



Original Research Article

Screening and production optimisation of alpha amylase from *Aspergillus* strains by using solid state fermentation

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ABSTRACT

Keywords

Aspergillus Strains, Solid-State fermentation, Alpha amylase production, lignocellulosic biomass.

Amylase is an amyolytic enzyme used in many food industries which is generally produced by *Aspergillus spp.* under solid state fermentation. By using different lignocellulosic biomass such as wheat bran, Rice bran, Corn, Rice Straw and sugar cane bagasses present a great potential as substrate and support the low production costs for amylase production under Solid-State fermentation by using *Aspergillus* Strains. The effect of various factors on Alpha amylase production was examined. Cultivation was carried out at different temperatures, 20, 25, 30, 35 and 40⁰ C from 24-144 hrs with 24 hrs. Study of influence of pH initial in SSF was conducted with pH 3.0, 4.0, 5.0, 6.0 and 7.0. The results were showed that wheat bran and sugar cane bagasses powder at the ratio of 1:1 was the best for optimum production of Alpha-amylase. The maximum yield was achieved with optimized process parameters such as Solid substrate (Wheat Bran), incubation period (72 hours), moisture (60%), pH (5.0) and fermentation temperature (30°C).

Introduction

Enzymes being the most important products obtained for human needs have stimulated renewed interest in the exploration of industrially relevant enzymes from several natural sources including plants, animals and microorganism. Amylases are important enzymes employed in the starch processing industries for the hydrolysis of starch into simple sugars by the breakdown of 1, 4- α - D glucosidic linkages between adjacent glucose units

there by hydrolyzing single glucose units from the non-reducing ends of amylose and amylopectin in a stepwise manner. This enzyme is extensively used in starch liquefaction, paper industries, food, pharmaceutical and sugar industries.

Amylase catalyses the breakdown of starch into sugars. alpha-amylase can breakdown long-chain carbohydrates, ultimately yielding maltose from amylose, or maltose, glucose, and "limit dextrin"

from amylopectin. Amylases are produced by a wide spectrum of organisms, although each source produces biochemical phenotypes that significantly differ in parameters like pH and temperature optima as well as metal ion requirements. Current developments in bio-technology are yielding new applications for enzymes. In the last decades, there has been an increasing trend towards the utilization of the solid state fermentation (SSF) to produce several enzymes from micro-organisms (Sodhi et al., 2005). The food, beverage and agro industries produce large quantities of residues that pose serious problems of disposal, in spite of them being sources of biomass and nutrients. These substrates are used for production of valuable compounds such as enzymes and various secondary metabolites (Soccol et al., 2003). Agro-industrial residues are generally considered the best substrates for SSF processes and use of SSF for the production of enzymes is no exception to that (Ellaiah et al., 2002). Use of suitable low cost fermentation medium for production of alpha amylase using agricultural products has been reported (Haq et al., 2003). Wheat bran, paddy husk, rice processing waste and other starch containing wastes have gained importance as supports for growth during enzyme production (Anto et al., 2006). Hence, the present study was aimed to accomplish the objective of production of amylase and gluco-amylase enzyme from various agricultural waste products by *Aspergillus* strains using solid state fermentation technique and its process optimization.

Materials and Methods

Micro organism and primary Screening

The fungal strain obtained from cow dung was screened for the amylase production

on starch containing plates.

For the primary screening of amylase production, the fungal strains were spot inoculated on medium consists of peptone (0.3%), starch (0.1%) and agar (2%) and the growth was dominant after 48 hrs of incubation at 37⁰C. To observe the production of amylase, 1% of iodine solution was added in Culture plate. Disappearance of blue color around the colony shows the secretion of extra cellular enzyme amylase. The fungal strain secretes an amylase, it breaks down the starch into glucose and it was utilized as a carbon source for the growth. When the iodine solution was added, the starch which was present in the culture plate was converted into blue color and the starch hydrolyzed spot was transparent.

