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Characterization of Alpha Amylase from *Bacillus* sp.1 isolated from paddy seeds

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ABSTRACT

Objective: This work was conducted with the aim of isolating promising α-amylase producing bacteria from different sources, using both solid-state and submerged fermentations.

Methodology and results: Two bacterial species were isolated from paddy seeds as promising candidates. The most promising one, *Bacillus* sp.1, was identified under submerged fermentation (SmF) and further characterized. Different carbon and nitrogen supplements were used to enhance production of the amylase enzyme. The maximum amount of enzyme was obtained with supplements of 1% starch or urea. Further attempts to enhance α-amylase production by *Bacillus* sp.1 were assessed by UV induced mutagenesis. One of the mutants (*Bacillus* sp.1m20) showed increased enzyme activity than the parental strain, *Bacillus* sp.1. However, the survival rate of this mutant decreased with increased duration of UV exposure. Partial purification of the enzyme using ammonium sulphate fractionation resulted in 1.06 fold increase in the enzyme activity. The enzyme had a molecular weight of ~ 40KDa by SDS-PAGE. The enzyme activity, which was optimal at 30°C and pH 6.8, was increased by the presence of Ca^{2+} and Co^{2+} ions.

Application of findings: The results suggest that *Bacillus* sp.1m20 could be exploited for industrial production of alpha amylase at relatively low temperatures.

Keywords: alpha amylase, *Bacillus* sp.1, submerged fermentation, mutagenesis, partial purification.

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Introduction

Amylases are produced in a variety of living organisms like bacteria, fungi, plants and animals. Microbial amylases are used for industrial purposes (Alva *et al*., 2007). Using current technology, microbial amylases are commercially produced and these synthetic types have completely replaced chemical hydrolysis of starch in industries. With the discovery of new strains of microorganisms and development of more efficient production strategies, microorganisms have substantial potential to contribute to a number of industrial applications. Such industrially important microorganisms are found within the *Bacillus*

species because of their rapid growth rates that lead to short fermentation cycles, their capacity to secrete proteins into extra cellular medium, and general handling safety (Pandey *et al*., 2001). However, catabolic repression of enzyme synthesis has been reported in submerged fermentations (SmF) of bacteria and fungi (Tomanga 1966; Heinekes *&* O'Connor 1972). This poses serious problems to the economics of SmF for the production of alpha-amylase and other enzymes (Emanuilova & Toda, 1984). However, solid-state fermentation (SSF) significantly overcomes catabolite repression of alpha amylase production by *Bacillus licheniformis* M 27 (Ramesh *&* Lonsane, 1991).

Amylases have potential application in several industrial processes, e.g. food, fermentation, detergent, textile, pharmaceutical, chemical, brewing and paper industries (Moreira *et al*., 1999; 2001; Kathiresan *&* Manivannan, 2006). In earlier days, amylase was produced by submerged fermentation (SmF) technique (Pandey *et al*., 2001). However, in recent years solid-state fermentation (SSF) process has been used more extensively, as it is more advantageous than SmF in processes using crude enzyme. Agricultural and industrial wastes are considered as good substrates for SSF to produce enzymes (Kunamneni *et al*., 2005).).

The objective of the present study was to isolate bacteria from different sources and screen them for the production of α-amylase, select the best one, optimize the culture conditions for enzyme production and characterize the enzyme for industrial applications.

Materials and Methods

Isolation of Bacteria: The microorganisms were isolated using three methods. In method 1, the Standard Blotter Method (Baki *&* Anderson, 1973) was used to isolate bacteria from seeds of different plants seeds from various locations. The organisms that were isolated were identified on the basis of morphological characteristics, and maintained on nutrient agar slants at 4°C. In the second method (settle plate technique), a petridish containing a suitable agar medium was horizontally exposed for 5 – 10 min; plates were then incubated at 37ºC for 2 days (Aneja, 2002). In the third method, a moist part in the garden was selected. The debris from the top of the soil was collected and brought to the laboratory in a Ziploc bag. One gram each of soil was suspended in a test tube with 9ml of sterile distilled water and mixed thoroughly. This was serially diluted and 0.1 ml of the diluted samples were spread on Nutrient agar in petridishes and incubated at 37ºC for 24 h (Abe, 1988).

