DESCRIPTIONS OF ALPHA AMYLASE PRODUCED FROM *ASPEGILLUS NIGER*

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ABSTRACT: Alpha amylase is one of the most significant enzymes in modern biotechnology. The aim of this research was to isolate, purify and characterize the strain *Aspergillus niger* and one of its enzyme like alpha amylase. For this purpose potato, corn, wheat and rice were used as substrates for the production of alpha amylase. The optimum conditions for enzyme production were standardized using various parameters such as substrate, pH and temperature. The results showed that the production of alpha amylase was highest in case of potato starch in order followed by corn, wheat and rice starches. Optimum production of enzyme was recorded at 50°C temperature and 6.0 pH during 72 to 96 hours. The maximum activity of enzyme was observed in the presence of calcium chloride as compared to other metal ions. The study suggested that potato starch could be used as a potential raw material for the alpha amylase production.

Key words: Amylase, pH, Temperature, Potato, Rice, Optimum.

INTRODUCTION

Amylases are among the major enzymes having highest significance in biotechnology. In recent years, microbial production has tremendously increased due to its wide spread use in paper, fine chemical, leather and detergent industries (Asghar *et al.,* 2000). The enzyme is also used for desizing of textile fibers and for the pharmaceutical industries (Mishra and Dadhich, 2010). Alpha amylase particularly produced from *Aspergillus niger* has been utilized for food industry (Alva *et al.,* 2007). Amirul *et al.* (1996) produced alpha glucosidase and alpha amylase from *Aspergillus niger* cultivation on a broth medium which contained starch as the source of carbon. Most starch processing enzymes were obtained from *Aspergillus niger*, *Arxula adeninivorans*, *Candida japonica* and *Bacillus sp.* (Gupta *et al.,* 2003). Several alpha amylase producing strains of yeast, fungi and actinomycetes were isolated from soil, (Chadha *et al.,* 1997). *Aspergillus* and *Rhizopus spp*. were more studied in the developing countries due to alpha amylase production probably because of their ubiquitous nature and non-particular nutritional requirements of these organisms. Pandey *et al.* (2005) reported that a strain of *Aspergillus niger* produced different types of enzymes, alpha amylase was one of them. The *Aspegillus sp*. produced a high quality of alpha amylase which has significant industrial importance (Chengyi *et al.,* 1999). Different fermentation processes such as solid state fermentation and submerged fermentation have been used for the production of alpha amylase but literature has shown that the cost of submerged fermentation was high as compared to solid state fermentation (Baysal *et al.,* 2003). Some researcher developed the low cost fermentation medium for the production of alpha amylase by using different agricultural products (Ikram *et al.*, 2003). A limited number of studies on isolation, purification and characterization of alpha amylase from fungal species have been conducted so far (Imran *et al.*, 2011). The aim of this research was the purification and characterization of alpha amylase produced from *Aspergillus niger* through the process of solid state fermentation by using potato starch as inducer substrate.

MATERIALS AND METHODS

Sample collection: A total of 100 soil samples were collected through Potato Baiting Technique. In this technique potatos pieces were buried about four inches deep under the soil. After 8 days, these pieces of potato were brought out from the dig and taken to the lab in a plastic bag.

Isolation: For isolation of fungal species, suspension of 10 grams of decaying potatos in 90 ml sterile distilled water was made and pipette out 0.1 ml from above solution. It was spread on potato dextrose agar (PDA) plate for the isolation of fungus. After that it was incubated at 30^{o}C for 72 hours.

A. Identification Method for Fungus:

a) Scotch tape method: A piece of scotch tape was taken and touched with the fungal growth in petri dish. Then a glass slide was covered with that tape and examined microscopically.

Direct slide preparation method: A drop of potato starch agar medium was placed in the bottom of a petri

dish and a few spores of fungus were transferred into it. After that it was incubated at 30°C for 3-5 days. After incubation, a drop of lacto phenol cotton blue was added to it and examined under the microscope.

Selection of amylase producing fungi: For screening purpose, potato starch agar was prepared and the pH of the medium was calibrated to 6.5 with 1M HCL/1M NaOH. After autoclaving, it was poured into the sterile test tubes and these were kept in slant position. The spores of *Aspergillus niger* was inoculated aseptically under laminar air flow and then incubated for 24-72 hours at 30°C.

