PJN
ISSN 1680-5194

PAKISTAN JOURNAL OF NUTRITION

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorpjn@gmail.com

Pakistan Journal of Nutrition 12 (5): 427-432, 2013
ISSN 1680-5194

Optimization of Growth and Amylase Production by *Aspergillus flavus* Grown on Some Agricultural Raw Materials in Nigeria

Oyewale Mojeed Oladapo
Department of Applied Sciences, Osun State Polytechnic, P.M.B. 301, Iree, Osun State, Nigeria

Abstract: *Aspergillus flavus* exhibited high growth and amylase production. Various Polysaccharides, including soluble starch, sorghum, cassava peel and maize induces high amylase production with maximal amylase activity at 2% W/V. An initial linear increase in amylase activity with increase in substrate concentration was obtained up to 2% W/V. An antagonistic relationship was obtained between amylase activity and substrate concentrations higher than 2% W/V, in the culture filtrate of *Aspergillus flavus* grown in shake flasks. The maximum growth and amylase production was obtained on the sixth day of incubation at a pH of 7.0 temperature of 29±1°C and 80 rpm. It is concluded from this study that cassava peel as well as sorghum may be an alternative carbon substrate for large scale cultivation of *A. flavus* for amylase production.

Key words: Soluble starch, sorghum, cassava peel maize, amylase activity, growth, substrate concentration, *Aspergillus flavus*

INTRODUCTION

Microbial enzymes are gaining importance because of their varied applications in industries, bioprocess, biotechnology and solid waste management. Numerous explorations of the microbial world for identifying enzyme producers have yielded a number of potential strains for the production of amylases (Garg and Doelle, 1989; Pestana and Castillo 1985; Rai and Deshmukh, 2005). Biopolymers such as cellulose, starch, pectin, xylan and lignin constitute a major part of living matter. The capability to utilize these substrates as carbon and energy sources is widely distributed among animals, plants and micro organisms. A wide variety of bacteria, fungi and yeasts produce a large array of extra-cellular enzyme to degrade these substances in different environmental niches (Antranikian, 1992). The great diversity of Polysaccharide-hydrolyzing enzymes have fulfilled the requirement of enzymes industry over last few decades and also led to interest in further screening programmes to look for enzymes with novel properties (Sanjeer Kumar and Satyanarayana, 2005).

Starch is a major storage product of many economically important crops such as wheat, rice, maize, tapioca and potato. Starch can be extracted commercially from many raw materials including maize, wheat, barley, potato, rice, oat, cassava, sorghum and others. Several thermophilic moulds have been reported to secrete amylolytic enzymes and some of these have been purified and characterized.

Amylase enzymes have industrial, Biotechnological and pharmaceutical uses particularly as detergent additives at high Ph (Borkar and Bhosle, 2003; Oyewale, 2006; 2010).

The myriad of biochemical interconversion in Living system which constitute the metabolic atlas in mediated by protein molecules called enzymes. Enzymes of microbial origin including those of Fungi have been exploited in medical, food, pharmaceutical and textile industries as well as biotechnology based industries (Ali *et al*., 1989; Fawole, 1986 and Oyewale 2006). Yeast amylases production have been reported at different pH and temperatures of incubation (Lagzouli Mohammed *et al*., 2007).

This present investigation aims at optimization of a medium for maximum production of extracellular amylase by *Aspergillus flavus* using readily available carbon sources such as cassava peels, sorghum and maize as substitute for commercial soluble starch and also studying the effects of different concentrations of selected raw materials as medium for optimum production of amylase enzymes.

MATERIALS AND METHODS

Preparation of inoculum: With aid of sterile corkborer 5 mm disc of an advancing edge of a 4-day old fungal culture was inoculated on to an agar slant prepared with Czapekdox agar containing 2% (W/V) soluble starch. The cultures were then incubated at 30°C for 48 hrs before use.

Culture methods: Spores of 48 hrs old cultures of *Aspergillus flavus* was harvested using the methods described by Akinyosoye and Akinyanju (1989). The 48 hrs old cultures of *Aspergillus flavus* was washed with 10 mls of sterile distilled water, by shaking to obtain a suspension of spores. An aliquot (0.5 ml) of spore

suspensions was aseptically used to inoculate 50 mls of liquid medium, containing any of the carbon sources and then incubated at room temperatures (29±1)°C on a rotary shaker at 80 rev per minute. Cultures were then suction filtered through a preweighed No 1 whatmann Filter paper and the filtrate obtained was used as crude extract. At intervals of 24 hrs the protein content as well as the amylase activity in the culture filtrate were determined. The mycelium retained on the pre-weighted No 1 whatman's filter paper was used for growth measurements.

