

Original Research Article

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Screening of *Aspergillus* species for the Production of α -Amylase

Kavitha Sagar^{1*} and G. M. Vidyasagar²

¹Department of Botany, Vijayanagara Sri Krishnadevaraya University, Ballari,
Karnataka, India

²Department of Botany, Gulbarga University, Kalaburagi, Karnataka, India

*Corresponding author

ABSTRACT

Eight species were isolated from soil samples collected from different places of Kalaburagi city, Karnataka, India and identified as *Aspergillus terreus*, *A. ochraceus*, *A. ornatus*, *A. clavatus*, *A. flavus*, *A. niger*, *A. flavus (I)* and *Aspergillus* sps. for the production of α - and β - amylase at four different time intervals i.e., 96h, 120h, 144h and 168h. Highest activity for enzyme was observed in presence of *A. flavus* continuously after 96h, 120h and 144h of incubation. β - amylase was maximum produced by *A. ornatus* and *A. flavus* after 120h of incubation. Of eight *Aspergillus* sps, *Aspergillus ornatus* and *A. flavus* were selected after screening for the production of α - and β - amylase. Four different concentrations i.e. 0.5, 1.0, 1.5, 2.0 and 3.0 % of carbon sources viz., glucose, sucrose, lactose and maltose were used as supplements to check their effect on enzyme production. Considerable α -amylase was produced by *A. ornatus* at 2.0% after 144h of incubation in the presence of glucose and β - amylase was produced at 144h in the presence of lactose. Maximum α -amylase was produced by *A. flavus* (1.769 U/ml) in the presence of lactose at 2.0% concentration after 144h of incubation. Effect of various nitrogen sources were tested for production of α -amylase, of which Ammonium sulphate (1.0%) was found to be effective for α -amylase production by *A. ornatus* (3.559 U/ml) at 144h of incubation and Casein hydrolase (2.0%) was effective at 168h by *A. flavus* (3.663 U/ml). *A. ornatus* and *A. flavus* can be further investigated for bulk production of α - and β - amylase.

Keywords

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Introduction

Amylase is a hydrolytic enzyme capable of degrading starch, glycogen and related polysaccharides of 1, 4 and 1,6 glucosidic linkages.

Bacteria and fungi are the best known microorganisms for extracellular production of amylases in large quantities. Filamentous fungi are some of the most prolific producers of extracellular enzymes and are widely used for industrial enzyme

production (Barnett and Fergus, 1997). Among these yeasts, *Aspergillus niger* and *Aspergillus oryzae* are the most industrially important ones which produce includes α -amylase, glucoamylase, glucose isomerase and invertase. The enzymatic digestion of raw starch has attracted much attention in recent years (Hoshino *et al.*, 1991) due to their uses in food, pharmaceutical and fine industries. Today about 95% of food industries are working on enzyme based processes and an excellent number of extensive reviews are available particularly, on the application of immobilized enzymes and cell in processing of soya milk and soya food (Khara and Jha, 1995), lipase catalase, inesterification reaction and their industrial applications, micro encapsulated enzymes and their applications in food industry (Kausal and Anand, 1999). The marketing of beer, wine, cheese and bread using amylases is thousands of years of old process. The use of enzyme in laundry as detergent is also the most significant market outlet for industrial enzyme. There is a great demand for new and improved amylases and also for the fungal organisms which are potent in producing amylases. In this view an attempt was made to screen the species of *Aspergillus* isolated from the different soil samples of Kalaburagi for the production of amylases.

Materials and Methods

Isolation of fungi from soil

Soil samples collected from different places of Kalaburagi were brought to laboratory in sterile polythene bags. 2 g of soil sample was added in 10 ml of sterilized distilled water in a test tube. From this 1 ml of suspension was pipette out and spread uniformly over the medium in petriplate containing 30 ml Potat Dextrose Agar medium. The petriplates were incubated at $28\pm 2^\circ$ C for 6 days in the laboratory. The fungal colonies that appeared on the medium were isolated and identified by using the book "*The Genus, Aspergillus*" written y Kenneth Raper and Dorothy Fennel (1965).

Screening of *Aspergillus* species for Amylase production

Eight *Aspergillus* species isolated from different places in and around Kalaburagi city were inoculated in different petriplates of modified Czapek agar medium. The petriplates were incubated at $28\pm 2^\circ$ C for 7 days at laboratory temperature. On 8th day of incubation, the petriplates were flooded with iodine stain. Amylase production was detected by the disappearance of the color of the blue starch around fungal colonies. The transparent zone developed around the colony was measured by taking two perpendicular measurement of diameters using scale.