Growth kinetics of isolated fungi: The fungal species which gave the higher zone of hydrolysis were selected for study of the kinetics of its growth. For this purpose, we prepared 100 ml potato starch broth and divided in two flasks containing 20ml and 80ml respectively and autoclaved at a temperature of 110°C for 20 minutes. Then cooled it and added a loopful of fungal isolate into 80 ml flask. The incubation was carried out at 28°C at 100 rpm for seven days and the 2nd flask was kept as blank for control purpose. The growth of isolate was observed for seven days by noting the absorbance at 600nm against blank.

Fungal amylase production medium: Fifteen ml distilled water was poured on the PDA agar slant having spores. Spore suspension (0.5 ml) was transferred into medium which contained g/l KH_2PO_4 (1.4), NH_4NO_3 (0.5), KCl (0.1), $MgS0_47H_20$ (0.001) and $FeSO_47H_2O$ (10). Soluble starch and pH was maintained at 6.5. Incubation was carried out with shaking for 72 hours.

Extraction of alpha amylase: It is very easy to remove the fungal mycelium from the enzyme production medium. Pour the whole contents of the flask containing the growing fungus and 100 ml of 100 Mm sodium citrate buffer containing 1 ml Tween eighty of pH 6.5 and this crude filtrate wil be filtered through a Whattman number 1 filter paper. The filtrate contained the crude amylase.

Measurement of alpha amylase activity:

Gram iodine test: Gram iodine was used for qualitative and quantitative analysis of alpha amylase.

DNS methods: Transferred 1 ml of crude extract into a test tube and put 1 ml of 1% soluble of starch in buffer solution. Incubate at 40°C for 30 minute in water bath and took 1ml of crude enzyme extract in another test tube and boiled for 20 minutes. Added 1ml of starch solution in it and also added 2ml of DNS reagent in it. Boil for 5 minutes and determined the color intensity at 600 nm of wavelength.

Characterization of alpha amylase:

Effect of pH**:** Alpha amylase tested at different pH levels ranging from 4 to 10 by using different buffer systems including in succinate buffer, citrate buffer, phosphate buffer and sodium phosphate buffer.

Effect of temperature: Thermostablity of enzyme was checked by using different incubation temperatures range from 20 to 70°C at optimum pH of 6.0.

Effect of substrate concentration: For optimization, we used different substrates including potato, rice, corn and wheat.

Effect of enzyme activators and inhibitors: The activity of alpha amylase was demonstrated by using different metal ions e.g. $FeSo_4$, $MnSo_4$, $CaCl_2$, $ZnCl_2$, $AgNO_3$ and organic compounds including TEMED, EDTA.

RESULTS AND DISCUSSION

The genus Aspergillus produces a huge range of extracellular enzymes, of which amylases are mainly considerable for industrial significance (Murali *et al.*, 2011). The aim of the present study was the isolation, identification and partial characterization of crude alpha amylase produced by *Aspergillus niger*. *Aspergillus niger* grew well on potato starch agar and showed a good amount of alpha amylase production after 72-92 hours of incubation.

A. Identification of Fungus: Based on morphological studies and lactophenol cotton blue investigations the identified fungus was considered as *Aspergillus niger*. The conidia of isolate was dark brown and conidiophores were hyaline, smooth. *Aspergillus niger* required no flooding, no prior replication of colonies on slants; the zones were very sharp and contrast with the blue black background. The isolate of *Aspergillus niger* was identified using various techniques explained in the Handbook of fungi (Alexopoulos CJ 1962). The identification was done by observing morphological and cultural appearances of the isolate.

B. Growth kinetics of isolated fungi: The result of growth kinetics of *Aspergillus niger* was explained in the Graph 1 below. *Aspergillus niger* can maintain stationary phase between 96 to 120 hours. After that the decline phase was observed. This may be due to the depletion of nutrients and increases of waste materials. Almost similar results have been reported by different researchers around the world (Gupta *et al.,* 2010; Khan and Yadav, 2011).