Screening of bacterial isolates: Primary screening of bacterial isolates for production of alpha amylase was done by the starch agar plate method (Aneja, 2002). Plates containing Nutrient agar, modified to contain 2% starch as the sole source of carbon were prepared and spot inoculated with one isolate per plate. The plates were incubated for 48h at 37 $\pm$ 2ºC. The starch hydrolysis test was performed by adding a few drops of freshly prepared iodine into the plates after the incubation period. Where starch was not hydrolyzed a blue-black color was observed due to the formation of starch-iodine complex. A zone of clearance was observed around the cultures that were producing amylase.

Inoculum preparation: Each bacteria isolate was transferred from stock culture to 100-mL nutrient broth. The inoculated flasks were incubated overnight at 35 ± 2°C. The broths were then centrifuged at 10,000 rpm for 10 min. After centrifuging the pellet was re-suspended in 10mL of sterile distilled water and the absorbance (*A*) read at 660nm. Cell density in the inoculum suspension was adjusted to an OD of 0.5, which contains 4.5×10^5 cells/ml.

Media composition: The medium for solid substrate fermentation contained 5g of wheat bran as solid substrate and 8ml of Bushnell Haas (BH) medium, used to adjust the moisture content from 43 to 81% in 250-mL Erlenmeyer flasks. Fermentation was carried out for three days (72 h). The medium for submerged fermentation was composed of 2% peptone, 0.5% soluble starch, 0.3% K_2HPO_4 and 0.1% $MgSO_4.7H_2O$ (Ajayi *&* Fagade, 2003). A volume of 100mL of the medium

was put in a 250mL flask and inoculated with 1mL of bacterial cell suspension. Fermentation was carried out for 4 days.

Optimization of culture conditions for enzyme production: To ascertain the effect of culture conditions on amylase production, the study was carried out at different temperature conditions (25, 37 and 40ºC), medium pH (4.5, 6.0, 7.5 and 9), different carbon sources (glucose, maltose, sucrose and soluble starch) and nitrogen sources (beef extract, meat extract, casein and urea).

Enzyme extraction and assay: After solid substrate fermentation 22 ml of distilled water was added to the culture flasks and mixed well in a rotary shaker (200 rpm) at room temperature (22 ± 2ºC) for 30 min, followed by filtration and centrifugation at 8,000 rpm for 10 min. After 96h of submerged fermentation the broth was centrifuged at 8,000 rpm for 10 min. In both cases the cell free supernatant obtained after centrifugation was used as the source of enzyme.

Estimation of α-amylase activity was carried out according to the dinitro salicylic acid (DNS) method of Miller (1959). One hundred micro liter of 1% starch was incubated with 1ml of enzyme extract and 1ml of phosphate buffer (pH 6.5). The reaction mixture was incubated for 20 min before stopping the reaction by addition of 0.5 ml DNS reagent, and cooling in a water bath for 10 min. A volume of 2.5ml distilled water was added and the absorbance read at 540nm using a colorimeter against glucose as the standard. One unit of enzyme activity is defined as the amount of enzyme, which releases 1µmole of reducing sugar as glucose per minute, under the assay conditions (U/ml/min). The experiments were carried out in triplicates and standard error was calculated.

Protein estimation: Protein content of the enzyme extracts was estimated by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard. Enzyme activity is expressed as specific activity, which is equivalent to U/mg protein. All the experiments were carried out in triplicates and the standard error was calculated.

Mutagenesis: Five test tubes with the aforesaid bacterial suspension were taken and one of them (the control) was kept aside in the dark and the rest four were given UV radiation (2600 Å) for time periods varying from 5 - 20min with samples being collected at 5min intervals. After UV radiation they were kept in darkness for stabilization of thymine-thymine (T-T) dimers. The bacterial cell suspensions were used as inoculum for enzyme production by submerged fermentation. After four days of fermentation, bacterial enzyme was extracted and the activity was assayed. 0.1 ml of the UV-treated bacterial cell suspensions were then inoculated onto petridishes containing 25ml of Nutrient agar and incubated at 37°C till colony formation. These cultures were used for the analysis of percentage survival of the organisms after UV irradiation (Saha & Bhattacharya, 1990).