Preparation of culture for collection of amylase enzyme

The culture medium was used in the growth of the fungi for the production of amylase was modified Czapek medium and pH was adjusted to 5.0 – 7.0. About 100 ml of medium was poured in 250 ml Erlenmeyer flask and sterilized at 15 lb pressure at 121° C for 30 min. After cooling, medium was inoculated with a sterile loopful of fungus and grown at 28° C with shaking (20 strokes/min). Aliquots were drawn at different time intervals and centrifuged at 10,000 rpm for 15 mins in a cooling centrifuge to remove the mycelia and spores before the supernatants were used in enzyme assays.

Maltose calibration curve

Maltose calibration curve was drawn using 3,5-dinitrosalicic acid (DNS) as colour reagent. A standard solution of maltose was prepared with a concentration of 1 mg/l. Different aliquots of maltose solution from 0.1 to 1 ml were pipette out into a series of test tubes and the volume was made upto 2 ml with required amount of double distilled water. To each tube, 1 ml of DNS reagent was added. The contents of the tube were covered with glass marble and were kept on boiling water bath for 5 minutes, after which they are cooled and diluted. To 10 ml with double distilled water. The optical density of the orange color formed was measured at 540 nm against a reagent blank in

spectrophotometer. A standard graph was drawn. This was used to convert the Colorimeter readings into micrograms of maltose liberated.

Assay for activity of amylase

Amylase activity was assayed following the method of Bernfeld *et al.*, (1955). In this method 0.5 ml of enzyme aliquot was incubated for 5 min along with 0.5 ml of 1% starch prepared in 0.1 M acetate buffer (pH 5.6) at room temperature. Then the reaction was arrested by adding 1 ml DNS. The tubes were heated for 5 min on boiling water bath and cooled.

The solution was made up to 10 ml with distilled water. The OD was measured at 540 nm using spectrophotometer. In another set of test tubes fixed aliquot of enzyme was arrested at zero minute by adding DNS reagent and OD was read at 540 nm. Difference in OD of enzymes arrested at zero minute and enzyme made to act on substrate for 5 min was noted and was referred to maltose calibration curve. Then the concentration of maltose liberated was noted and calculated the enzyme activity using the formula:

$$\text{Activity (unit/ml/min)} = \frac{\text{Concentration of maltose X 2}}{\text{Mol. Wt. Of maltose X incubation period}}$$

Units of amylase activity

One unit of amylase activity is defined as the amount of enzyme that liberates one μ mole maltose under assay conditions.

Effect of carbon sources on α -amylase production by *A. ornatus* and *A. flavus*

In order to study the effect of carbon sources in addition to starch on α -amylase production, sucrose, glucose, lactose and maltose were added (1% w/v) in modified Czapek medium separately and pH was adjusted at 7. Then the medium was sterilized for 15 mins and 100 ml of the medium was poured in 250 ml of Erlenmeyer flasks.

A loopful of each fungus was taken and inoculated in separate conical flask. Amylase activity was assayed by DNS method at different intervals 96h, 120h, 144h 168 h. Of incubation at 28°C.

Effect of different concentrations of glucose and lactose on α -amylase production by *A. ornatus* and *A. flavus*

Different concentrations of glucose and lactose such as 0.5, 1, 1.5, 2.0, 2.5 and 3% were prepared using Czapek media and tested for their effect on α -amylase production. The enzyme activity was estimated at 144 h and 168h of incubation.

Effect of supplementary nitrogen sources on α -amylase production by *A.ornatus* and *A. flavus*

Effect of nitrogen sources such as ammonium sulphate, ammonium dihydrogen phosphate, ammonium nitrate, peptone, casein hydrolysate, potassium nitrate and beef extract at 1% (w/v) and was studied in submerged fermentation on α -amylase production.

Effect of different concentrations of ammonium sulphate and casein hydrolysate on α -amylase production by *A.ornatus* and *A. flavus*

Different concentrations of ammonium sulphate and casein hydrolysate such 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 % were prepared in Czapek media and tested their effect on the production of α -amylase.

Statistical analysis

All the experiments were carried out in triplicate but only the mean of each set is shown. The data was statistically analysed applying Analysis of Variance and Duncan's Multiple Range test was done as post-test.

Results and Discussion

Eight *Aspergillus* species namely *A. terreus*, *A. ochraceus*, *A. ornatus*, *A. clavatus*, *A. flavus*,

Aspergillus spp., *A. niger* and *A. flavus* I were isolated from the soil samples from different places of Kalaburagi city (Table-1).