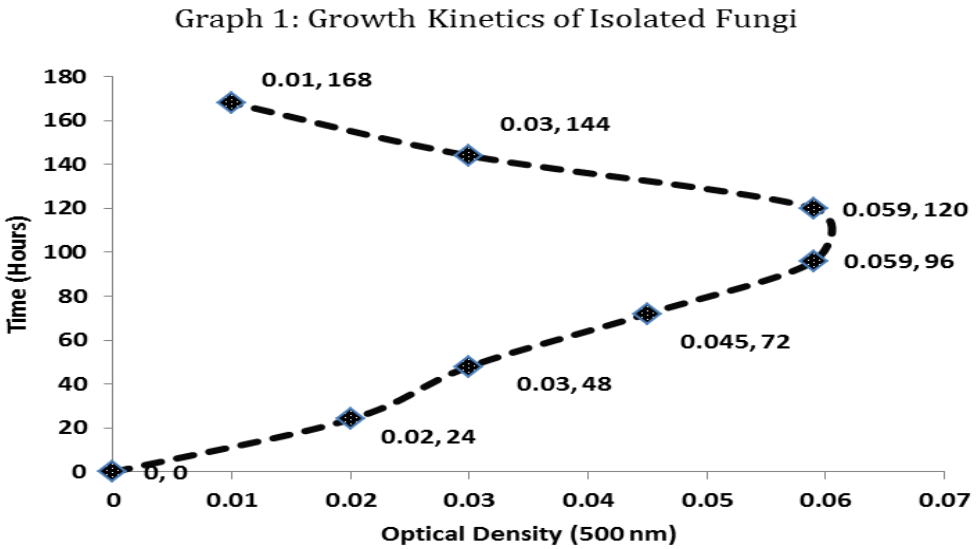
C. Effect of temperature: Temperature has direct effect on the growth activities of the microbes. Therefore, the maximum temperature depends on whether the microorganisms are of mesophilic, thermophilic or

psychrophilic nature. Amongst the different fungal species, most alpha amylase production studies have been completed with mesophilic fungi within the 25–37°C range of temperature (Francis *et al.,* 2003; Adejuwon *et al.*, 2012). The stability of alpha amylase was maintained at 40°C to 50°C. Some researchers have demonstrated the similar thermostablity pattern of some mesophilic fungi (Gupta *et al.,* 2003). *Aspergillus niger* species which have been isolated from marine water showed optimum temperature at 70°C (Mohaptera *et al.,* 1998). Some earlier studies on fungal and yeast sources of alpha amylase production have showed the optimum temperature of 30°C to 70°C (Gupta *et al.,* 2003; Sun *et al.,* 2009). But in the present study the alpha amylase reached at optimum level (50°C) after 72-96 hour of incubation as shown in Graph 2.

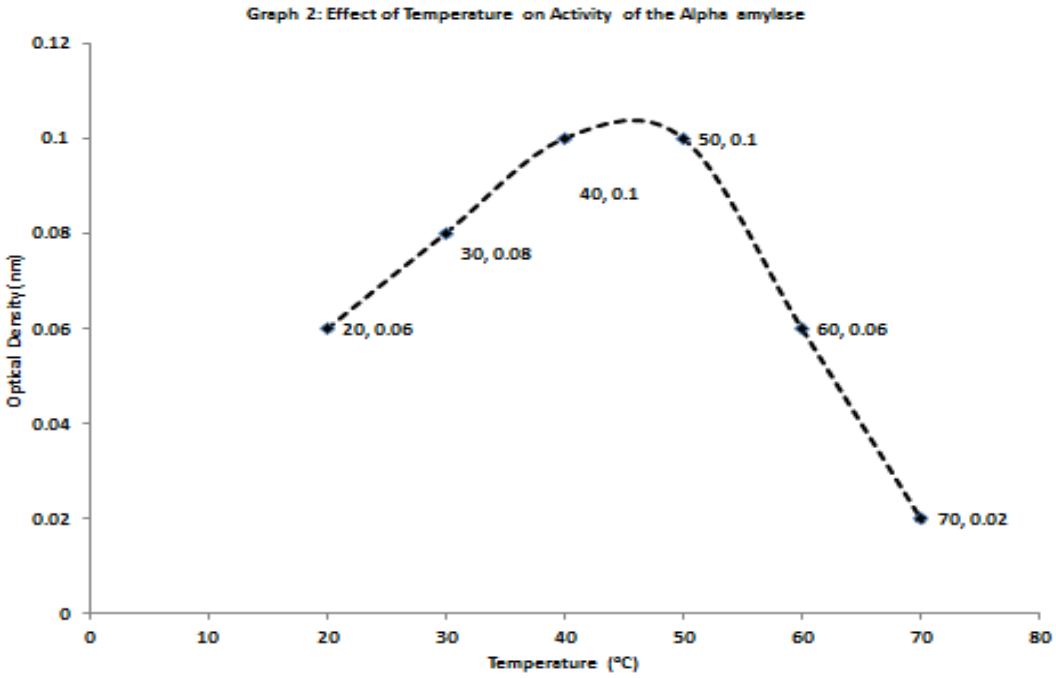
D. Effect of pH: The most important physical parameter which influenced the morphological changes in microbes and enzyme secretion was pH. Since alpha amylase was a pH sensitive enzyme (Joel and Bhimba, 2012). In the present study, the maximum production was achieved at pH 6 as shown in Graph 3. Some researcher reported different range of pH 4.5 to 6, in which *Aspergillus niger* showed maximum enzyme activity (Shafique *et al.,* 2009). Namasivayam and Nirma (2011) isolated *Aspergillus niger* in marine water, which showed maximum enzyme production at pH 9. *Aspegillus flaviceps* indicated optimum enzyme activity at pH 6.5 (Sukla, 2009). Irfan *et al.* (2012) reported that the *Aspergillus niger*-17 gave maximum enzyme production at pH 5.0.

