ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 7 (3): 551-556, 2008
ISSN 1682-296X

Production and Characterization of α-Amylase from *Aspergillus niger*

[1]A. Gupta, [2]V.K. Gupta, [3]D.R. Modi and [2]L.P. Yadava
[1]M.J.P. Rohilkhand University, Bareilly, U.P., India
[2]Central Institute for Sub-Tropical Horticulture, Lucknow-227 107, India
[3]Department of Biotechnology, B.B.A. University, Lucknow-226025, U.P., India

Abstract: The cultural and nutrient requirements of *Aspergillus niger* for production of α-amylase in production media containing different pH, temperature, incubation period, metal ion concentrations, surfactants, carbon sources and nitrogen sources were quantified in present study. The optimum pH, temperature and incubation period for enzyme production were 5.0, 30°C and 5th day, respectively. Of the carbon sources, starch at 0.5% was recorded to be the best carbon source for enzyme production. Peptone at 0.03% was ideal nitrogen source. However, surfactants Tween-80, Triton X-100 and Sodium dodecyl sulphate at 0.02, 0.002 and 0.0002% concentration were most effective for enhancement of α-amylase production. The main objectives of the present study were to use a suitable fungal strain for production of extra cellular alpha-amylase, to determine the time course for the production of alpha-amylase and to study the effects of external substances that may enhance the production of extra cellular alpha-amylase, including metal ions and surfactants.

Key words: *Aspergillus niger*, α-amylase, metal ion concentrations, surfactants, carbon sources

INTRODUCTION

α-amylases enzyme are all α-1, 4-glucan 4-glucanohydrolases (E.C. 3.2.1.1) that are important enzymes employed in the starch processing industries for the hydrolysis of polysaccharides such as starch into simple sugar constituents (Akpan *et al*., 1999b; Fogarty and Kelly, 1980; Haq *et al*., 2002; Nigam and Singh, 1995). Starch degrading enzymes like amylase have received great deal of attention because of their perceived technological significance and economic benefits. This enzyme is also used for the commercial production of glucose. In storage tissues such as seeds, starch a polysaccharide of glucose is hydrolyzing for utilization by the growing seedlings to meet its energy requirement. Now a days the new potential of using microorganism as biotechnological sources of industrially relevant enzymes has stimulated renewed interest in the exploration of extra cellular enzymatic activity in several microorganisms (Akpan *et al*., 1999b; Bilinski and Stewart, 1995; Buzzini and Martini, 2002). These enzymes are found in animals (saliva, pancreas), plants (malt), bacteria and molds (Abu *et al*., 2005; Omemu *et al*., 2005). Sources of amylases in yeast, bacteria and moulds have been reported and their properties described by Adebiyi and Akinyaanju (1998), Akpan *et al*. (1999b) and Buzzini and Martini (2002). The amylase of fungal origin was found to be most stable than the bacterial enzyme (Duochaun *et al*., 1997). To prepare these extra cellular enzymes on a commercial scale, many attempts have been made to specify cultural conditions and to select superior strains of the fungus (Abu *et al*., 2005). Few attempts have been made to elucidate the control mechanism involved in the formation and secretion of the extra cellular enzymes. Among mold species producing high levels of amylase, *Aspergillus niger* is used for commercially production of alpha-amylase. Studies on fungal amylase especially in the developing countries have concentrated mainly on *Aspergillus* spp. probably because of ubiquitous nature and non-fastidious nutritional requirement of these organism fungus (Abu *et al*., 2005; Gomes *et al*., 2005). Using thermo acidophilic and alkaline amylase it is possible to extent the use of amylases under extreme condition of pH and temperature (Kindle, 1983). α-amylases from *Aspergillus oryzae* was the first microbial enzyme to be manufacture for sale and was named by solid-state cultivation for many years. The manufacture had switched to submerged fermentation and such methods have been reviewed. Some difficulties were encountered in making this change since the most effective preparation of some applications contain other enzymes, especially amyloglucosidases and the submerged methods gives a narrower spectrum of additional additives. So, it is worthwhile to isolate a suitable strain of *A. niger* for efficient mechanism. Selection of suitable production media is very essential

Corresponding Author: Dr. L.P. Yadava, Central Institute for Sub-Tropical Horticulture, Lucknow-227 107, U.P., India
Tel: +91 522 2841023 Fax: +91 522 2841025

for growth of microorganism as well as production of enzyme. The production of alpha-amylase by mould has been greatly affected by cultural and nutritional requirement (Pedersen and Nielson, 2000; Prescott and Dunn, 1987). Therefore, the objective of this study was the characterization and optimization of cultural conditions for the production of alpha-amylase.