Amylase Purification and Molecular Mass Determination: Fourty milliliter of enzyme extract was used. Solid ammonium sulphate was slowly added to yield 60% saturation and the mixture was kept overnight at 4ºC. The precipitate was collected by centrifugation, dissolved in 0.1M phosphate buffer (pH 7) and dialyzed overnight against 0.01M phosphate buffer. All the above steps were conducted under freezing temperature. The samples thus obtained were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for molecular weight determination. Phosphorylase *b* (97 kDa), albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa) and lysozyme (14.3 kDa) were used as molecular mass markers (Young *et al*., 1995). Enzyme activity was estimated in the purified enzyme samples and protein content was also measured by the method of Lowry *et al.* (1951). The purified bacterial enzymes were subjected to activity staining by allowing the native gel to act on 1% starch solution for 30min and amylase activity was visualized by staining with iodine solution (0.05g iodine and 0.5g of KI in 100ml distilled water) (Shih & Labbe, 1995).

Evaluation of factors affecting enzyme activity

Thermostability of Amylase: The thermal stability of the enzyme was assessed by incubating the enzyme without the substrate fractions at various temperatures between 30 to 60ºC for 1h. At 10min intervals, aliquots of 1ml of the incubated enzyme were assayed for activity.

pH: The effect of pH on amylase activity was assessed by incubating the reaction mixture at pH values ranging from 3.5 to 9.5.

Effect of Metal Ions: Enzyme activity was assayed in the presence of 10mM concentrations of various metal ions (Na^+, Mg^{2+}, Ca^{2+} and Co^{2+}). The chloride salts of these metal ions were used

($NaCl$, $MgCl_2$, $CaCl_2$, $CoCl_2$). The relative activity of the enzyme was compared with the activity obtained in 0.1M phosphate buffer.

Results and Discussion

Bacterial isolates from different sources were tested for production of amylase by the starch hydrolysis test. On the basis of the area of clearance, several bacterial isolates were selected, after which two out of ten isolates were selected for further evaluation. The two bacterial species, identified as *Bacillus* sp.1 and *Bacillus* sp.2, produced more enzyme by submerged fermentation than in solid substrate fermentation. Isolate *Bacillus* sp. 1 was selected for further studies on optimization of culture conditions by submerged fermentation. The enzyme activity observed for this strain was much higher than that reported by Ajayi *&* Fagade (2003) comparing different *Bacillus* sp. The production of more enzyme by SmF than SSF as observed in this study was contrary to the report of Dey *&* Agarwal (1999), who described 3 – 4 times higher productivity of a heat stable α-amylase by *Streptomyces megasporus* through SSF.

Incubation at room temperature (24 – 28°C) was more favorable for enzyme production in both SSF and SmF. Bacterial amylases are produced at a much wider range of temperature. *Bacillus licheniformis* and *B. subtilis* are among the most commonly reported *Bacillus* that produce alpha amylase at 37 - 60°C (Mishra *et al*., 2005; Mendu *et al*., 2005). Padmanabhan *et al.* (1992) has reported that recovery of -amylase through SSF depends on the temperature of extraction.

With *Bacillus* sp.1, and using SmF, an increase in pH from 4.5 to 6.0, increased enzyme production but pH levels above 6 caused a decrease in enzyme production. pH is one of the most important factors for in any fermentation process and each microorganism has a range for growth and activity. Alpha amylases are generally stable over a wide range of pH from 4 to 11 (Fogarty *&* Kelly, 1979), though some are only stable within a narrow pH range (Coronado *et al.,* 2000). Most Bacillus isolates, e.g. *B. subtilis*, *B. licheniformis* and *B. amyloliquefaciens* seem to require an initial pH of 7.0 (Syu *&* Chen, 1997; Tanyildizi *et al*., 2005; Haq *et al*., 2005).

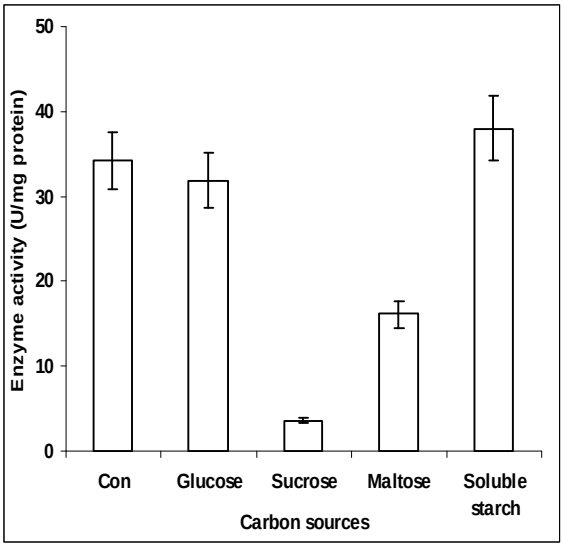

Fig. 1: Effect of different carbon sources on enzyme production by SmF.