E. Effect of different substrate: The Graph 4 shows a comparison between the alpha amylase activity in the medium containing different substrates including potatos, wheat, corn and rice starch used in the present study. Potatos starch represented the optimum amylase activity as compared to other substrates. Puri *et al.,* (2013) used agricultural residues including paddy husk, rice bran and wheat bran for the optimum production of alpha amylase from *Aspergillus oryzae*. Khan and Yadav (2011) used different industrial and vegetable waste as substrate for the production of alpha amylase, but the optimum enzyme activity was found in wheat bran. Rice was the best substrate for the maximum production of alpha amylase (Adejuwon *et al.,* 2012). Similar findings have been reported by using different substrates including's wheat bran, wheat straw, rice, for the maximum production of alpha amylase by using *Humicola lanuginose* species in solid state fermentation. Among these substrates wheat bran has been proved as a best substrate for the optimum production of alpha amylase (Singh *et al.,* 2009).

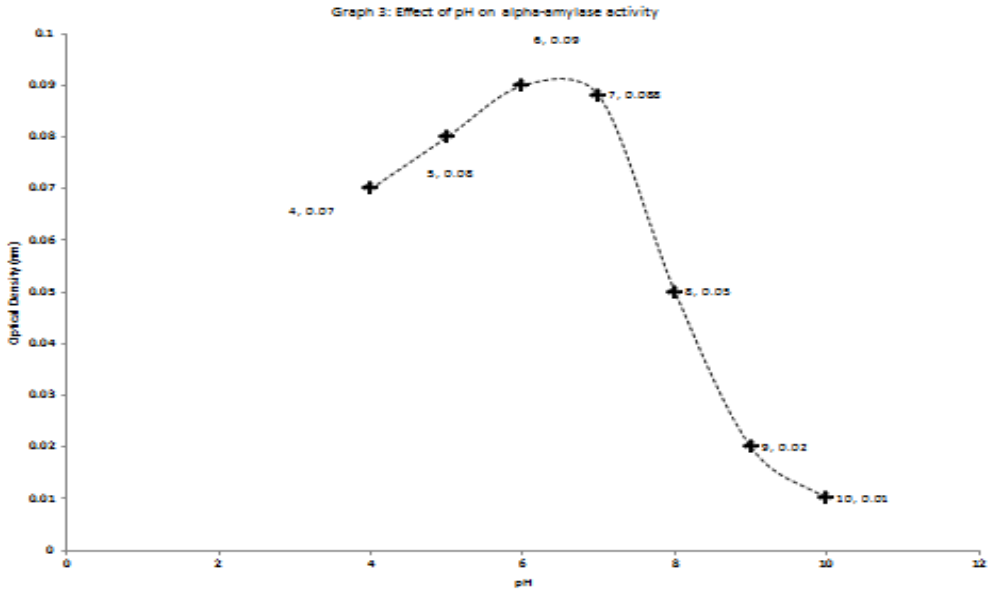
F. Effect of enzyme activators and inhibitors: It was assumed that hydrolytic enzymes particularly alpha amylases are known to be metalloenzymes. So the presence of these ions has a suitable effect for the growth of microorganisms as well as their enzyme production. For that purpose we evaluated different activator and inhibitor including $FeSo_4$, $MnSo_4$, $CaCl_2$, $ZnCl_2$, EDTA, TEMED and $AgNO_3$. Among these sources $CaCl_2$ gave the best result for the optimum production of enzyme and $AgNO_3$ was the strongest inhibitor for the production of alpha amylase. These results have been predicted in Graph 5. Similar findings have been observed in many studied in the past (Abou Zeid, 1997; Mishra *et al.,* 2005; Varalakshmi *et al.,* 2009). Various past studies indicated that $ZnCl_2$ and EDTA have strong ability to inhibit the enzyme production (Ahmad *et al.,* 2010; Hailemariam *et al.,* 2013).



