity

For determination of the extracellular enzymatic activity in the respective fermentation set, the supernatant was assayed calorimetrically (Systronics-166) following the method of Bernt (Bernt, 1974) in glycine-NaOH buffer (pH 8.0) at 37^0C using the substrate di-sodium salt of *para*- nitrophenyl phosphate (*p*-NPP), Sigma USA. One unit of phosphatase =10 µg of para-nitrophenol (*p*-NP) formed per ml per min at 37^0C**.**

RESULTS AND DISCUSSION

16S rDNA sequence similarity and phylogenetic analysis of the organism

Molecular identification of the isolated gram positive sporulating bacilli was carried out based on its 16S rDNA sequence analysis. The 1.5 kb sequences obtained from the strain (Fig.1) were aligned with all the presently available 16S rDNAsequences in the GenBank and RDP database. As a result, a phylogenetic tree was constructed using the neighbor joining method, and is shown in Fig.2. Phylogenetic analysis using the 16S rDNA sequences indicated that the organism was a strain of *Bacillus* and it had 99% 16S rDNA sequence-similarity with the *B. flexus* strain LF-3, strain SL21 and strain FFA4 (Table1).

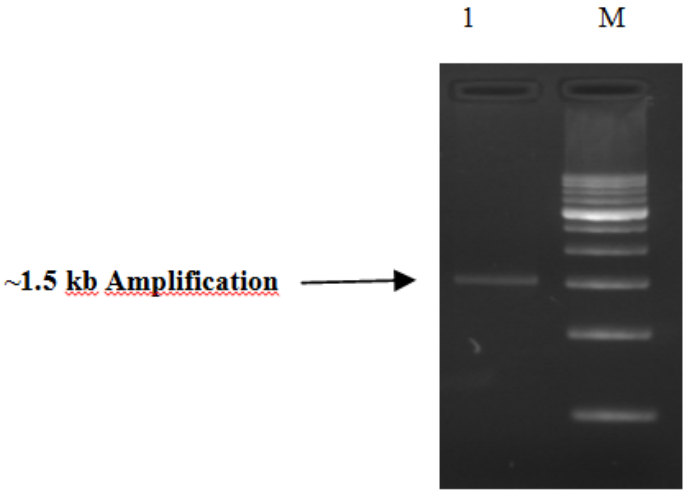


Figure 1 PCR amplification. The PCR products (~1.5 kb) were loaded on 1.0% agarose gel along with StepUp^{TM}500bp DNA ladder. Lane 1: PCR Amplification of 16srDNA sample from BLY01 and Lane M1: DNA ladder

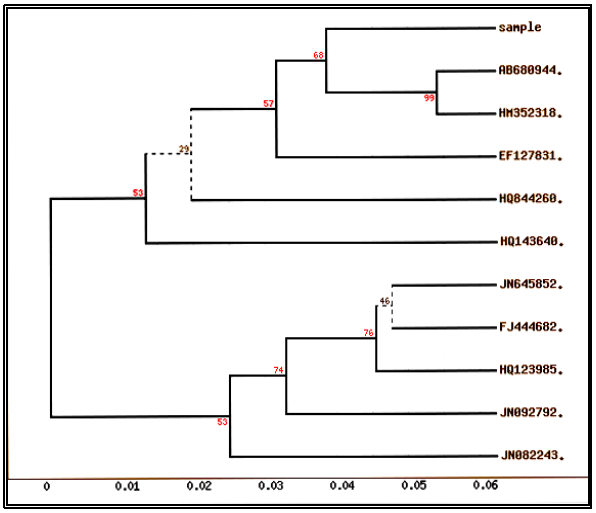


Figure 2 Phylogenetic tree based on partial 16S rDNA sequences, showing the relationship between the isolated strain BLY01 and other species belong to the genus *Bacillus*. The ID numbers of organisms are in parentheses. The tree was constructed using the neighbour-joining method. Numbers at nodes indicate levels of bootstrap support (%) determined from resampled data.