Submerged and Solid state fermentation

Preinoculum Preparation

For preinoculum preparation, Cultures were inoculated in a production medium containing NH₄NO₃-0.6%, KH₂PO₄-0.2%, MgSO₄-0.2%, Starch-2%, FeSO₄.7H₂O-0.0001% and incubated for 24hrs at room temperature at 150rpm. The lag phase culture was treated as a preinoculum for submerged and solid state fermentation.

Submerged fermentation

10ml of preinoculum was transferred into 90 ml of sterilized production medium containing conical flask. Flasks were kept in an orbital shaker and samples were collected at frequent time intervals in a sterile condition. The collected samples were centrifuged at 7,500rpm at 40⁰C for 15mins. The obtained cell free extract was used to estimate reducing sugar and soluble proteins.

Solid State Fermentation

Different lignocellulosic substrates such as wheat bran, rice bran, rice straw, sugarcane bagasses and corn were used to study the need of proteinaceous substances for enzyme production. These substrates were milled and sieved to get a particle size of 500 to 1000 μm , which were used for the further experiment.

10gms of different agricultural waste material was weighed into a conical flask and moistened with a nutrient salt solution containing NH_4NO_3 -0.2%, KH_2PO_4 -0.6%, and MgSO_4 -0.2%. The substrate was mixed thoroughly after the addition of nutrient salt solution and then the solid substrates were sterilized for 15minutes at 121°C at 15lbs. The flasks were inoculated with the inoculums after reaching the room temperature while the moisture content was maintained at 70% in all the flasks. The collected samples at different time intervals were used to analyze the dry weight determination the supernatant of the fermented substrate extract was used to estimate the Amylase, reducing sugar and total soluble protein estimation.

Dry weight determination

A known quantity of the fermented substrate was taken in a Petri dish and its dry weight was determined by placing the samples in a hot air oven at 50°C for overnight. Wet weight and the Dry weight of the sample was noted down and the dry weight was calculated as shown below.

$$\text{Dry weight of per g of substrate (g/g)} = \frac{[(\text{Wt of Petri dish Dry wt of the substrate}) - (\text{Wt of empty Petri dish})]}{[(\text{Wt of Petri dish} + \text{Wet wt of the substrate}) - (\text{Wt of empty Petri dish})]}$$

Optimization of Moisture content and pH

The effect of moisture content on enzyme activity was optimized by inoculating the two different strains at different moisture content such as 50%, 55%, 60%, 65%, 70% and 75%. 10gms of substrate was taken in to the 250 ml conical flask, the moisture content was calculated and different volumes of nutrient salt solution was added into the flask. Then flasks were sterilized for 15minutes at 121°C . Constant volume of inoculums was added to all the flasks. Samples were collected at regular intervals.

The effect of pH on enzyme activity was measured by changing the pH of substrate at different pH such as 3, 4, 5, 6, and 7. The pH of the nutrient solution was adjusted with 0.1N HCl and added into the conical flask which contains substrate and sterilized for 15minutes at 121°C . All the flasks were inoculated with the constant volume of the inoculums with constant moisture content. Samples were collected at different time intervals in a sterilized condition and were analyzed for the enzymatic assay and soluble protein estimation.

$$\% \text{ of Moisture} = \frac{(\text{Initial weight} \times \text{final weight})}{(\text{Final weight} - \text{initial weight})} \times 100$$

Optimization of Temperature

The enzyme activity was observed at different temperature 20°C , 25°C , 30°C , 35°C and 40°C respectively. 10gms of substrate was taken into a flask with constant moisture content, pH. The Solid substrate was sterilized and inoculated with a 12ml of preinoculum. Now the flasks were incubated at different temperature and the samples were collected at different time intervals. The

collected samples were used for the further analysis.

Soluble Protein Estimation

The soluble protein content of the enzyme extracts was estimated by the Lowry et al. (1951), bovine serum albumin was used as a standard. All the experiments were carried out in triplets and standard deviation was calculated.