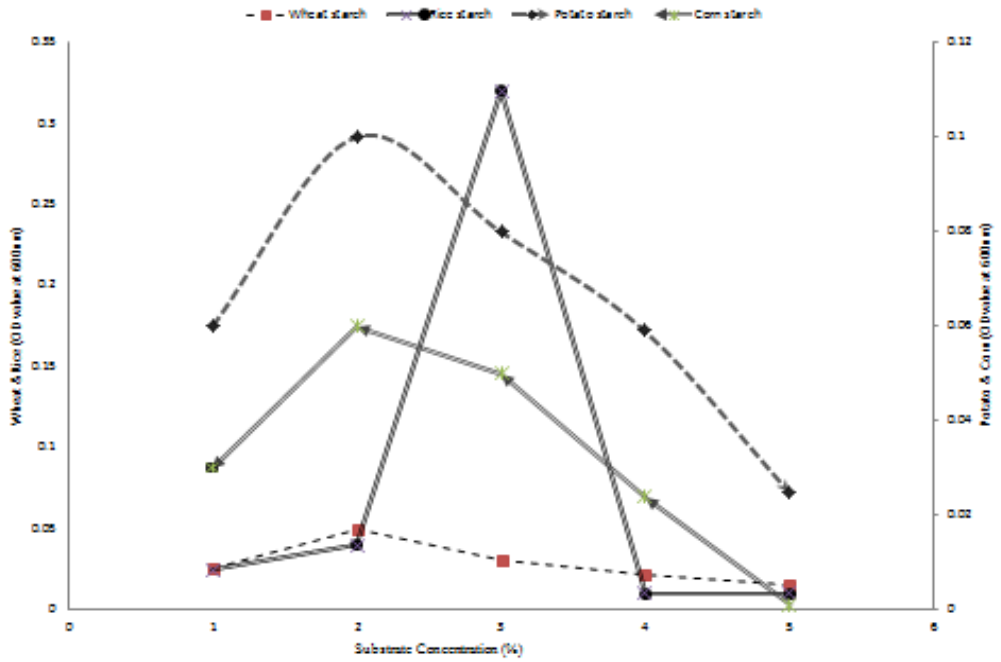
Graph 1: Showing the growth kinetics of isolated fungi



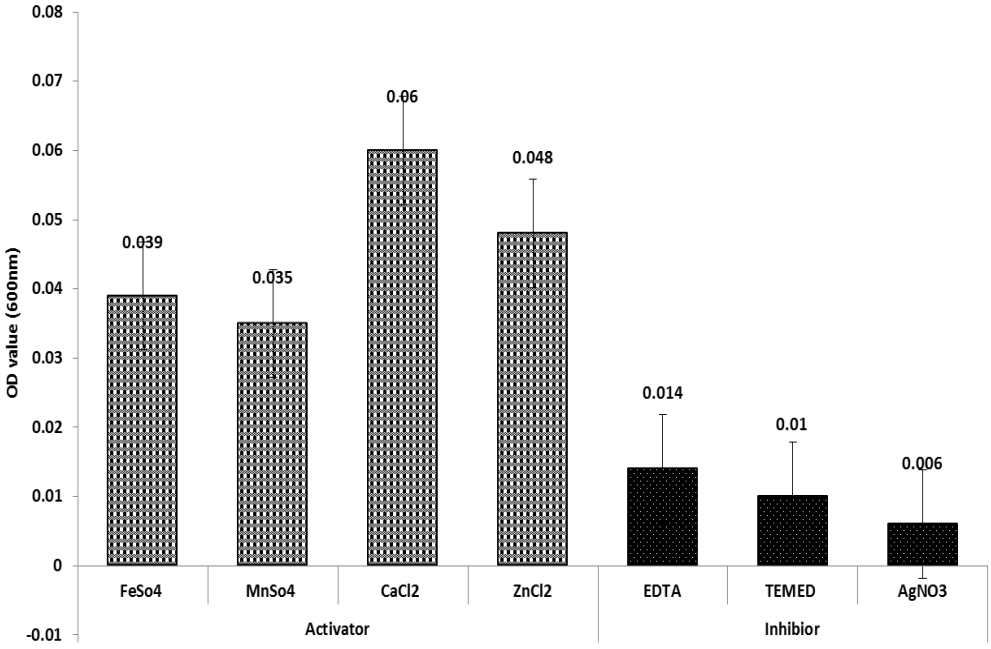
Graph 2: Showing the effect of Temperature on the Activity of Alpha amylase