Determination of fungal growth: Growth of fungus was determined at intervals of 24 hrs over a period of seven days by the dry mycelial weight method. The filter pads used to harvest the mycelial were first oven dried at 80°C to a constant weight. After filtration the mycelia on the filter paper were oven dried at 80°C. The weight was expressed in milligrams/50 ml of culture.

Determination of amylase activity: The amylase activity in the culture filtrate of *Aspergillus flavus* was determined by the D.N.S.A. methods (Bernfeld, 1955; Ogundero, 1979; 1982a,b; Ettalibi and Baratti, 1988); 1 ml of culture filtrates was added to 3 mls of standard soluble starch in 0.002 M Na_2 HPO_4 and 0.006M NaCl (Ph 6.9) and incubated at 45°C for one hour (Bernfield, 1955; Ogundero, 1979). The reducing sugars produced were determined by addition of 3 mls of Dinitro salicylic acid D.N.S.A reagent which contained D.N.S.A (1.0 g); 2M NaOH, (20 mls); Potassium sodium tartarate (30 g); in 100 mls of distilled water (Fergus, 1969) and boiled for five minutes to complete the reactions. The Absorbance of the cooled solution was then measured at 540 n.m with a W.P.A. S106 spectrophotometer. The reaction mixtures of the uninoculated control was used to set absorbance readings at zero. The amylase activity of the culture filtrates was expressed as total reducing sugars released/min/mg protein.

Determination of protein contents: The methods of Lowry *et al.* (1951) was used for the determination of soluble proteins in the culture filtrates of *Aspergillus flavus*.

Determination of Hydrogen ion concentration (pH): This was carried out at intervals of 24 hrs over a period of seven days. The pH values of the culture filtrates were determined with PYE unicam pH meter (model 292 MK2).

Effects of different concentrations of carbon sources on growth and amylase production: 50 cm^3 of Basal salt medium containing 0.4%, 0.8%, 1.2%, 2%, 3%, 4% and 5% (W/V) concentrations respectively, of soluble starch, maize, cassava peel and sorghum as sole carbon source were prepared in 250 cm^3 flasks. These were buffered to a Ph 7.0 with acetate buffer, after which they were all sterilized. After sterilization and cooling the media were each aseptically inoculated with 0.5 cm^3 spore suspension of *Aspergillus flavus*.

All culture were incubated at room temperature (29±1°C) on a rotary shaker at 80 revolutions per minute for six days, at the expiration of the sixth day, culture were analyzed growth, amylase activity and protein contents.

RESULTS

Aspergillus flavus was found to produce extracellular amylase into culture medium during growth on cassava peel, sorghum and soluble starch respectively.

Growth of *A. flavus* on soluble starch, showed a steady increase in mycelial dry weight, from 50 mg/50 ml culture to 67 mg/50 ml culture after five days incubation and then dropped to 63 mg/50 ml culture on the seventh day (Fig. 1).

During growth on soluble starch, amylase activity in the culture filtrate of *A. flavus* increased from 0.02 mg Total Reducing Sugars released/min/mg protein to 0.61 mg T.R.S released/min/mg protein after six days of incubation (Fig. 1). The pH of growth medium containing soluble starch increased from 5.7 to 7.6 over a period of seven days.

A gradual increase in mycelial dry weight, from 20 mg/50 ml culture on the first day to a maximum of 30 mg/50 ml culture on the fifth day of incubation was obtained (Fig. 2). Amylase activity in the culture filtrate of *Aspergillus flavus* grown on 2% (w/v) cassava peel increased from 0.03 mg T.R.S. released/min/mg protein to 0.48 mg/T.R.S. relased/min/mg protein on the third day of growth (Fig. 2) this is followed by a decrease in amylase activity on the fourth and fifth day respectively which later increased to a maximum of 0.50 mg T.R.S. released/min/mg protein on the sixth day of incubation (Fig. 2).

The pH of growth medium containing cassava peel increased from 5.5 to 7.6 within seven days of incubation.