Screening of *Aspergillus* spp. For amylases production by plate culture method

Amylase production by eight *Aspergillus* spp. was detected by the disappearance of the blue iodine color around the fungal colonies. Evaluation of the clear zones was done by taking two perpendicular measurement of diameter by using scale.

Of the eight species screened, *A. flavus* showed the wide distinct clear zone of 6.49 cm followed by *A. ornatus* (6.0), *A. ochraceus* (5.79cm), *A. terreus* (5.65 cm), *A. clavatus* (5.6cm), *A. niger* (5.5cm), *A. flavus* (I) (5.28cm) and *Aspergillus* spp. (4.96cm).

Assay of α -amylase activity

α -amylase activity was assayed followed the method of Bernfeld *et al.*, (1955) at four time intervals i.e. after 96h, 120h, 144h and 168h of incubation. The results are depicted in table -3. After 96h of incubation, maximum activity was recorded in *A. flavus* (1.769 U/ml) followed by *A. terreus* (1.250 U/ml) and *A. ochraceus* (1.117 U/ml). Minimum activity was recorded in *A. niger* (0.214 U/ml). The statistical analysis results show that at 96h of incubation, *A. clavatus*, *A. flavus* (I), *A. ornatus*, *A. ochraceus* and *Aspergillus* spp. showed homogeneity for α -amylase production.

A. flavus when compared to 96h, showed lesser activity after 120h of incubation but among others highest activity was recorded (1.635 U/ml). This was followed by *A. ornatus* and *A. clavatus* (1.058 and 0.922 U/ml). Least α -amylase was produced by *A. terreus* and *A. niger* (0.414 and 0.436 U/ml). *Aspergillus* spp. and *A. clavatus* formed a homogenous group at this hour of incubation.

α -amylase activity was increased after 144h of incubation which was found to be the highest (2.294 U/ml) in *A. flavus* and *A. flavus* (I), followed by *A. ornatus*, *A. terreus* and *A. ochraceus* (1.525, 1.428

and 1.369 U/ml). Minimum activity was expressed by *A. niger* (0.932 U/ml).

A. terreus showed the highest activity (1.458 U/ml) after 168 h of incubation followed by *A. niger* (1.050 U/ml) and *A. ornatus* (0.984 U/ml). Least activity was found in *Aspergillus* spp. (0.266 U/ml).

Among eight species *A. flavus* showed the highest activity continuously after 96h, 120h and 144h of incubation (1.769, 1.635 and 2.294 U/ml). Though activity of *A. terreus* was found to be less when compared to 96h, 120h and 144h of incubation, but at 168h higher α -amylase yield was found in *A. terreus* (1.458 U/ml) and least activity was resulted in *Aspergillus* spp., and *A. flavus* (I) (0.266 and 0.318 U/ml). Data analysis reveal that at 168h of incubation *A. flavus* (I) and *A. ochraceus* are not different statistically. *A. terreus* is different from the other eight species with respect to α -amylase production.

Though after 96 h of incubation *A. flavus* produced higher α -amylase it decreased at 120 h, but at 168h there was sudden fall in enzyme activity, whereas *A. terreus* produced highest α -amylase at this hour. *A. ochraceus*, *A. terreus* and *A. ornatus* formed homogenous group.

Assay of β -amylase activity

After 96h of incubation, *A. flavus* showed the highest production (1.161 U/ml). Least activity was recorded in *Aspergillus* spp. (0.18 U/ml). *A. ochraceus* and *A. ornatus* form a homogenous group of production of β -amylase production.

Maximum β -amylase production at 120h of incubation was recorded in *A. ornatus* (1.753 U/ml) and *A. flavus* (1.265 U/ml). Again least activity was expressed in *A. flavus* (I) (0.251 U/ml). Almost similar β -amylase yield was obtained from *A. flavus* (I), *A. terreus*, *Aspergillus* spp., *A. clavatus*, *A. niger* and *A. ochraceus* which expressed homogeneity.

A. flavus (I) showed highest yield (1.465 U/ml) after 144h of incubation followed by *A. niger* (0.643

U/ml), *Aspergillus* sps, (0.639 U/ml). Minimum activity was recorded in *A. terreus* (0.192 U/ml). Nearly same amount of β -amylase was produced by *A. terreus*, *A. ochraceus*, *A. ornatus*, *A. flavus*, *A. clavatus*, *Aspergillus* sps, and *A. niger* according to the analysis which shows they are homogenous group at 144h of incubation.