Graph 3: Showing the effect of pH on the Activity of Alpha amylase



Graph 4: Showing the effect of Different Substrates on the Activity of Alpha amylase



Graph 5: Showing the effect of Various Activators and Inhibitors at Alpha amylase Production.

Conclusion: A specific culture *Aspergillus niger* was used for the optimum production of alpha amylase through submerged fermentation using different substrates. Out of the different starch substrates tested for optimum alpha amylase production, potato starch showed the best results. Many other factors were also examined for obtaining maximum enzyme production. Enzyme was stable at 50°C temperature and 6.0 pH. Among different metal ions $CaCl_2$ gave best results for the optimum production of enzyme and $AgNO_3$ was the strongest inhibitor for the production of alpha amylase. Therefore, it can be concluded that *Aspergillus niger* can be used on a large scale production of alpha amylase.

REFERENCES

AbouZeid, A. M. Production, purification and characterization of an extracellular á-amylase enzyme isolated from *Aspergillus flavus*. Microbios., 89: 55-66 (1997).

Adejuwon, A. O., O. Olajide and O. Adewole. Production of amylase from *Aspergillus niger* using a defined synthetic growth medium and also rice (*Oryza sativa*) as growth substrate. J. Med. Res., 7: 91-94 (2012).

Ahmad, A., C. J. Khan and L. Milad. A novel thermostable, acidophilic alpha-amylase from a new thermophilic Bacillus sp. Ferdowsicous. Int. J. Biol. Macromol., 46: 289-297 (2010).

Alexopoulos, C. J. Introductory Mycology. John Wiley and Sons Inc., New York, London. Pp 263-273 (1962).

Alva, S., J. Anumpama, J. Savla, Y. Y. Chiu, P. Vyshali, M. Shruti, B. S. Yogeetha, D. Bhavya, J. Purvi, K. Ruchi, B. S. Kumudini and K. N. Varalakshmi. Production and characterization of fungal amylase enzyme isolated from Aspergillus sp. JGI 12 in solid state culture. Afr. J. Biotechnol., 6: 576-581 (2007).

Amirul, A., S. L. Khoo., M. N. Nazalan, M. S. Razip and M. N. Azizan. Purification and properties of two forms of glucoamylase from *Aspergillus niger*. Folia Microbiol., 41: 165-174 (1996).

Asghar, M., M. Yuqub, M. A. Sheikh and A. R. Baraque. Optimization of cellulase production by Arachniotus sp. using corn stover as substrate. J. Anim. Plant. Sci., 10: 37-40 (2000).

Baysal, Z., F. Uyar and C. Aytekin. Solid-state fermentation for production of alpha -amylase by a thermo tolerant *Bacillus subtilis* from hot-spring water. Proc. Biochem., 38: 1665-1668 (2003).

Chadha, B. S., S. Singh and G. Vohra. Shake culture studies for the production of amylase by *thermomycece lanuginous*. Acta Microbio. Immunol. Hung., 44: 181-185 (1996).

Chengyi, W. H., M. Ming and R. Jiang. Studies on the properties of α-amylase produced by *Bacillus pumilus* 289 (PBX96). Acta. Micropalaeon. Sinica., 32: 400-404 (1999).

Francis, F., A. Sabu, K. M. Nampoothiri, S. Ramachandran, S. Ghosh, G. Szakacs and A. Pandey. Use of response surface methodology for optimizing process parameters for the production of alpha amylase by *Aspergillus oryzae*. Biochem. Eng. J., 15: 107-115 (2003).

Gupta, A., N. Gautam and D. R. Modi. Optimization of alpha amylase production from free and immobilized cells of *Aspergillus niger*. E3 J. Biotechnol. Pharmaceut. Res., 1: 001-008 (2010).