MATERIALS AND METHODS

The study was carried out at Biotechnology Laboratory, B.B.A. University, Lucknow, U.P., India during 2006-2007.

Isolation and identification of *Aspergillus niger* strain: The *Aspergillus* culture were isolated from soil by serial dilution method of Clark *et al.* (1988), 1 g soil sample was dissolved in 100 mL sterilized distilled water. The soil suspension was diluted up to 10^{+3} to 10^{-3} and 0.5 mL of diluted suspension was used and micro-organism producing starch digesting α-amylase screened according to method described by Bergmann *et al.* (1988) and Akpan *et al.* (1999a). *Aspergillus niger* colonies producing large clear zone were picked up and purified by streaking on PDA. Identification was based on cell and colony morphology characteristics as per method described by Rasper and Fennel (1965). The young colonies of *Aspergillus niger* were aseptically picked up and transferred to PDA slants and incubated at 27°C for 4-5 days for maximum growth.

Growth medium: The medium contained (% w/v): Starch (1%); KH_2PO_4 (0.2%); $(NH_4)_2SO_4$ (0.14%); $CaCl_2$ (0.03%); $MgSO_4.7H_2O$ (0.03%), Urea (0.03%), Peptone (0.1%), Trace element solution (0.01%), Triton X-100 (0.02%). Trace element solution contain in 500 mL (2.5 g $FeSO_4$, 1.0 g $CoCl_2$, 1.76 g $ZnSO_4$, 0.98 g $MnSO_4$). The pH of the medium was adjusted to 4.8 with concentrated HCl, medium was sterilized by autoclaving at 121°C and 15-17 psi for 30 min.

Inoculum and fermentation: A certain inoculum size of conidia (Each mL of cells suspension contained 2.0×10^6 cells) was transferred from a stock culture in 250 mL flask containing 50 mL of growth medium. The flasks were incubated for 72 h at 28±2°C on a rotatory shaker at 150 rpm. On the last day of incubation period (72 h), the fungal mass was separated by centrifugation at 4500 rpm for 10 min. The clear supernatant (Crude enzyme) was used for estimation of α-amylase, the enzyme activity was expressed in number of units. One unit of enzyme was defined as the amount of enzyme (protein) in milligram required for hydrolysis of starch to produce a millimolar of reducing sugar (glucose) in 1 h under assay conditions. The specific activity was defined as number of units per gram protein.

Preparation of enzyme: The enzyme was precipitated from clear supernatant at 4°C by adding solid ammonium sulphate to achieve 85% saturation. The ammonium sulphate was added slowly, keeping the solution in ice and the protein was allow to precipitate by keeping it overnight at 4°C. The protein was separated by centrifugation at 2000 x g for 30 min at 4°C, dissolved in minimum volume of phosphate buffer (50 mM and pH 7.2) and used immediately for activity determination.

Enzyme assay: Enzyme activity was determined by DNS method described by Mandels *et al.* (1976) using starch as the substrate. The reaction mixture contained the following in a total volume of 2 mL: 10 mg starch, 1 to 1.8 mg protein and 50 mM Sodium-phosphate buffer of pH-7. The reaction mixture was incubated at 37°C for 30 min and the reaction was terminated by addition of 3 mL of DNS solution (3, 5-dinitro Salicylic Acid). After stopping the reaction the tubes were placed in boiling water bath for 5 min and then cooled and absorbance was determined at 540 nm. The amount of glucose produced was calculated by referring to the standard plot using glucose as the reducing sugar (The standard plot was prepared simultaneously).

Nutritional requirement for extra cellular α-amylase production: Growth and incubation period for maximum alpha-amylase production was quantified along with the effect of carbon, nitrogen source on productivity of enzyme was studied on different percentage of carbon (0.5 to 2.0%); nitrogen source (0.3%); variable temperature range (20 to 50°C) and pH range of (3 to 8) for productive fermentation media. The initial pH of medium was adjusted using concentrated HCl. Effect of Ca^{++} metal ions concentration (10 to 100 mM) and surfactants (Tween-80, Tween-20, Triton X-100 and SDS at the rate of 0.0002 to 0.2% by w/v) were studied.

RESULTS

Twenty-six isolates of *Aspergillus niger* were isolated from different soil sample of different places in India and were screened for maximum production of alpha-amylase by observing clear zone of starch hydrolysis in Petridishes. The *Aspergillus niger* were identified according to Onion *et al.* (1987). Among all the isolates tested the culture No. AN-9 showed maximum production of alpha-amylase and was selected for characterization studies of alpha-amylase.