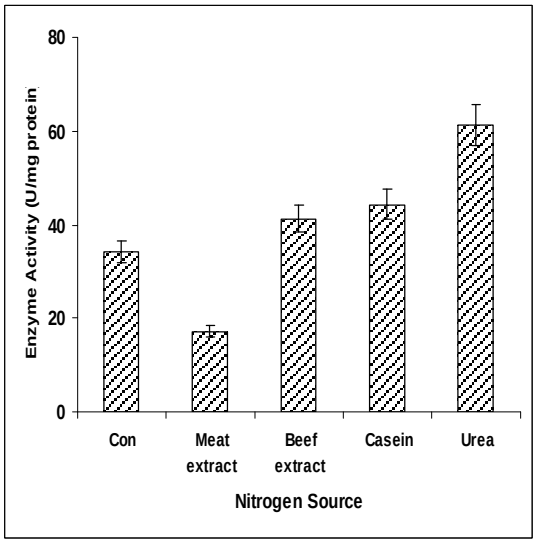

Fig. 2: Effect of different nitrogen sources on enzyme production by SmF.

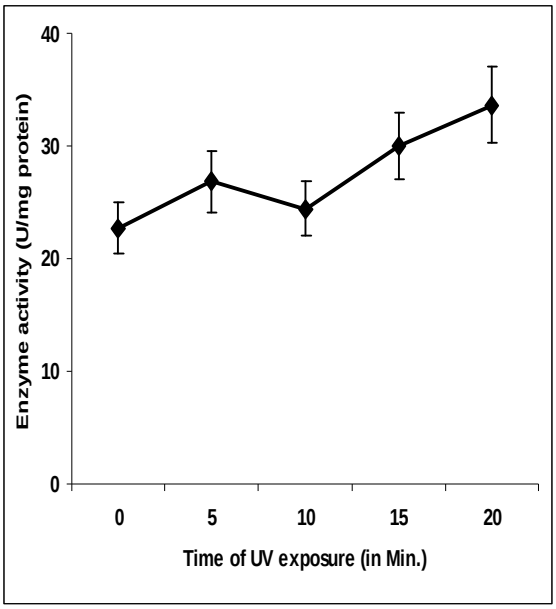

Figure 3: Effect of UV induced mutation on enzyme production (SmF).

Supplementation of carbon sources in the form of monosaccharides, disaccharides and polysaccharides showed substantial difference in effect to alpha amylase production. Supplementation with soluble starch gave maximum amylase activity of 38 U/mg of protein compared to glucose (31.8 U/mg of prote; Maltose (16.1) and sucrose (3.06 U/mg of protein) repressed the production of enzyme (Fig. 1). Dharani (2004), reported highest amylase production in sucrose medium. Addition of organic nitrogen sources such as casein, beef extract and urea to the medium resulted in a considerable increase in the production of -amylase (Fig. 2). Media supplemented with urea gave the maximum amylase activity (61.33), followed by beef extract (41.33), casein (44.35) compared to the control's 22.7 U/mg of protein. Supplementation of meat extract resulted in a decrease in enzyme production, contrary to the findings of Dharani (2004), of increased enzyme production by *B. licheniformis* SPT 27 after addition of meat extract.

Results showed an increase in α-amylase production with increased duration of exposure to UV radiation. The mutant had increased amylase production (33.65 U/ mg protein) compared to the parent strain (22.7 U/mg of protein) (Fig. 3). However, the survival rate of bacteria decreased with increased duration of exposure to UV light.

The enzyme extracted from the bacteria was assessed for reaction with regard to pH, temperature and substrate concentration. After partial purification by ammonium sulphate precipitation the purity of bacterial amylase was 1.06 fold higher than the crude enzyme (Table 1). The molecular mass of the amylase was estimated to be ~40 kDa by SDS-PAGE. The bacterial enzyme was highly stable at 30ºC and after 1 hour, the activity actually doubled when compared to its activity after 10 min at 30º C. At 60º C the enzyme showed least stability, retaining only half of its original activity (Fig. 4 A, B, C & D). Inactivation of enzymes at high temperature due to incorrect conformation as a result of hydrolysis of the peptide chain, destruction of amino acid or aggregation, have been reported previously (Schokker & van Boekel, 1999; Alva *et al.,* 2007).