Gupta, R., P. Gigras, H. Mohapatra, V. K. Goswami and B. Chauhan. Microbial Alpha amylases: a biotechnological perspective. Proc. Biochem., 38: 1599-1616 (2003).

Hailemariam, A. T., T. B. Gezmuand and G. D. Haki. Thermostable alpha amylase from geothermal sites of ethiopia (afar region): isolation, purification and characterization. Green J. Biolog. Sci., 3: 061-073 (2013).

Ikram-ul- Haq, H. Ashraf, J. Iqbal and M. A. Qadeer. Production of alpha amylase by *Bacillus licheniformis* using an economical medium. Biores. Technol., 87: 57-61 (2003).

Imran, M., M. J. Asad, M. Gulfraz, N. Mehboob, N. Jabeen, S. H. Hadri, M. Irfan, Z. Anwar and D. Ahmed. Hyper production of glucoamylase by *Aspergillus niger* through chemical mutagenesis. Int. J. Physical Sci., 6: 6179-6190 (2011).

Irfan, M., M. Nadeem and Q. Syed. Media optimization for amylase production in solid state fermentation of wheat bran by fungal strains. J. Cell Molec. Biol., 10: 55-64 (2012).

Joel, E. L. and B. V. Bhimba. Production of alpha amylase by mangrove associated fungi Pestalotiopsis microspore strain VB5 and *Aspergillus oryzae* strain VB6. Indian J. Geo-Marine Sci., 41:279-283 (2012).

Khan, J. A. and S. K. Yadav. Production of alpha amylases by *Aspergillus niger* using cheaper substrates employing solid state fermentation. Int. J. Plant Anim. Environm. Sci., 1: 100-108 (2011).

Mishra, B. K. and D. Dadhich: Production of amylase and xylanase enzymes from soil fungi of Rajasthan. J. Adv. Dev. Res., 1: 21-23(2010).

Mishra, S., S. B. Noronda and G. K. Suraishkumar. Increase in enzyme productivity by induced oxidative stress in *Bacillus Subtilis* cultures and analysis of its mechanism using microarray data. Proc. Biochem., 40: 1863-1870 (2005).

Mohapatra, B. R., U. C. Banerjee and M. Bauji. Characterization of fungal amylase from Mucor sp. Associated with the marine sponge Spirastrella spp. J. Biotechnol., 60: 113-117 (1998).

Murali, K. C., S. Chellu and C. Suresh. Fermentative Production and Thermostability Characterization of á Amylase from Aspergillus Species and Its Application Potential Evaluation in Desizing of Cotton Cloth. Biotechnol. Res. Int., 26: 1-8 (2011).

Namasivayam, S. K. R. and D. Nirma. Enhanced production of alpha amylase using vegetable waste by *Aspergillus niger* strain SKO1 marine

isolate. Indian J. Geo-Marine Sci., 40: 130-133 (2011).

Pandey, A., C. Webb, C. R. Soccol and C. Larroche. Rice bran as a substrate for proteolytic enzyme production. Enzyme Technology, New delhi: Asiatech Publishers, Inc. 197 (2005).

Puri, S., M. Arora and L. Sarao. Production and optimization of amylase and glucoamylase using *Aspergillus oryzae* under solid state fermentation. Int. J. Res. Pure Appl. Microbiol., 3: 50-56 (2013).

Shafique, S., R. Bajwa and S. Shafique. Screening of *Aspergillus niger* and *Aspergillus flavus* strains for extra cellular α-amylase activity. Pakistan J. Bot., 41(2): 897-905 (2009).

Singh, R. K., S. Kumar and S. Kumar. Production of alpha amylase from agricultural byproducts by *Humicola lanuginosa* in solid state fermentation. Curr. Trends Biotechnol. Pharm., 3: 172-180 (2009).

Sukla, K. Potato peel as a solid state substrate for thermostable alpha amylase production by thermophilic bacillus isolate. World J. Microbial. Biotechnol., 25: 417-422 (2009).

Sun, H., X. Ge, L. Wang, P. Zhao and M. Peng. Microbial production of raw starch digesting enzymes. Afr. J. Biotechnol., 8: 1734-1739 (2009).

Varalakshmi, K. N., B. S. Kumudini, B. N. Nandini, J. Solomon, R. Suhas, B. Mahesh and A. P. Kavitha. Production and characterization of α-amylase from *Aspergillus niger* JGI 24 isolated in Bangalore. Polish J. Microbiol., 58: 29-36 (2009).