Amylase activity Assay

Amylase activity was determined as described by Okolo et al. (1995). The reaction mixture consisted of 1.25 ml of 1% soluble starch, 0.25 ml acetate buffer (pH 5.0), 0.25ml of distilled water, and 0.25 ml of crude enzyme extract. Substrate blank was prepared without adding enzyme and enzyme blank was prepared without adding substrate. After 10 min of incubation at 50°C, dinitrosalicylic acid (DNS) was added to stop the reaction and kept it in boiling water bath for 15 mins. 1 ml of a 40% potassium sodium tartrate (Rochelle salt) solution was added to stabilize the color. After cooling to room temperature in a cold water bath, the color developed was read at 575nm using a Shimadzu UV-160A spectrophotometer. Glucose was used as the standard. The blank contained 0.5ml of 0.1M acetate buffer (pH 5.0), 1.25ml 1% starch solution and 0.25ml distilled water. One unit (IU) of alpha-amylase was defined as the amount of enzyme releasing one μmol glucose equivalent per minute under the assay conditions.

$$\text{Enzyme activity} = \frac{\text{Test} - (\text{enzyme blank} + \text{substrate blank})}{\text{Volume of enzyme extract}}$$

Statistical analysis

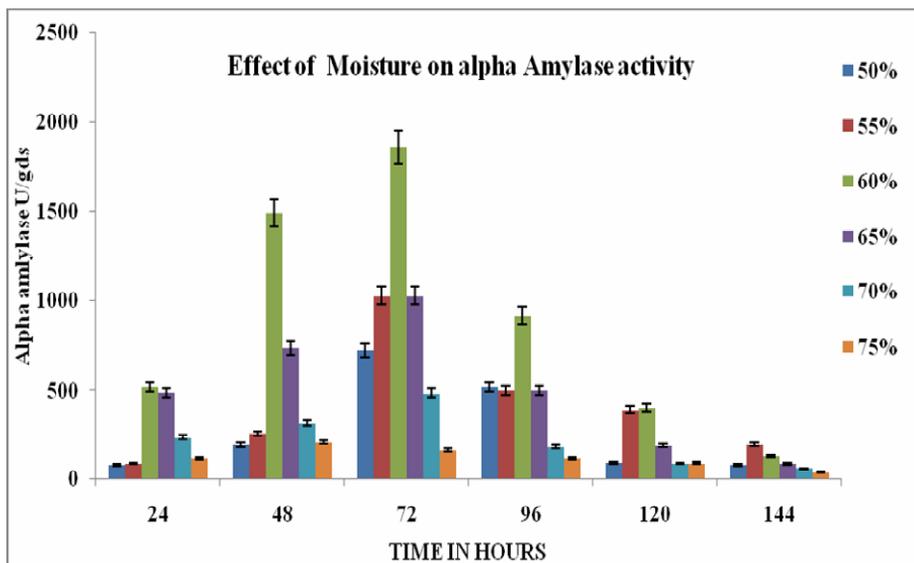
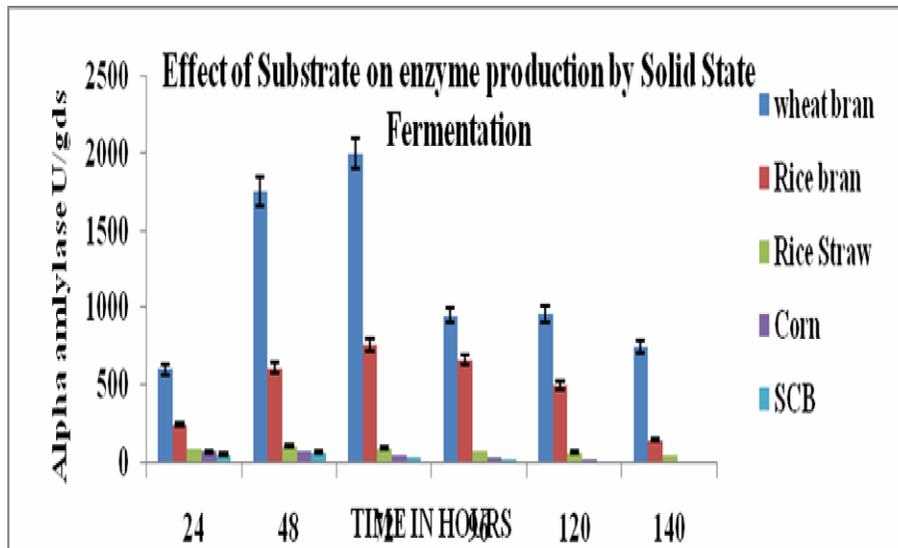
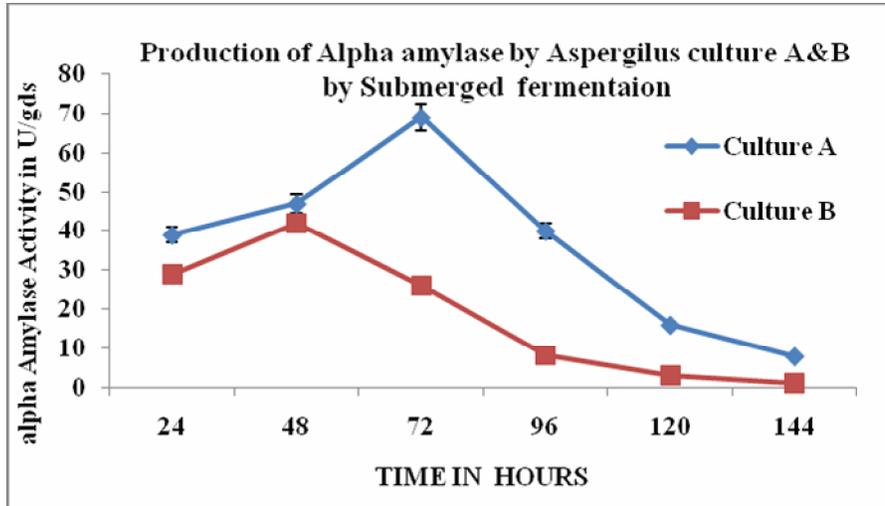
Experimental data of different parameters were analyzed by using Descriptive