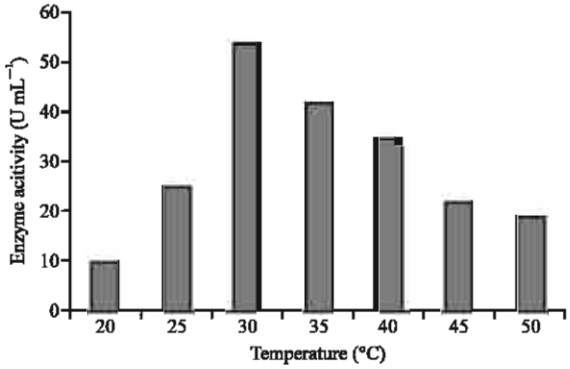

Fig. 1: Effect of temperature on extra cellular α-amylase production

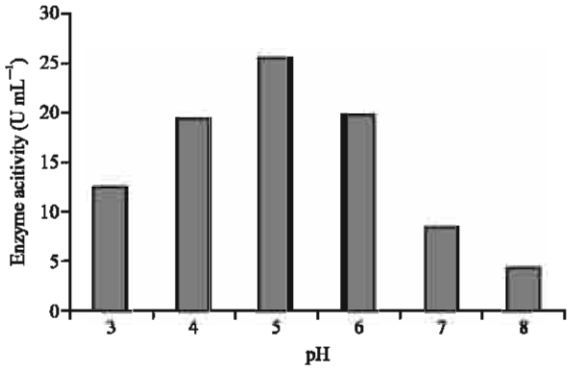

Fig. 2: Effect of pH on extra cellular α-amylase production

Assay of enzyme production was carried out at various temperature ranges 20 to 50°C for 24 h. It was found that *Aspergillus niger* should considerable amount of growth at 20°C but there was no enzyme production. However, the optimum temperature for enzyme production was 30°C (Fig. 1).

The initial pH of medium was adjusted to variable pH range by adding the 0.1 N HCl. Enzyme purified was tested in the pH range (pH 3 to 8). The production of alpha-amylase was found to be the best at pH 5.0. Below and above this pH production of alpha-amylase was significantly lower (Fig. 2).

The alpha-amylase activity was determined after every 24 h of incubation in order to determine the optimum incubation period for maximum production of extra cellular alpha-amylase. The enzyme production however, started after 24 h of inoculation and showed maximum production on 5th day of incubation period at 30°C (Fig. 3).

Flask containing production media supplemented with carbon sources (glucose, sucrose, starch, carboxymethyl cellulose, fructose, sorbitol, xylose, galactose and dextrin). The influence of these carbon

Table 1: Enzymatic production of α-amylase in U mL^{-1} as influenced by different carbon sources and their concentration

	Concentration of carbon source (%)		
Carbon sources	0.5	1.0	2.0
Sucrose	44.40	34.60	27.20
Starch	48.80	36.80	28.80
Glucose	10.44	7.52	1.88
Dextrin	42.60	33.50	26.60
Fructose	37.50	30.40	24.20
Carboxymethyl cellulose	11.82	8.12	2.42
Sorbitol	32.20	28.50	22.80
Xylose	35.50	29.60	23.60
Galactose	41.40	31.20	25.50

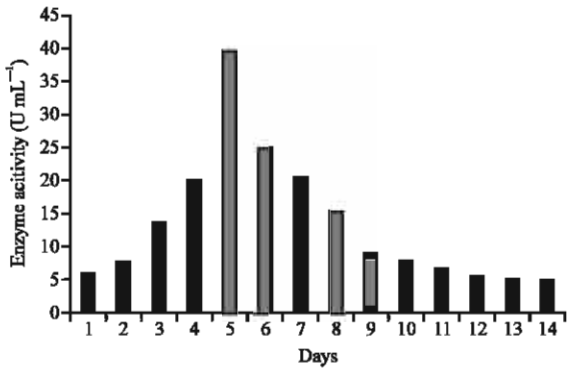

Fig. 3: Effect of incubation period on extra cellular α-amylase production

sources were tested at different concentrations (0.5 to 2.0%). Starch, Sucrose and dextrin and galactose were good carbon sources for amylase production. Xylose, fructose and sorbitol could be considered as moderate source while carboxymethyl cellulose was poor producer of amylase as it was found that 0.5% concentration is good carbon source for amylase production but carboxymethyl cellulose and glucose at 0.5% concentration produced low amount of α-amylase (Table 1).