Table1 Alignment view of strain BLY01 using combination of NCBI GenBank and RDP database

Alignment View	ID	Alignment results	Sequence Description
	BLY01	1.00	Studied Sample
	HQ844260.1	0.99	*Bacillus sp. AS6*
	HQ143640.1	0.99	*Geobacillus stearothermophilus strain*
	EF127831.1	0.99	*Bacillus flexus strain LF-3*
	JN645852.1	0.99	*Bacillus flexus strain SL21*
	JN082243.1	0.98	*Bacillus sp. ce45*
	AB680944.1	0.98	*Bacillus flexus gene*
	JN092792.1	0.99	*Bacillus flexus strain FFA4*
	HM352318.1	0.98	*Bacillus sp. CmNA4*
	HQ123985.1	0.99	*Bacillus sp.*
	FJ444682.1	0.99	*uncultured Bacillus sp. clone 2y-54*

Growth and synthesis of enzymes

ime scale study of α- amylase activity of the BLY01 cells (Plate1) resulted in the accumulation of utilizable reducing sugar in the medium and enzyme increased steadily along with growth up to 25h of the incubation period and thereby decreased in the late phase of growth (Fig. 3). The maximum amylase synthesis (56.4 unit/ml) was shown by the stationary phase cells of the strain. Synthesis APase had started at an early stage of growth and continued to attain the maximum concentration of 16.5 unit/ ml after 30 h during the late stationary phase of growth, followed by a slow decline of the activity.

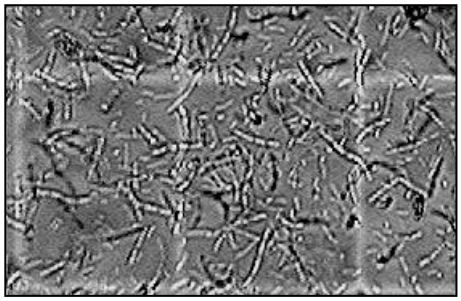


Plate 1 Cells of *B. flexus* BLY01 at their log phase before the onset of sporulation. Cells were grown in modified Stockdale medium under shake flask condition at 37^{0}C.

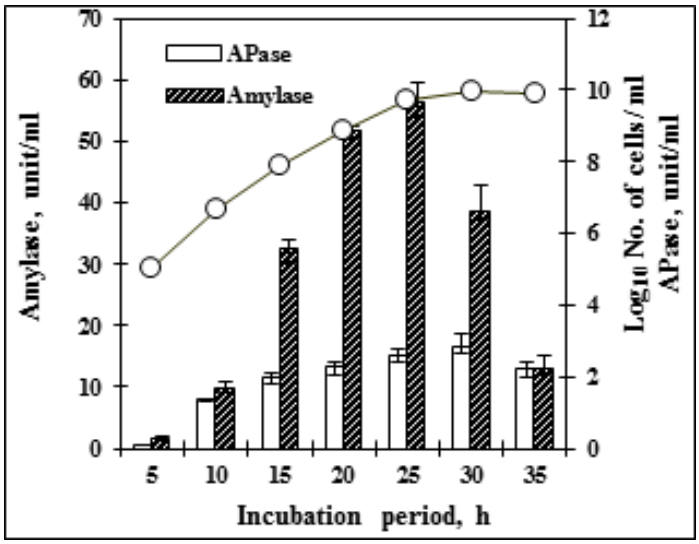
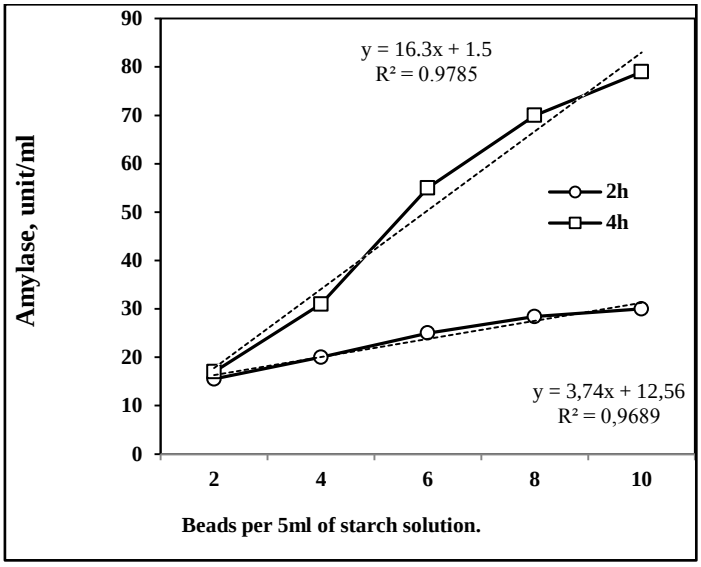