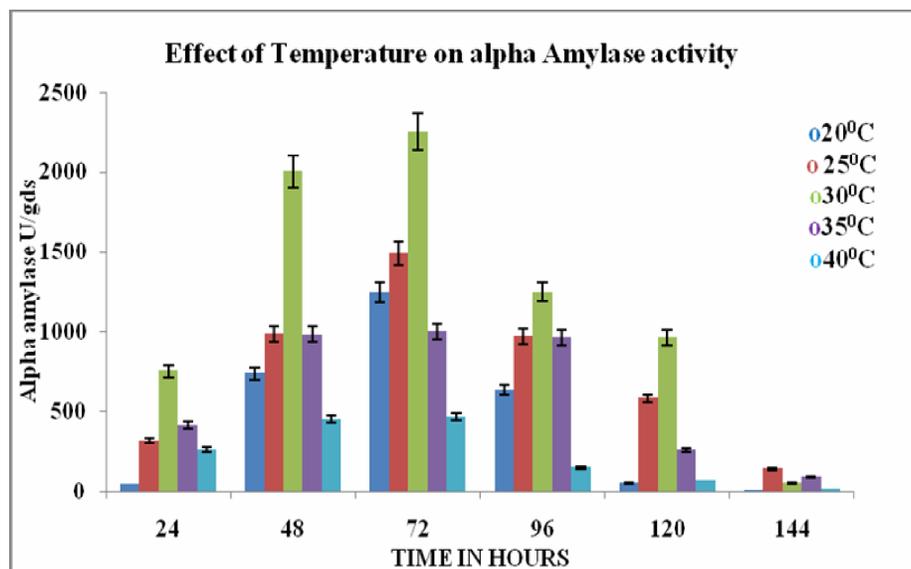
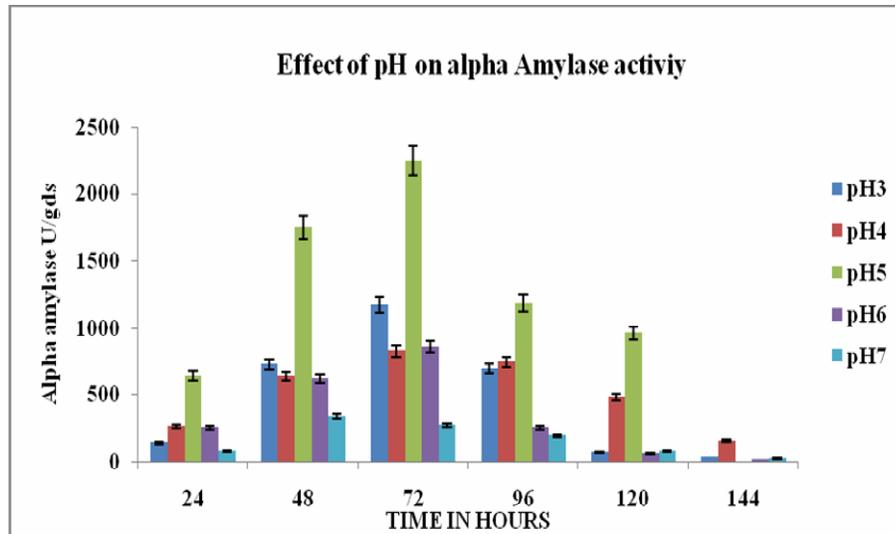
Statistics in MS-EXCEL-2007 software.