Effect of different nitrogen sources on the production of alpha-amylase was studied, it was observed that casein and gelatin caused poor enzyme production. Peptone supported maximum production of enzyme whereas urea produced considerable amount of alpha-amylase. The optimum concentration of peptone was 0.03% (Fig. 4).

Ca^{++} is a divalent ion, which is known to affect the permeability of the cells. Since alpha-amylase is an extra cellular enzyme, it was considered important to investigate the effect of Ca^{++} on the production of extra cellular enzyme. In a typical experiment the enzyme production and the cell biomass were determined after 5 days of incubation which gave maximum yield of the enzyme. The results indicate that Ca^{++} in the production medium have adverse effect on the production of alpha-amylase, it was

Table 2: Enzymatic production of α-amylase in U mL^{-1} as influenced by different surfactants sources and their concentration

Surfactants (W/V)	Concentration of surfactants source (%)			
	0.0002	0.002	0.02	0.2
Tween-80	52.20	56.10	63.40	60.80
Triton X-100	52.40	62.80	53.20	48.80
SDS	64.40	63.20	52.20	48.40

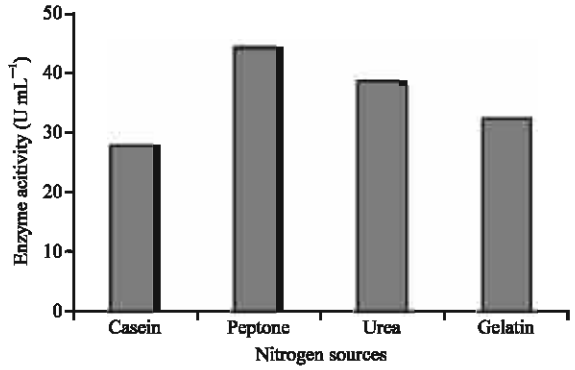

Fig. 4: Effect of different nitrogen sources at 0.03% concentrations on extra cellular α-amylase production

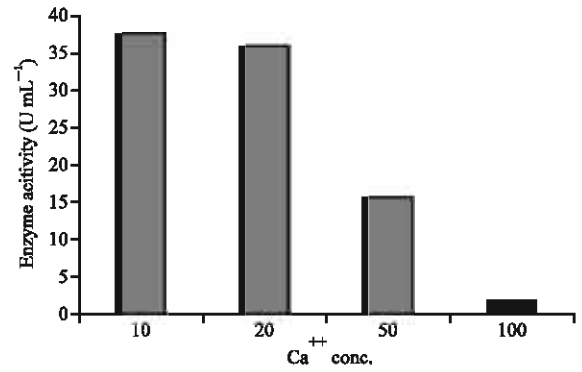

Fig. 5: Effect of Ca^{++} at different concentrations on extra cellular α-amylase production

observed that at 100 mM concentration of Ca^{++}, nearly 50% inhibition was observed at 50 mM concentration of Ca^{++} (Fig. 5). The detergent Tween-80, Triton X-100 and SDS favored more amylase production in culture media. Tween-80 at 0.02% caused maximum enhancement where as Triton X-100 and SDS increased amylase activity at 0.002 and 0.0002%, respectively (Table 2).