Table 1: Results of partial purification of bacterial amylase

Purification method	Volume (ml)	Total activity (µM/min)-U	Total Protein (µM/min)	Specific activity U/mg of protein	% Amylase recovery	Fold purification
Crude Enzyme	40	9.77	39.295	9.94	100	1
Ammonium sulphate precipitation	0.8	5.85	0.55	10.63	59.87	1.06

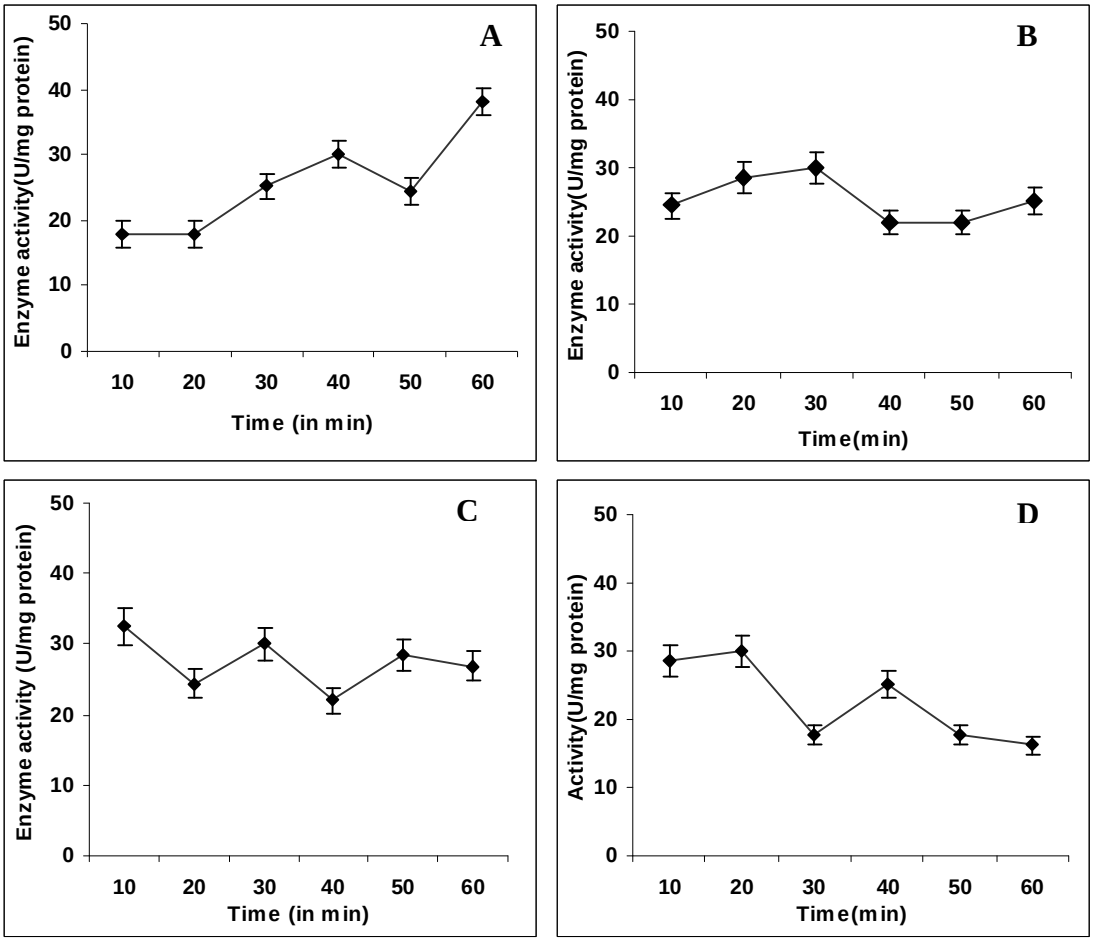

Figure 4: Stability of amylase at (A), 30 (B), 40 (C) 50 and (D) 60°C.