Results and Discussion

Different Fungal colonies were isolated from cow dung samples enriched for amylase producing microorganisms by serial dilution process where in PDA (potato dextrose agar) media was prepared, autoclaved and poured in sterile Petri plates. 50 μl of serially diluted samples diluted up to 10^{-5} dilutions were spread on respective solidified PDA plates. The inoculated petriplates were incubated at 28°C for 48 hours. All the strains were screened for the Amylase activity by plate assay method. Two fungal isolates were selected and differentiated as *Aspergillus* Strains on the basis of physical characteristics (J. I. Pitt et al., 1985). The obtained *Aspergillus* were named as Strain A and B. The isolates were further inoculated on sterile PDA plates by point inoculation and incubated at 28°C for 48 hours in order to obtain pure fungal plates. Optimization of process parameters is very important for overproduction of enzymes to meet industrial demand (anyildizi M.S et al 2005). Production of amylase in submerged fermentation was observed with the best two strains at different time intervals, maximum enzyme activity was obtained by culture A in 72 hrs. Enzyme production was observed from 24 hrs and no enzyme activity was found after 96hrs in culture B and 120 hrs in culture A. From this experiment Culture A showed maximum activity of 69 U/gds and culture B showed 35U/gds activity at 48hrs.

Different lignocellulosic substrates such as wheat bran, rice bran, rice straw, corn, and sugar cane bagasses were used for the solid state fermentation. Wheat bran shown to the best substrate for the alpha amylase activity as compared with other substrates. Maximum enzyme activity 1990 U/gds was observed in 72hrs





followed by rice straw 760U/gds. Amylase activity was almost negligible in Rice straw, Corn and Sugar Cane Bagasses and enzyme activity was reduced after 72hrs of incubation.

When compared with solid state and submerged fermentation with two different strains. Maximum Amylase activity was obtained in solid state fermentation wheat bran as a substrate. Further experiment was carried out with the wheat bran as a substrate in solid state fermentation.

Production of Amylase was studied by varying the percentage of moisture content in the medium at different moisture content like 50% ,55% , 60%, 65% ,70% and75% and the samples were collected at 24hrs interval.

Maximum activity 1861U/gds was observed at 60% of moisture at 72hrs of incubation. After 72hrs enzyme activity was reduced drastically and at 14hrs activity was not considerable.