An increase in the mycelial dry weight from 34 mg/50 ml culture to 58 mg/50 ml culture was observed in the basal medium containing 2%(w/v) sorghum. Amylase activity in the culture filtrate of *Aspergillus flavus* grown on 2% (w/v) sorghum increased from 0.03 mg T.R.S. released/min/mg protein on zero day to 0.42 mg T.R.S. released per min/mg protein on fourth day. A slight decrease in amylase activity was observed on the fifth day but reached a maximum of 0.51 mg T.R.S. released/min/mg protein after six days of incubation (Fig. 3). The pH values increased from 6.2 to 7.5 after six days of incubation and later decreased to 7.4 on the seventh day.

Growth of *Aspergillus flavus* on maize, showed a steady increase in mycelial dry weight from 25 mg/50 ml culture to a maximum of 38 mg/50 ml culture on the fifth day. It then decreased to 35 mg/50 ml culture on the seventh

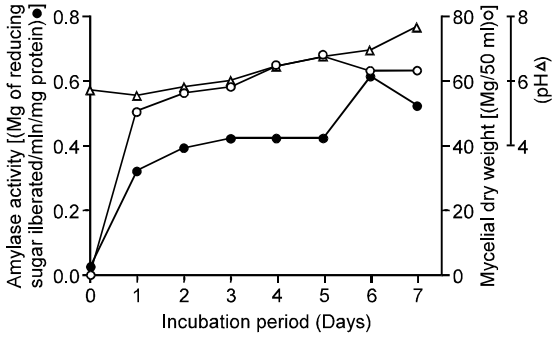

Fig. 1: Growth and amylase production by *A. flavus* grown on 2% (w/v) soluble starch

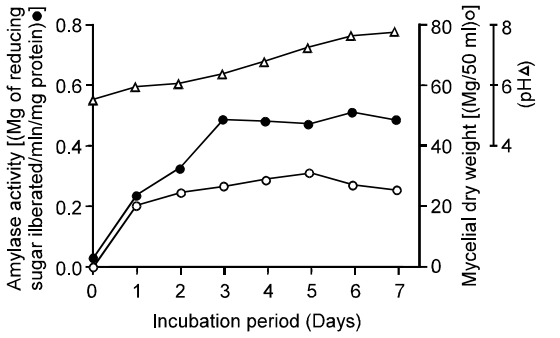

Fig. 2: Growth and amylase production by *A. flavus* grown on 2% (w/v) cassava peel

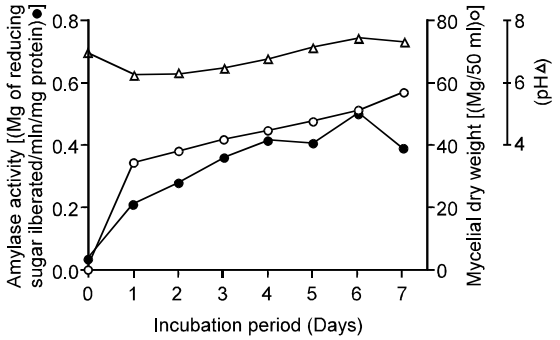

Fig. 3: Growth and amylase production by *A. flavus* grown on 2% (w/v) sorghum

day (Fig. 4). During growth of *A. flavus* on 2%(w/v) maize, amylase activity in the culture filtrates increased from 0.02 mg T.R.S released/min/mg protein to a maximum of 0.47 mg T.R.S. released/min/mg protein on the sixth day, after which it dropped to 0.36 mg T.R.S. relased/min/mg protein on the seventh day (Fig. 4). The pH of growth medium increased from 6.1 on the first day to 7.5 on the seventh day of incubation.

During the course of this investigation, Polysaccharides of different concentrations varying from 0.4% to 5% (W/V) were incorporated into the basal medium and their