Figure 3. Time course depiction of growth as $\log_{10}$ number of cells per ml (-o-), α -amylase (▨) and APase (□) synthesis by *B. flexus.* BLY01 cells in starch–calcium phosphate medium under shake flask condition at 37^{0}C.

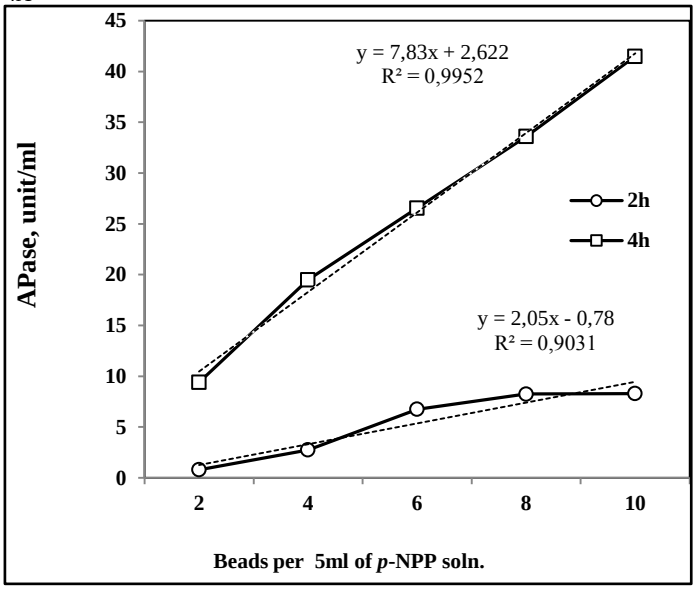
Fermentation with immobilized cells

For both of the amylase and Apase enzymes synthesis much quicker response (within 2 and 4 h) of immobilized cells (Plate2), compared to the fresh cells, were observed when the supernatant was taken for assay after beads incubation. Maximum enzymatic activities were found in the supernatants taken from 10 beads per 5ml fermentation sets (Fig.4 Aand B) *i.e.* 79 units and 41.5 units of amylase and APase respectively in per unit volume of reaction mixtures.

Plate 2 Fresh Ca-alginate beads containing *B. flexus* cells. Beads were prepared following modified method of Kierstan and Bucke (1977).



4A



4B

Figure 4A and 4B. Effect of beads number and incubation period on enzyme activities of *B. flexus* BLY01. Cells were immobilized in calcium alginate gel and incubated in starch solution at 37 ^{0}C under shake flask condition at 80 rpm. **Figure 4A** Amylase activity. **Figure 4B** APase activity

Repeated fermentation with Immobilized cells

The respective beads after a cycle of fermentation for 4 h were transferred aseptically to the fresh starch-buffer and to the *p*- nitrophenol phosphate buffer solution for determination of α- amylase and APase activity respectively and the process was repeated up to the third fermentation batch cycle considering it as the semi-continuous fermentation with immobilized cells of the strain BLY01. After each cycle of fermentation the efficiency of activity of either of the enzymes was reduced to some extent (Table 2). However, 84% and 80% of activity was retained in the immobilized cells for of α-amylase and APase, respectively, after 3rd fermentation cycle. Data from these incubations, however, were still higher than the activity of free cells of similar amount.