DISCUSSION

The occurrence of amylolytic organism from the soil agrees with earlier reports of Rehana and Nand (1989). Adebiyi and Akinyanju (1998) also reported that the soil is known to be a repository of amylase producer. *Aspergillus niger* requires no flooding, no prior replication of colonies on slants; the zones are very sharp and contrast with the blue-black background (Akpan *et al*., 1999a). Studies of Brgmann *et al.* (1988) reveals that enzyme preparation of *Aspergillus* spp. consisted of at least two enzymes, glucoamylase and α-amylase. These thermostable α-amylases differ in their pH optimum, temperature optimum, temperature stability and in several other physiochemical depending on the species origin. Hence different enzymes have found specific applicability in different industries. A number of reports exist regarding the influences of various environmental conditions like effect of pH value and temperature optimum, incubation period, carbon sources, nitrogen sources and metal ion on the production of thermostable α-amylase by *Aspergillus niger* (Okolo *et al*., 2006; Nagamine *et al*., 2003; Francis *et al*., 2002; Carlsen and Nielson, 2001; Pedersen and Nielson, 2000; Abou-Zeid, 1997). Increase in the incubation period resulted decrease in the production of α-amylase by culture of *Aspergillus niger*. It may be due to the fact that after maximum production of α-amylase enzyme (maximum incubation time), the production of other by product and depletion of the nutrients. These byproducts inhibited the growth of fungi and hence enzyme formation (Duochaun *et al*., 1997). The enzyme is very sensitive to pH. Therefore, the selection of optimum pH is very essential for the production of α-amylase (McMohan *et al*., 1999). It was concluded that both organic and inorganic were required for the optimum growth of organism as well as for the production of α-amylase. Acidic medium was required for optimum production of α-amylase as the *Aspergillus niger* was used as organism. Enzyme production started at pH 3.0 and cease at pH 8.0 maximum enzyme production of enzyme occurred at pH 4 to 5, very little growth was observed without enzyme production in medium at initial pH 3 to 4. At higher pH value (10-11), growth was quite high but enzyme production was low. The effect of different carbon and nitrogen source for α-amylase production by *Aspergillus niger* (Bajpai and Bajpai, 1989). Viswanathan and Surlikar (2001) reported the effect of calcium ions on production of α-amylase by *Aspergillus* spp. Agger *et al.* (2001) suggested the ability of filamentous fungi to secrete large amounts of extra-cellular proteins makes them well suited for protein production. Van der Maarel *et al.* (2002) concluded the properties of starch converting enzyme of the α-amylase family. Since the enzyme was precipitated by Ammonium Sulphate. It may be ruled out that Ca^{++} directly inhibit the activity of enzyme. We believe the effect of Ca^{++} is the inhibition of production of α-amylase *in vivo* by *Aspergillus niger*

cells. There is also inhibition in cell mass. Interestingly the specific activity of the enzyme was reduced to 1.6 units/mg from about 5.5 units/mg protein, by 50 mM, Ca^{++} concentration in the production medium. Indicating that the inactive protein excreted into the medium was enhanced causing reduction in the specific activity of the enzyme. Thus from these results we conclude that the presence of Ca^{++} in the production medium while enhances the amount of total protein secreted into the medium the total amount of α-amylase activity was greatly reduced.

Therefore, it was concluded in our present study that both nutritional and cultural conditions were required for optimum growth and production of α-amylase from *A. niger*.

ACKNOWLEDGMENT

We are thankful to Department of Biotechnology, BBA University for financial support to complete this research. Dr. HD Kumar, Former Head, Department of Botany, BHU and Late. Dr. CB Sharma for providing the research guidance.

REFERENCES

Abou-Zeid, A.M., 1997. Production, purification and characterization of an extracellular alpha amylase enzyme isolated from *Aspergillus flavus*. Microbias, 89: 55-56.

Abu, E.A., S.A. Ado and D.B. James, 2005. Raw starch degrading amylase production by mixed culture of and *Saccharomyces cerevisae* grown on sorghum pomace. Afr. J. Biotechnol., 4: 785-790.

Adebiyi, C.A.B. and J.A. Akinyaanju, 1998. Thermophilic amylase producers from the soil. Nig. J. Sci. Technol., 11: 30-38.

Agger, T., A. Spohr and J. Nielsen, 2001. Amylase production in high cell density submerged cultivation of *Aspergillus oryzae* and *nidulans*. Applied Microbiol. Biotechnol., 55: 81-84.

Akpan, I., M.O. Bankole and A.M. Adesemowo, 1999a. A rapid plate culture method for screening of alpha-amylase producing microorganism. Biotechnol. Tech., 13: 411-413.

Akpan, I., M.O. Bankole, A.M. Adesemowo and G.O. Lantunde-Data, 1999b. Production of alpha amylase by *Aspergillus niger* in a cheap solid medium using rice bran and agricultural material. Trop. Sci., 39: 77-79.

Bajpai, B.P. and P.K. Bajpai, 1989. High temperature alkaline alpha-amylase from *Bacillus licheniformis* TCRDC-B13. Biotechnol. Bioeng., 33: 72-78.

Bergmann, F.W., J. Abe and S. Hizukuri, 1988. Selection of microorganism which produce raw-starch degrading amylase. Applied Microbiol. Biotechnol., 27: 443-446.

Bilinski, C.A. and G.C. Stewart, 1995. Yeast Protease and Brewing. In: Yeast Biotechnology and Biocatalyst, Verachtert, H. and Mot (Eds.). Marcel Dekker, New York, pp: 147-162.