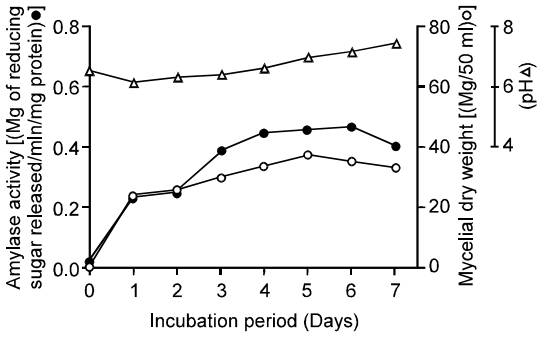

Fig. 4: Growth and amylase production by *A. flavus* grown on 2% (w/v) maize

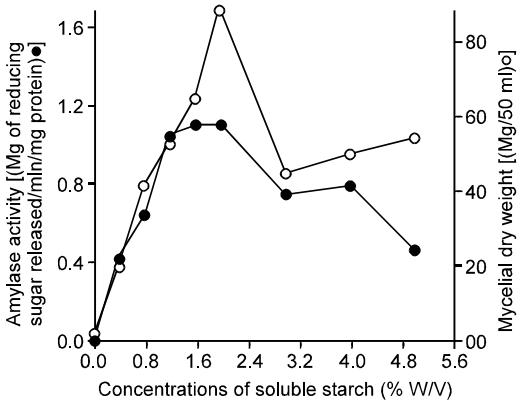

Fig. 5: Effect of different concentrations of soluble starch on growth and amylase production by *A. flavus*, incubated at 30°C for six days

effects on growth and amylase production by *Aspergillus flavus* were also determined.

In a medium containing soluble starch growth increased with increase in substrate concentrations with a maximum of 90 mg/50 cm^3 culture at 2% (W/V) concentrations (Fig. 5). Subsequent increase in concentrations lead to a decrease in mycelia dry weight (Fig. 5).

Similarly, amylase activity increased sharply with increase in substrate concentration reaching a maximum of 1.1 mg Total Reducing Sugars released/min/mg/proteins at 2% (W/V) concentrations. Subsequent increase in substrate concentrations, led to a decree in amylase activity.

On sorghum, an increase in growth with increase in substrate concentration was observed, reaching a maximum of 60 mg/50 ml culture at a of 2% (W/V) concentration

Furthermore increase in substrate concentration brought a decline in mycelial dry weight (Fig. 6). While an increase in amylase activity, with increase in substrate concentrations was obtained reaching a maximum of 0.80 mg Total Reducing Sugars released/min/mg

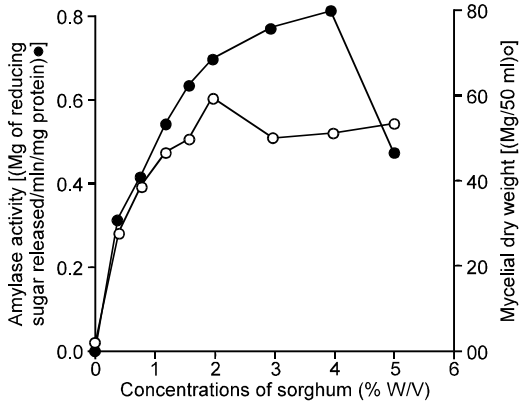

Fig. 6: Effect of different concentrations of sorghum on growth and amylase production by *A. flavus*, incubated at 30°C for six days

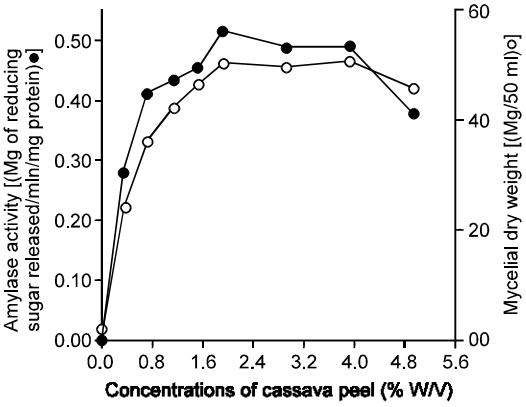

Fig. 7: Effect of different concentrations of cassava peel on growth and amylase production by *A. flavus*, incubated at 30°C for six days

proteins at 4% (W/V) which then decreased to 0.43 mg, Total reducing sugar released/min/mg proteins during growth at 5% W/V concentrations (Fig. 6).

In a medium containing cassava peel, growth increased with increase in substrate concentration, with a maximum yield of 50 mg/50 ml culture, at 2% (W/V) concentrations, of cassava peel (Fig. 7). Cassava peel, at concentrations greater than 2% (W/V) did not significantly alter the maximum growth observed at 2% W/V concentrations. Similarly amylase activity in the culture filtrate, increased with increase in substrate concentration releasing a maximum of 0.51 ml. Total Reducing Sugars released/min/mg protein at 2% (W/V) concentrations. Concentrations higher than 2% (W/V) led to a decrease in amylase activity (Fig. 7).