Table 2 Efficiency of free and immobilized cells of *B. flexus* BLY01 at different fermentation cycle.

Fermentation with	Amylase, unit/ml*	APase, unit/ml**
Free cells as high cell density culture (0.1 mg/5ml approx.)	56.8	16.5
Immobilized beads, 1st cycle	72.2	37.4
Immobilized beads, 2nd cycle	68.5	35.0
Immobilized beads, 3rd cycle	61.0	29.6

Legend: Beads were incubated and transferred aseptically. In each case 5 beads were incubated in 5 ml fermentation culture for 4 h at 37^{0}C under shake flask condition at 80 rpm.
*Amylase activity was determined following the method of **Miller (1959).**
APase activity was determined following the method of **Bernt (1974).

DISCUSSION

The gram-positive bacterium *Bacillus* is capable of secreting large amounts of endogenous proteins as enzymes into the extracellular medium. The strains of *Bacillus* have been reported as able producer of α-amylase (**Dey *et al*., 2003; Kumar *et al*., 2012**) as well as APase enzyme (**Bookstein *et al*., 1990; Mahesh *et al*., 2010**) as their primary metabolites in presence of the respective substrates. It is evident that the secretion activity is rather low during organisms' exponential growth and increases substantially at the onset of stationary phase (**Priest, 1977**) which corroborates with the findings derived from the strain *B. flexus* BLY01 fresh cells (Fig.3). The sharp decline in the amylase activity by the strain BLY01 in its late phase had been depicted which might be due to the presence of a negligible amount of residual starch in the growth medium, compare to that of in the early phase of culture. Similarly, the data generated during determination of APase activity the strain BLY01 showed the repressive effect after the period when cells entered the stationary phase at 30th hour of incubation. It may happen due to the shifting of pH of the medium from alkaline to acidic (data not shown) as the results of accumulation of cellular metabolites and excess inorganic phosphate ions derived from APase activity.

The results of the amylase and APase activities by the cells of BLY01 entrapped in calcium alginate beads were found excellent. The same beads seemed to be the effective for either α- amylase activity or for APase activity. The proportional increment of enzyme activity with increase in immobilized bead number per volume of substrate-buffer solution had been noted. The activity of enzymes also increased with duration of incubation at 37^0C. Similar enhancement in amylase synthesis has been described using immobilized beads of *B. macerans* (Ahmed, 2008) and cellulose synthesis by immobilized *B. pumilus* EWBCM1 (**Kumar *et al*., 2012**). More than 27% and 126% increments in the efficiency of α-amylase and APase respectively, were observed from the results obtained in the first cycle cultivation of immobilized BLY01 cells compare to those of same amount fresh cells activity. Similar enhancements in the amylase activity were described in case of *B. circulans* (**Dey *et al*., 2003**) and *B. subtilis* (**Konsoula and Liakopoulou-Kyriakides, 2006**) when cells were immobilized in Ca-alginate gel. However, in batch cultivation APase activity of the beads become limited in presence of inorganic phosphate ions which affect the integrity of calcium alginate beads (**Han *et al*., 2011**) unless the product is removed from the reaction site.

As the microbial enzymes amylase and APase as well have immence importance in food and pharcutical industries (**de Souza** and **de Oliveira Magalhães, 2010; Raja *et al*., 2011; Christopher and Kumbalwar, 2015),** the technology of cell immobilization of *Bacillus flexus* BLY01 is aptly suited for dual enzymes synthesis using the same beads for quicker and vigor action.