Again after 168h of incubation, maximum yield was obtained by *A. niger* (0.583 U/ml). *A. flavus* (I) and *A. niger* were found to produce β -amylase better (0.540 and 0.583 U/ml) than *A. terreus*, *A. ochraceus* and *A. flavus* (0.362, 0.274 and 0.273 U/ml). Lower β -amylase was yielded by *A. ornatus* (0.214 U/ml).

Of the eight *Aspergillus* species *A. ornatus* and *A. flavus* were found to be good producers of β -amylase. *A. ornatus* (1.753 U/ml) was better than *A. flavus* (1.265 U/ml) after 120 h of incubation, whereas sudden decrease in the activity was recorded in *A. ornatus* (0.214 U/ml) after 168 h of incubation (Table-4). Optimum incubation period for all the species was 144 h.

Effect of different supplementary carbon sources on the production of α -amylase by *A. ornatus*

The effect of four supplementary carbon sources viz., glucose, sucrose, lactose and maltose on α -amylase production was determined in *A. ornatus* (Table-5).

α -amylase activity determined at 96h of incubation in the medium supplemented with sucrose was high (0.348 U/ml) followed by glucose, maltose and lactose.

At 120 h also sucrose supplemented medium showed high activity (0.384 U/ml) and less activity was recorded in the medium supplemented with glucose. Lactose and maltose were not effective as inducers for α -amylase production.

After 144h of incubation glucose was found to be effective with 1.117 (U/ml) activity, followed by sucrose (547 U/ml) and least in lactose (303 U/ml).

At this hour lactose, maltose and sucrose formed a homogenous group for α -amylase production. The enzyme production increased with increase in incubation period. But decreased at 168 h of incubation.

At 168h decrease in α -amylase activity was recorded. Among the C- sources, glucose was good which showed 0.962 (U/ml) α -amylase production. Least was produced, in the presence of lactose (0.266 U/ml). The reduction of enzyme production might be due to the decline in nutrients and sugar contents. Moreover, it may also be due to the inhibitors generated by the fungus (Ramesh & Lonsane, 1990; Kirshna & Chandrasekaran, 1996).

Among C-sources used, glucose was found to be the better inducer for enzyme production at 144 h of incubation for *A. ornatus*.

Effect of different supplementary carbon sources on the production of α -amylase by *A. flavus*

After 96h of incubation, *A. flavus* produced high α -amylase in the presence of sucrose (0.140 U/ml) whereas, glucose was found to be very poor (0.059 U/ml). Lactose and maltose showed 0.096 U/ml and 0.103 U/ml α -amylase activity, respectively.

α -amylase activity at 120h of incubation was high in the presence of lactose (0.429 U/ml) followed by maltose (0.273 U/ml), sucrose (0.199 U/ml) and glucose (0.185 U/ml) (Fig.1)

The results depicts that carbon sources (1%) when used as supplements in the medium containing 1% starch were not found to be good inducers, in turn they repressed the α -amylase enzyme production in both *A. ornatus* and *A. flavus*. Addition of 1% sucrose, glucose, lactose and maltose to the medium of 1% starch brought about an immediate fall α -amylase production in *A. ornatus* and *A. flavus*. The results are in agreement with the findings of Adinarayana Reddy and Abouzied (1986) who reported that for the highest amylase activity in *Aspergillus niger* NRRL 330, *A. awamorii* NRRL 3112 and *A. foetidus*, 1-2% of starch was effective

(15 U/ml), but glucose was found to be inhibitory for amylase production. This effect of glucose might be due to direct inhibition of enzyme of glucose or by catabolite repression of α -amylase by glucose. Though supplementary C-sources were rapidly utilized by fungi and growth was seen, α -amylase production was inhibited. Similar results were reported by Chiou and Jeang (1995) where they observed that glucose, fructose, maltose and soluble starch did not positively affect the amylase production in *Cytophaga* sps., Nandakumar *et al.*, (1999) found the catabolite repression by glucose on biosynthesis of α -amylase by *A. niger* CFTRI 1105. Ghosh *et al.*, (1990) also reported that in *A. terreus* strain 4, extracellular glucoamyl production is subject catabolite repression, when starch medium was supplemented with glucose. Similarly Flore *et al.*, (1993) reported that glucose (2%) negatively effected α -amylase production in submerged cultures of *Streptomyces kanamyceticus*.