Table.1 Total Soluble Protein obtained by solid state fermentation by *Aspergillus* Strain-A

Time in Hrs	Soluble Protein (mg/ml) of Culture - A																					
	Screening of Strain	Type of Substrate					Moisture Content (%)						pH					Temp (°C)				
		WB	RB	RS	CN	SCB	50	55	60	65	70	75	3	4	5	6	7	20	25	30	35	40
24	0.165±0.001	0.018±0.00	0.031±0.00	0.073±0.00	0.015±0.00	0.043±0.02	0.055±0.001	0.073±0.00	0.078±0.00	0.111±0.001	0.113±0.001	0.133±0.001	0.167±0.00	0.075±0.00	0.135±0.01	0.032±0.00	0.035±0.00	0.013±0.00	0.021±0.00	0.146±0.01	0.045±0.00	0.092±0.00
48	0.137±0.002	0.071±0.00	0.044±0.00	0.079±0.00	0.031±0.00	0.0175±0.01	0.0655±0.02	0.049±0.00	0.048±0.00	0.028±0.00	0.073±0.00	0.056±0.00	0.035±0.00	0.005±0.00	0.025±0.00	0.467±0.04	0.459±0.04	0.183±0.02	0.015±0.00	0.199±0.03	0.021±0.00	0.081±0.00
72	0.215±0.01	0.094±0.00	0.027±0.00	0.071±0.00	0.020±0.00	0.0944±0.02	0.043±0.001	0.043±0.00	0.063±0.00	0.102±0.002	0.075±0.00	0.087±0.00	0.164±0.01	0.126±0.00	0.024±0.00	0.327±0.01	0.063±0.00	0.217±0.02	0.023±0.00	0.229±0.02	0.029±0.00	0.084±0.00
96	0.888±0.06	0.062±0.00	0.006±0.00	0.008±0.00	0.035±0.00	0.0765±0.03	0.025±0.001	0.065±0.00	0.098±0.00	0.063±0.00	0.045±0.00	0.366±0.04	0.065±0.00	0.098±0.00	0.147±0.00	0.098±0.00	0.157±0.01	0.138±0.01	0.430±0.02	0.314±0.03	0.017±0.00	0.053±0.00
120	0.166±0.001	0.028±0.00	0.094±0.00	0.045±0.00	0.074±0.00	0.0484±0.04	0.019±0.00	0.058±0.00	0.084±0.00	0.049±0.00	0.062±0.00	0.022±0.00	0.051±0.00	0.083±0.00	0.102±0.00	0.010±0.00	0.106±0.03	0.091±0.03	0.371±0.04	0.311±0.04	0.010±0.00	0.046±0.00
144	0.140±0.001	0.198±0.04	0.096±0.00	0.072±0.00	0.047±0.00	0.072±0.05	0.006±0.00	0.491±0.02	0.063±0.00	0.038±0.00	0.041±0.00	0.016±0.00	0.012±0.00	0.721±0.05	0.095±0.01	0.009±0.00	0.102±0.01	0.052±0.03	0.296±0.00	0.216±0.00	0.006±0.00	0.038±0.00

WB= wheat Bran, RB= Rice Bran, RS= Rice Straw, CN= Corn, SCB= Sugar Cane Bagasses

Determination of alpha Amylase production at different pH

Among the physical and chemical parameters, optimum temperatures, pH range, are the most important for enzyme production by microbes (Bose & Das 1996 Gupta et al. 2003). Production of amylases was studied by varying the pH of the medium. pH have profound effect on enzyme activity. The samples were collected at 24 hrs interval. The enzyme activity was observed at different pH like 3, 4,5,6,7, where the highest enzyme activity (2247U/gds) was observed at pH 5 in 72 hrs of incubation. Previous reports showed that fungi required slightly acidic pH and bacteria required neutral pH for optimum growth (Gangadharan et al. 2006)

Production of Amylase was studied by using different temperature of the medium. The samples were collected at 24hrs interval. The enzyme activity was observed at different temperature 20⁰C, 25⁰C, 30⁰C, 35⁰C, 40⁰C.the highest enzyme activity was observed at temperature 30⁰C.Below the temperature 30⁰C was shown very poor enzyme activity. It is reported that optimal temperature for enzyme production in *A.niger* at room temperature in Submerged Fermentation and Solid State Fermentation (Varalakshmi et al., 2009) and reported 30⁰C be the best for enzyme production by *Penicillium fellutanum* (Kathiresan & Manivannan, 2006). Some reports such as *S.cerevisiae* and *S.kluyveri* were also produce amylase at 30⁰C K (Moller, M. Z. et al., 2004).