CONCLUSION

The organism *Bacillus flexus* BLY01 thus proved to be a potent source for any of the enzymes α-amylase and APase which showed higher efficiency as entrapped cells in calcium alginate gels specially for APase activity and there was a minimum loss after 3rd cycle of fermentation. This type of reusable immobilized cells could be beneficial as stable and commercially viable biotechnological tool for biocatalyst activity.

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REFERENCES

AHMED, S.A. 2008. Invertase production by *Bacillus macerans* immobilized on calcium alginate beads. *Journal of Applied Science and Research*., 4, 1777-1781.

AMANO, H., MATSUNAGA, A., YAMAGUCHI, S. 2009. β-amylase, gene coding and manufacturing method. US patent 20110059491 A1. Endorsed on 10 March

BERNT, E. 1974. Alkaline Phosphatase in Milk. In *Methods on Enzymatic analysis*,. Vol. II 868-870 p. Academic Press, Inc., New York, NY. ISBN: 978-0-12-091302-2. http://dx.doi.org/10.1016/b978-0-12-091302-2.50070-0.

BOOKSTEIN, C., EDWARDS, C.W., KAPP, N.V., HULETT, F.M. 1990. The *Bacillus subtilis* 168 alkaline phosphatase III gene: impact of a pho AIII mutation on total alkaline phosphatase synthesis. Journal of Bacteriology, 172, 3730–3737. PMCID: PMC213351 http://dx.doi.org/0021-9193/90/073730-08$02.00/0.

CHRISTOPHER, N., KUMBALWAR, M. 2015. Enzymes used in Food Industry A Systematic Review. *International Journal of Innovative Research in Science, Engineering and Technology*, 4 (10): 9830-9836. http://dx.doi.org/10.15680/IJIRSET.2015.0410073.

DE SOUZA P.M., DE OLIVEIRA MAGALHÃES P. 2010. Application of microbial α-amylase in industry. *Brazilian Journal of Microbiology*, 41(4):850-61. http://dx.doi.org/10.1590/S1517-83822010000400004.

DEY, G., SINGH, B., BANERJEE, R. 2003. Immobilization of α-Amylase Produced by *Bacillus circulans* GRS 313. *Brazilian Archives of. Biology and Technol*ogy, 46, 167-176. http://dx.doi.org/10.1590/s1516-89132003000200005.

DHANASEKARAN, D., SIVAMANI, P., RAJAKUMAR, G., PANNEERSELVAM, A., THAJUDDIN, N. 2006. Studies on free and immobilised cells of *Bacillus* species on the production of alpha-amylase *The Internet Journal of Microbiol*ogy, 2 (2), 70-76. http://dx.doi.org/10.5580/18bf.

GOKSUNGUR, V., ZORLU, N. 2001. Production of ethanol from beet molasses by Ca-alginate immobilized yeast cells in a packed-bed bioreactor. *Turkey Journal of Biol*ogy, 25, 265–275.

HAN, Y.U., LEE, W.S. , LEE, C.G. , PARK, S.J., KIM, K.W., KIM, S.B. 2011. Entrapment of Mg-Al layered double hydroxide in calcium alginate beads for phosphate removal from aqueous solution. *Desalination and Water Treatment* 36, 178-186. http://dx.doi.org/10.5004/dwt.2011.2254.

KONSOULA, Z., LIAKOPOULOU-KYRIAKIDES. M. 2004. Hydrolysis of starches by the action of an α-amylase from *Bacillus subtilis*. *Process Biochemistry* 39, 1745–1749. http://dx.doi.org/10.1016/j.procbio.2003.07.003.

KUMAR, R.S., SHANKAR, T., ANANDAPANDIAN, K.T.K., ISAIARASU, L. 2012. Effect of *Bacillus pumilus* EWBCM1 whole cell immobilization in various matrices on cellulase enzyme production and saccharification of sugarcane bagasse. American-Eurasian *Journal of Agriculture and. Environmental Science*, 12 No.1, 128-132.