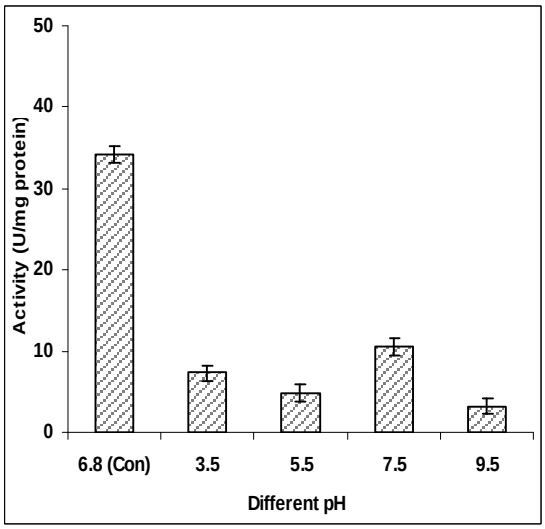

Figure 5: Effect of pH on activity of amylase.

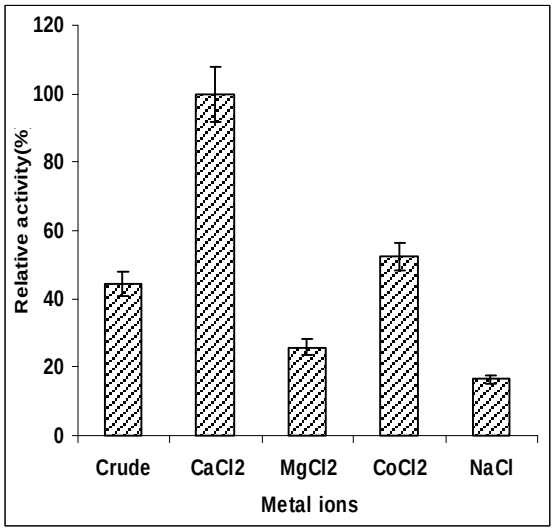

Figure 6: Effect of metal ions on amylase activity.

Phosphate buffer at pH 6.8 resulted in the highest enzyme activity of 34.21 U/mg of protein, as compared to pH of 3.5, 5.5, 7.5 and 9.5 (Fig. 5). With regard to metal ions, enzyme activities were enhanced by calcium and cobalt, while magnesium and sodium ions inhibited enzyme activity (Fig. 6). Ca^{2+} ions have been reported to stabilize alpha amylase at higher temperatures (Chung *et al.,* 1995). .

References

Abe J, Bergman FW, Obata K, Hikuri S, 1988. Production of raw starch digesting amylase by *Aspergillus* K-27. Applied Microbiology and Biotechnology 27: 447 - 450

Ajayi AO, Fagade OE, 2003. Utilization of corn starch as substrate for β- amylase by *Bacillus* spp. African Journal of Biomedical Research 6(1): 37-42.

Alva S, Anumpama J, Savla J, Chiu YY, Vyshali P, Shruti M, Yogeetha BS, Bhavya D, Purvi J, Ruchi K, Kumudini BS, Varalakshmi KN, 2007. Production and characterization of fungal amylase enzyme isolated from *Aspergillus* sp. JGI 12 in solid state culture. African Journal of Biotechnology 6(5): 576-581.

Aneja KR, 2002. Experiments in microbiology, plant pathology, tissue culture and mushroom production technology. Pp 169 -171.

Baki A, Anderson, 1973. Vigour determination in soyabean seed by multiple criteria. Crop Science 13: 630 - 633.

Chung YC, Kobayashi T, Kanai H, Akiba T, Kudo T, 1995. Purification and properties of extracellular amylase from the hyperthermophilic Archaeon *Thermococcus profundus* DT 5432. Applied and Environmental Microbiology 61(4): 1502 -1506.

Coronado M, Vargas C, Hofemeister J, Ventosa A, Nieto JJ, 2000. Production and biochemical characterization of an á-amylase from the moderate halophile *Halomonas meridiana*. FEMS Microbiolgy Letters 183: 67 - 71.

Dey S, Agarwal SO, 1999. Characterization of a thermostable alpha amylase from a thermophilic *Streptomyces megasporus* strain SD12. Indian Journal of Biochemistry and Biophysics 36: 150 - 157.