There are number of fungi reported for producing Alpha Amylase. These have been found to be useful in the food industry. The isolation of fungal strains

and cultural conditions and composition of media for optimal production of amylase by *Aspergillus* strains has been developed in this study. Agro waste material such as wheat bran led to a reduction in the culture medium cost for Amylase production, which usually ranges from 25% to 50% of the total production cost. The optimum time for production was 72hrs and after optimization of process parameters such as moisture, Temperature and pH enzyme production was increased significantly. .

The optimum enzyme production by the *Aspergillus* strain was found at 30⁰C. It can be concluded that, *Aspergillu* strain A can be a potential producer of extracellular amylase which could find applications in industry. Due to the importance of these findings, further studies need be carried on in order to commercialize the production process.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

- Anto, H., U. Trivedi, and K. Patel. 2006. Alpha amylase production by *Bacillus cereus* MTCC 1305 using solid-state fermentation. Food Technol. Biotechnol. 44: 241-245.
- Bose K, Das D (1996) Thermostable α -amylase production using *B. licheniformis* NRRL B1438. Indian J Exp Biol 34:1279-1282.
- Ellaiah P, Adinarayana K, Bhavani Y, Padmaja P, Srinivasula B (2002). Optimization of process parameters for glucoamylase production under solid state fermentation by a newly isolated *Aspergillus* species. Process. Biochem. 38: 615–620.

- Gangadharan D, Sivaramakrishnan S, Namboothiri KM, Pandey A (2006) Solid culturing of *Bacillus amyloliquefaciens* for α -amylase production. *Food Technol Biotechnol* 44(2):269-274 .
- Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B (2003) Microbial α -amylases: a biotechnological prospective. *Process Biochem* 38:1599-1616.
- Harmeet K. Sodhi, Kanupriya Sharma, Jugal K. Gupta, Sanjeev K. Soni. Production of a thermostable-amylase from *Bacillus* sp. PS-7 by solid state fermentation and its synergistic use in the hydrolysis of malt starch for alcohol production *Process Biochemistry* 40 (2005) 525-534.
- J. I. Pitt, A. D. Hocking, *Fungi and Food Spoilage*, Academic Press, Australia, 1985.
- Kathiresan K and Manivannan S. 2006. Amylase production by *Penicillium fellutanum* isolated from Mangrove rhizosphere soil. *African Journal of Biotechnology*. 5: 829-832.
- K. Moller, M. Z. Sharif, L. Olsson, J. *Biotechnol.*, 2004, 111 (3), 311.
- Lowry O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the Folin- Phenol reagents. *J. Biol. Chem.* 48: 17-25.
- M. S. Tanyildizi, D. Ozer, M. Elibol, *Process Biochem.*, 2005, 40 (7), 2291.
- Okolo BN, Ezeogu LI, Mba CI (1995). Production of raw starch digesting amylase by *Aspergillus niger* grown on native starch sources. *J. Sci. Food Agric.* 69:109-115.
- Haq U.I, H. Ashraf, J. Iqbal, and M. A. Qadeer, Production of alpha amylase by *Bacillus licheniformis* using an economical medium, *Bioresource Technology*, vol. 87, no. 1, pp. 57-61, 2003.
- Soccol C R, Luciana P S, Vandenberghe (2003). Overview of applied solid state fermentation in Brazil. *Biochem. engg.* 13: 205-218.
- Varalakshmi KN, Kumudini BS, Nandini BN, Solomon J, Suhas R, Mahesh B and Kavitha AP. 2009. Production and characterization of alpha amylase from *Aspergillus niger* JGI 24 isolated in Bangalore. *Polish Journal of Microbiology.* 58: 29-36.