MAHESH, M., GULERIA, N., RAJESH,.T.S., SOMASHEKHAR, R. PUTTAIAH,.E.T. 2010. Isolation and characterization of extracellular thermostable alkaline phosphates enzyme from *Bacillus spp*. *International Journal of Applied Biology and Pharmaceutical Technology*, 1, 22-33.

MILLER, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 31, 426-429. http://dx.doi.org/*10.1021/ac60147a030*.

MUGINOVA, S.V., ZHAVORONKOVA, A.M., POLYAKOV, A.E., SHEKHOVTSOVA, T.N. 2007. Application of alkaline phosphatases from different sources in pharmaceutical and clinical analysis for the determination of their cofactors; zinc and magnesium ions. *Analytical Sciences*, 23, 357-363. http://dx.doi.org/10.2116/analsci.23.357.

PANDEY, A., NIGAM, P. SOCCOL, C.R., SOCCOL, V.T., SINGH, D., MOHAN, R. 2000 Advances in microbial amylases. *Biotechnology and Applied. Biochemistry*, 31, 135–152. http://dx.doi.org/10.1042/ba19990073.

PATEL, R.F., SHARMA, M.C. 2012. Effect of different parameters on alkaline Phosphatase productivity from facultative alkaliphile *Bacillus flexus* FPB17 isolated from an alkaline lake sediments in India. *Asian Journal of Microbiology, Biotechnology and Environmental Science*, 14, 451-456.

PATEL, R.F., SHARMA, M.C. 2013. Optimization of metal salt requirement for production of alkaline phosphatase by a halotolerant facultative alkaliphile *Bacillus flexus* FPB17 using response surface methodology. *International Journal Agricultural Technol*ogy, 9, 81-93.

PRIEST, F.G. 1977. Extracellular enzyme synthesis in the genus *Bacillus*. *Bacteriological Review*, 41**,** 711-753.

RAJA, M.M.M., RAJA A. IMRAN M.M, SANTHA A.M.I., DEVASENA, K. 2011. Enzymes Application in Diagnostic Prospects. *Biotechnology*, 10: 51-59. http://dx.doi.org/ 10.3923/biotech.2011.51.59.

ŠARAPATKA, B. 2003. Phosphatase activities (ACP, ALP) in agroecosystem soils. Doctoral thesis. ISSN 1401-6249, ISBN 91-576-6403-X

SINGH NIGAM, P. 2013 Microbial enzymes with special characteristics for biotechnological applications. *Biomolecules*,. 3, 597-611. http://dx.doi.org/10.3390/biom3030597.

STOCKDALE, H., RIBBONS, D.W., DAWES, E.A. 1968. Occurance of poly-β–hydroxybutyrate in Azotobacteriaceae, *Journal of Bacteriol*ogy 95, 1798-1803.

TAMBEKAR, D.H., DHUNDALE, V.R. 2013. Multienzyme producing halophilic bacteria from Lonar lake. *International Journal of Pharma and Biosciences*, 4(B), 1271 – 1276.

TANAKA, A., KAWAMOTO, T. 1999. Cell and enzyme immobilization. In Manual of industrial microbiology and biotechnology. 2nd ed. Washington, DC: ASM Press; 94–102 p. ISBN 1-55581-128-0. http://dx.doi.org/10.1002/abio.370200110.

YANG, R.W., SHAO, Z.X., CHEN, Y.Y., YIN, Z., WANG, W.J. 2005. Lipase and pancreatic amylase activities in diagnosis of acute pancreatitis in patients with hyperamylasemia. *Hepatibiliary Pancreatic Disease.Internatinal.* 4, 600-603.

ZHAO, J, LAN, X., SU, J., SUN, L., RAHMAN, E. 2008. Isolation and identification of an alkaliphilic *Bacillus flexus* XJU-3 and analysis of its alkaline amylase. *Wei Sheng Wu Xue Bao*, 48(6), 750-756.