On maize, growth (measured as mycelial dry weight increased steadily with increase in substrate concentrations reaching a maximum of 45 mg/50 ml culture at 2% (W/V). Subsequent increase in substrate concentrations retarded the growth (Fig. 8). Amylase

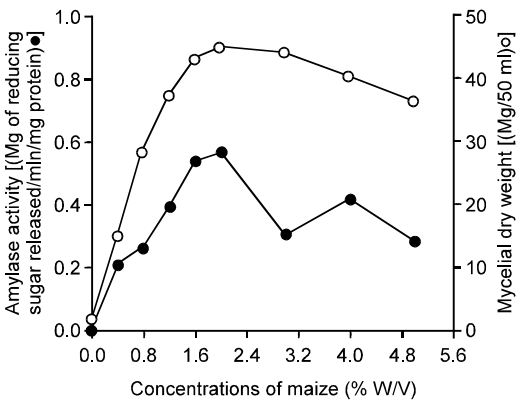

Fig. 8: Effect of different concentrations of maize on growth and amylase production by *A. flavus*, incubated at 30°C for six days

activity increases sharply with increasing substrate concentrations (Fig. 8).

The maximum activity of 0.56 mg Total reducing sugars released/min/mg protein was observed at 2% W/V concentrations (Fig. 8) subsequent increase in concentrations brought about a decrease in amylase activity (Fig. 8).

DISCUSSION

The results obtained in this investigation showed that *Aspergillus flavus* is capable of producing extracellular amylase into the culture medium when grown on basal medium containing various carbon sources as substrates. During growth on commercial soluble starch, sorghum, cassava peel and maize maximum amylase activity was observed in the culture filtrates after active growth had ceased (Fig. 1-4).

A commensurate increase in biomass with period of incubation was also observed during growth of *A. flavus* on soluble starch, sorghum, cassava peel and maize. This may suggest that *A. flavus* is capable of utilizing these carbon sources as substrates for the synthesis of cellular materials.

The pH changes of 5.5 to 7.6 was observed in the culture media throughout the incubation period. The increase in pH of the medium may also be as a result of an increase in the amount of enzymes and other metabolites released into the medium (Fig. 1-4).

The effects of different concentrations of carbon sources on amylase production showed that maximal level of amylase was produced during growth at 2% (W/V) (Fig. 5-8). The linear increase in amylase activity with increase in substrate concentration reported in this investigation was similar to the findings of Pestana and Castillo (1985); Garg and Doelle (1989); Ali *et al.* (1989). The reasons for this linear increase in amylase activity may be due to the secretion of more enzymes into the medium to hydrolyze more of the starch into a form that can be readily utilized for synthesis of cellular materials.

The antagonistic relationship obtained between amylase activity and substrate concentration higher than 2% (W/V), in the culture medium of the *Aspergillus flavus* (Fig. 5-8), could be as a result of the ability of the organism to utilize excess reducing sugars, produced into the culture medium, without necessarily requiring the synthesis of higher levels of amylase enzymes.

High concentrations of carbon sources in the growth medium may also be repressive to amylase synthesis, this may also be as a result of competition for active sites of the enzymes molecules by too many substrate molecules, thus the enzymes may be saturated hence, less effective.

It could also be as a result of catabolite repression, because of high concentrations of starch or by accumulation of rapidly metabolized carbon source. This result is also similar to that reported by Demot and Verachtert (1987).

Mountfort and Asher (1988), observed that amylase production by Neocallimastix frontalis was highest in 2.5 mg/cm^3 starch concentration and that subsequent increase in substrate concentration reduced amylase production.

Growth (expressed as Mycelial dry weight) increased substantially with increase in concentration of carbon substrate (Fig. 5-8).

In conclusion sorghum and cassava peel have been found to be better substitute for commercial soluble starch, for amylase production by *Aspergillus flavus*.

With regards to the ever increasing number of small and medium scale food processing industries in Nigeria there is an increase in agricultural raw materials and waste products, which can be readily utilized for large scale cultivation of *Aspergillus flavus* for amylase production.