Dharani Aiyer PV, 2004. Effect of C:N ratio on alpha amylase production by *Bacillus licheniformis* SPT 27. African Journal of Biotechnology 3(10): 519 - 522.

Emanuilova EI, Toda, K, 1984. Alpha - amylase production in batch and continuous cultures by *Bacillus caldolyticus*. Applied Microbiology and Biotechnology 19: 301- 305.

Fogarty WM, Kelly CT, 1979. Developments in microbial extra cellular enzymes. In: Wiseman A, (Ed.). Topics in Enzyme and Fermentation Biotechnology 3: 45 -108.

Haq I, Ashraf H, Qadeer MA, Iqbal J, 2005. Pearl millet, a source of alpha amylase production by *Bacillus licheniformis*. Bioresource Technology 96: 1201 - 1204.

Heinekes FG, O'Connor RJ, 1972. Continuous culture studies on the biosynthesis of alkaline protease, neutral protease and alpha-amylase by *Bacillus subtilis NRRL-B3411*. Journal of General Microbiology 73: 35 - 44.

Kunamneni A, Perumal K, Singh S, 2005. Amylase production in solid state fermentation by the thermophilic fungus *Thermomyces Lanuginosus*. Journal of Bioscience and Bioengineering 100(2): 168 - 171.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, 1951. Protein measurement with the Folin-Phenol reagents, Journal of Biological Chemistry 48: 17 - 25.

Mendu DR, Ratnam BVV, Purnima A, Ayyanna C, 2005. Affinity chromatography of - amylase from *Bacillus licheniformis*. Enzyme and Microbial Technology 37: 712 - 717.

Miller GL, 1959. Use of Dinitro salicylic acid reagent for determination of reducing sugar. Analytical Chemistry 31: 426 - 429.

Mishra S, Noronha SB, Suraishkumar GK, 2005. Increase in enzyme productivity by induced oxidative stress in *Bacillus subtilis* cultures and analysis of its mechanism using microarray data. Process Biochemistry 40: 1863 - 1870.

Padmanabhan S, Ramakrishna M, Lonsane BK, Krishnaiah MM, 1992. Letters in Applied Microbiology 15: 235 - 238.

Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D, Mohan R, 2000. Advances in microbial amylases. Biotechnology and Applied Biochemistry 31: 135 -152.

Pandey A, Soccol CR, Rodriguez-Leon JA, Nigam P, 2001. Solid-state fermentation in biotechnology, Fundamentals and Applications. 1st edition. pp 8 - 29.

Ramesh MV, Lonsane BK, 1991. Ability of a solid state fermentation technique to significantly minimize catabolite repression of alpha-amylase production by *Bacillus licheniformis M27*. Applied Microbiology and Biotechnology 35: 591 - 593.

Saha N, Bhattacharyya BC, 1990. Effect of pH, temperature, carbon and nitrogen source on the production of amylase in liquid culture by *Rhizopus oryzae,* Proceedings of All India Seminar on the scope of Alternate Energy Sources in chemical process industries at Harcout Butler Technological Institute, Kanpur.

Schallmey M, Singh A, Owen PW, 2004. Developments in the use of *Bacillus* sp. for industrial production. Canadian Journal of Microbiology 5: 1 -17.

Schokker EP, Van Boekel AJS, 1999. Kinetics of thermal inactivation of extracellular proteinase from *Pseudomonas flourescens* 22F, influence of pH, calcium and protein. Journal of Agriculture and Food Chemistry 47: 1681 - 1686.

Shih NJ, Labbe GR, 1995. Purification and characterization of an extracellular α-amylase from *Clostridium perfringens* type A. Applied and Environmental Microbiology 61(5): 1776 -1779.

Syu MJ, Chen YH, 1997. A study on the -amylase fermentation performed by *Bacillus amyloliquefaciens*. Chemical Engineering Journal 65: 237- 247.

Tanyildizi MS, Ozer D, Elibol M, 2005. Optimization of a-amylase production by *Bacillus sp*. using response surface methodology. Process Biochemistry 40: 2291- 2296.

Tomanaga G, 1966. Preferential synthesis of extracellular protease by *Aspergillus niger* in sulphur deficiency. Journal of General and Applied Microbiology 12: 267-276.

Vihinen M, Mantsala P, 1989. Microbial amylolytic enzymes. Critical Reviews in Biochemistry and Molecular Biology 24: 329 - 418.