Buzzini, P. and A. Martini, 2002. Extracellular enzymatic activity profiles in yeast and yeast like strains isolated from tropical environments. J. Applied Microbiol. 93: 1020-1025.

Carlsen, M. and J. Nielson, 2001. Influence of carbon sources on alpha amylase production by *Aspergillus oryzae*. Applied Microbiol. Biotechnol., 57: 346-349.

Clark, H.E., E.F. Geldrich, P.W. Kabler and C.B. Huff, 1988. Applied Microbiology. 1st Edn. International Book Company, New York, pp: 53.

Duochaun, L., Y. Yijun, P. Youliang, S. Chongyao, Z. Peijin and H. Yicum, 1997. Purification and properties of thermostable alpha amylase from thermophilic fungus *Thermomyces lanuginosus*. Acta. Microbiol. SIN., 37: 107-117.

Fogarty, W.M. and C.T. Kelly, 1980. Amylase, Amyloglucosidase and Related Glucanases. In: Microbial Enzymes and Bioconversions, Rose, A.H. (Ed.). Vol. 5, Academic Press, London, pp: 115-170.

Francis, F., A. Sabu, K.M. Nampoothiri, S. Szakaas and A. Pandey, 2002. Synthesis of alpha amylase by *oryzae* in solid state fermentation. J. Basic Microbiol., 42: 3206-3206.

Gomes, E., S.R. Souza, R.P. Grandi and E.D. Silva, 2005. Production of thermostable glucoamylase by newly isolated *flavus* A. 1.1 and *Thermomyces lanuginosus* A 13.37. Braz. J. Microbiol., 36: 75-82.

Haq, I., H. Ashraf, R. Abdullah and A.H. Shah, 2002. Isolation and screening of fungi for the biosynthesis of alpha amylase. Biotechnology, 2: 61-66.

Kindle, K.L., 1983. Characteristics and production of thermostable α-amylase. Applied Biochem. Biotechnol., 8: 153-153.

Mandels, M., R.E. Andreotti and C. Roche, 1976. Measurement of Saccharifying cellulose. Biotechnol. Bioeng. Symp., 6: 21-33.

McMohan, E.M., C.T. Ketty and W.M. Forgarty, 1999. High maltose producing amylotic system of a *Streptomyces* spp. Biotechnol. Lett., 21: 23-26.

Nagamine, K., K. Murashima, T. Kata, H. Shimoi and K. Ito, 2003. Mode of alpha amylase producing the Schochu Koji mold *Aspergillus kawachii*. Biosci. Biotechnol. Biochem., 67: 21191-21202.

Nigam, P. and D. Singh, 1995. Enzymes and microbial system involved in starch processing enzyme. Microb. Technol., 17: 770-778.

Okolo, B.N., L.I. Ezeogu and C.N. Mba, 2006. Production of raw starch digesting amylase by grown on native starch sources. J. Sci. Food Agric., 69: 109-115.

Omemu, A.M., I. Akpan, M.O. Bankole and O.D. Teniola, 2005. Hydrolysis of raw tuberstarches by amylase of *Aspergillus niger* AM 07 isolated from soil. Afr. J. Biotechnol., 4: 19-25.

Onion, A.H.S., D. Allsopp and O.W. Eggins, 1987. Smiths Introduction to Industrial Mycology. 7th Edn. Edward Arnold Ltd., London, pp: 187-188.

Pedersen, H. and J. Nielson, 2000. The influence of nitrogen sources on alpha amylase productivity of *Aspergillus oryzae* in continuous culture. Applied Microbiol. Biotechnol., 53: 278-281.

Prescott, S. and A. Dunn, 1987. Industrial Microbiology. 4th Edn. CBS Publishers and Distributers, New Delhi, India, pp: 550-565.

Rasper, K.B. and D.J. Fennel, 1965. The Genus *Aspergillus*. 1st Edn. Williams and Wilkins, Baltimore.

Rehana, F.V. and K. Nand, 1989. Preliminary studies on the production of thermostable α-amylase by a mesophilic strain of *Bacillus licheniformis*. Chem. Microbiol. Technol. Leberism., 12: 8-13.

Van der Maarel, M.J.E.C., B. Van der Veen, J.C.M. Uitdehaag, H. Leemhuis and L. Dijkhuizen, 2002. Properties and applications of starch converting enzymes of the alpha amylase family. J. Biotechnol., 94: 137-155.

Viswanathan, P. and N.R. Surlikar, 2001. Production of alpha amylase with *flavus* on *Amaranthus* grain by solid state fermentation. J. Basic Microbiol., 41: 57-64.