ACKNOWLEDGEMENT

My gratitude goes to my Supervisor, Prof. Alhassan Sanni, of the Department of Microbiology, University of Ilorin for his constructive criticism and members of the enzyme Research Group under the leadership of Prof. M.O. Fawole and Prof. J.A. Akinyanju of Department of Microbiology, University of Ilorin for making available some of the chemicals and reagents used for this research work.

REFERENCES

Akinyosoye, F.A. and J.A. Akinyanju, 1989. Effects of different Carbon sources on growth and sporulation of *Geotrichum candidum*. Nig. J. Botany, 2: 89-95.

Ali, S., Z. Hossain, Mahmood and R. Alam, 1989. Culture conditions for the production of glucoamylase from Rice brain by *Aspergillus terreus*. MIRCEN J. Appl. Microbiol. Biotechnol., 5: 525-532.

Antranikian, G., 1992. Microbial degradation of starch in Microbial Degradation of Natural products. Winkel, G. Ed., VcH, weinhein (Germany), pp: 27-51.

Bernfield, P., 1955. In method in Enzymology (Edited by colowick, S.P. Kaplan, N.O) Academic press New-York, 1: 149-158.

Borkar, S. and S. Bhosle, 2003. Alkaliphiles microbes in alkaline environments in Advances in Microbiology, P.C. Triveddi Scientific Publishers India.

Demot, R. and H.C. Verachtert, 1987. Purification and crystallization of extracellular amylolytic enzymes from yeast *Filobasidium capsuligenum*. Appl. Environ. Microbiol., 50: 1474-1482.

Ettalibi, M. and J.C. Barratti, 1988. Isolation and characterization of an amylolytic yeast, candida edax, MIRCEN. J. Appl. Microbiol. Biotech., 136, 193-202.

Fawole, M.O., 1986. The place of enzymology in industrial Development, a paper presented at second Annual conference of the Biotechnology society of Nigeria, May 16-18, university of Ilorin, Ilorin, Nigeria.

Fergus, C.L., 1969. Production of amylase by thermophilic fungi, Mycologia, 61: 1171-1175.

Garg, S.K. and H.H. Doelle, 1989. Optimization of cassava starch conversion to glucose by *Rhizopus oligosporus*. MIRCEN J. Appl. Microbiol. Biotechnol., 5: 297-305.

Lagzouli Mohammed, Mannane Zakaria, Aitounejjar Ali, Wafae Senhaji, Ouhssine Mohammed, Elyachioui Mohammed, Berny EL Hassan and Jadal Mohammed, 2007. Optimization of growth and extracellular glucoamylase production by Candida famata isolate, Afr. J. Biotech., 6: 2590-2595.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randal, 1951. Protein measurements with folin phenol reagent. J. Biol. Chem., 192: 265-275.

Mountfort, D.O. and R.A. Asher, 1988. Production of α-amylase by the ruminal anaerobic fungus (*Neocaellimastix frontalis*). Appl. Environ. Microbiol., 54: 2293-2299.

Ogundero, V.W., 1979. Amylase and cellulase activities of thermophilic fungi causing deterioration of Tobacco products in Nigeria. Mycopathologia, 69: 131-135.

Ogundero, V.W., 1982a. The production and activity of Hydrolytic exoenzymes by toxigenic species of *Aspergillus* from Garri. Nig. J. Sci., 16: 11-20.

Ogundero, V.W., 1982b. Cellulose hydrolysis by thermophilic fungi from composting plant materials. Nig. J. Sci., 16: 83-93.

Oyewale Mojeed Oladapo, 2006. Pharmaceutical microbiology, for the tropics (Postharvest and Biotechnology Research Centre, Osogbo).

Oyewale Mojeed Oladapo, 2010. Growth and amylase production by *Aspergillus flavus* grown on commercial soluble starch and cassava peel. J. Pure Appl. Sci., 7: 53-56.

Pestana, F. and F.J. Castillo, 1985. Glucoamylase production by *Aspergillus awamori*, on rice flour medium and partials characterization of the enzyme. MIRECN J. Appl. Microbiol. Biotechnol., 1: 225-237.

Rai, M.K. and S.K. Deshmukh, 2005. Fungi Diversity and Biotechnology, Scientific Publishers, India-510.

Sanjeer Kumar and T. Satyanarayana, 2005. Starch hydrolyzing enzymes of Thermaphilic moulds. In Fungi Diversity and Biotechnology, M.K. Rai and S.K. Deshmukh Eds. Scientific Publishers, (India), pp: 229-250.