Effect of different concentrations of glucose and lactose

Effect of different concentrations of glucose and lactose on the production α -amylase in *A. ornatius* and *A. flavus* is represented in fig. 2. Among the glucose concentrations maximum activity of 1.472 and 1.376 U/ml was recorded in the medium supplemented with 2.0% glucose and minimum of 0.628 and 0.392 U/ml in the medium supplemented with 0.5% (w/v) glucose at 144h and 168h of incubation, respectively. Similarly lactose showed maximum activity of 1.769 U/ml at 144h of incubation. The data represented show that at both 144 h and 168h of incubation, 2.0% concentration was effective on the production of α -amylase by *A. ornatius* and *A. flavus*. (Fig. 2).

Effect of nitrogen sources

Effect of seven N-sources on α -amylase production was determined in *A. ornatius* and *A. flavus* the results are presented in fig. 3 & 4. In *A. ornatius* maximum of 3.620 U/ml activity at 144h of incubation was recorded in ammonium sulphate. Similarly, at 168 h of incubation maximum activity of 1.887 U/ml was

recorded in ammonium sulphate, followed by 1,858 U/ml in casein hydrolysate, 1.657 U/ml ammonium nitrate, 1.627 U/ml in ammonium di hydrogen phosphate, 1.576 U/ml in beef extract, 1.391 U/ml in peptone and 1.146 U/ml in potassium nitrate. In *A. flavus* at 144 h of incubation maximum of 1.798 U/ml activity was recorded in casein hydrolysate, followed by 1.776 U/ml in ammonium sulphate, 1.694 U/ml in beef extract, 1.628 U/ml in potassium nitrate, 1.553 U/ml in peptone, 1.539 U/ml in ammonium di hydrogen phosphate and 1.354 U/ml in ammonium nitrate.

Similarly at 168 h of incubation, maximum activity (1.835 U/ml) was recorded in casein hydrolysate, followed by 1.828 U/ml in ammonium di hydrogen phosphate, 1.798 U/ml in ammonium sulphate, 1.694 U/ml ammonium nitrate, 1.672 U/ml in potassium nitrate, 1.657 U/ml in peptone and 1.598 U/ml in beef extract. Further, in this examination it was observed that ammonium nitrate, peptone, potassium nitrates have not served any significant changes on the enzyme production comparatively. It may be because as the ammonium and potassium ion is taken-up the ionic balance of the cell is maintained by the extrusion of hydrogen ions, resulting in more acidic environment which made fungi not to grow. The marginal effect of peptone, ammonium nitrate and potassium nitrate on α -amylase production may be due to presence of free ammonium in the medium.

The results show that for α -amylase production, ammonium sulphate proved to be the best N-source in *A. ornatius* whereas, Casein hydrolysate was good for *A. flavus*. However, a detailed study is required to investigate the effect of these N-sources on α -amylase production by *A. flavus* and *A. ornatius*.

Effect of different concentrations of ammonium sulphate and casein hydrolysate on the production of α -amylase

At 1.0% concentration of ammonium sulphate maximum of 3.559 U/ml and 1.887 U/ml activity was produced by *A. ornatius*. Similar result was reported by Gopika and Srikant (1995) where they

found that 95% α -amylase was secreted extracellularly by *A. oryzae* when ammonium sulphate was used as N-source.

Similarly in *A. flavus*, maximum activity of 3.544 U/ml and 3.663 U/ml in 2.0% of casein hydrolysate concentration was recorded. Pedersen and Nielsen (2000) reported that Casein hydrolysate when used as N-source even at 0.05 g/l, 35 % increase of α -amylase production resulted by *A. oryzae* in Solid State fermentation. The nitrogen is metabolized to produce primarily amino acids, nucleic acid, proteins and cellular component in fungi. As the production of α -amylase is affected by selected and low nitrogen concentration, it is required to identify proper type and percentage of necessary nitrogen compound.

Amylases are being voluminously used in many industries. Out of many industrially important enzymes, about 50% of amylases are derived from fungi and yeast. The present investigation highlights on effects of various carbon and nitrogen sources on the production of α -amylase production by *A. flavus* and *A. ornatius* at small scale production. The present study shows that *A. ornatius* and *A. flavus* consistently proved to be the best at 1% starch as substrate, 144h and 120h of incubation periods supported high yield of α - and β - amylase, respectively. Again of the C- sources used as supplements to the starch medium, though glucose and lactose were fairly good, they brought catabolite repression in *A. ornatius* and *A. flavus*. Hence it indicates that all the four C-sources viz., glucose, sucrose, lactose and maltose are not suitable as inducers for α -amylase production in these species. Of the N-sources used, ammonium sulphate (1.0%) and casein hydrolysate (2.0%) can be used as N-sources which were excellent inducers at 144 h.

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