Utilization of Sugarcane Bagasse For Solid-State Fermentation and Characterization of α- Amylase From Aspergillus flavus Isolated From Muthupettai Mangrove, Tamil Nadu, India.

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Abstract: In the present study, four fungal isolates from a mangrove soil were screened for their ability to produce α-amylase using solid state fermentation (SSF). Aspergillus flavus proved to be the best α-amylase producer. Various agronomic wastes such as molasses bran, maize meal, rice bran, sugarcane bagasse and wheat bran were used as solid substrates. The highest titer of enzyme activity was obtained when A. flavus was grown on sugarcane bagasse. Different carbon and nitrogen supplements were used to enhance the enzyme production and the highest yield was obtained with soluble starch and yeast extract (1% w/w) as supplements. The optimum pH, temperature, and incubation period for amylase production by the isolate was found to be 6.0, 30°C and 120 h respectively. Partial purification of the amylase using ammonium sulphate fractionation resulted in 5 fold increase in the enzyme activity. Sodium dodecyl sulphate polyacrylamide gel electrophoresis revealed the apparent molecular weight of the enzyme to be 55 kDa. The partially purified enzyme was optimally active at pH 5 and 55°C when 1% of starch and 80.64 µg/ml of enzyme concentrations were used. Presence of Mn²⁺ and Ca²⁺ salts enhanced the enzyme activity in contrast to Hg²⁺ and EDTA, which completely reduced the enzymatic activity. The results obtained in the present study suggest that the mangrove isolate of A. flavus may act as a potent strain for industrial production of α- amylase using SSF with sugarcane bagasse as the substrate.

Key words: Aspergillus flavus, α- amylase, solid state fermentation, sugar cane bagasse, enzyme activity.

INTRODUCTION

Various enzymes with different specificities are involved in the hydrolysis of starch. α- amylase (endo-1, 4, α- D- glucan glucohydrolase) is an extracellular enzyme that randomly cleaves the 1, 4, α- D glucosidic linkages between adjacent glucose units and hydrolyses single glucose units from the non-reducing ends of amylose and amyllopectin in a stepwise manner. Although amylase can be obtained from several sources such as plants, animals and microorganisms, the enzyme from microbial sources generally meet industrial demand (Pandey et al., 2000).

Microbial amylases have successfully replaced chemical hydrolysis of starch in starch processing industries. The amylase family of enzyme has been well characterized through the study of the various microorganisms. Fungal α- amylase is preferred for use in formulation for human or animal consumption involving application under acidic condition and around 37°C due to this biocompatibility (Karuki and Imanaka, 1999). Similarly, due to its biocompatibility, fungal amylases are preferred in baking and food processing. Production of amylase by Aspergillus strains in both submerged fermentation (SmF) and solid state fermentation (SSF) by using different food wastes or agricultural residues has been thoroughly studied (Francis et al., 2002). With the advent of new frontiers in biotechnology the spectrum of amylose application has expanded to many other fields such as clinical, medical, industrial process like food, textile, paper and analytical chemistry (Pandey et al., 2000).

The cost of enzyme production by SmF is high which necessitates reduction in production cost by alternative methods such as SSF. The contents of synthetic media are very expensive and these contents might be replaced with more economically available agricultural by-products (Ellaiah et al., 2002). During the last decade, an increased attention was paid to the use of various agro-industrial wastes for value addition in solid-state fermentation (SSF) by filamentous fungi as the major conversion technology (Pandey et al., 2001). The SSF has numerous advantages over SmF system including simple technique, superior volumetric productivity, low energy requirement and less water output, better product recovery and lack of foam build up and reported to be the most appropriate process for developing countries (Stredansky et al., 1999).

Mangrove ecosystems are among the most productive natural ecosystems and the biomass released annually from mangrove trees is estimated to be 0.48% of carbon biomass per hectare (Gong and Ong, 1990). Mangrove water bodies contain heavy deposits of particulate organic matter (detritus) derived chiefly from the

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decomposition of litter. The primary productivity of the mangrove water ranges from 2.0 to 3.6 g C m\(^{-3}\)/day (Nair and Gopinathan, 1983); the sediment microbial population is about seven fold higher than that of littoral zone, and the suspended particulate matter 20 times greater than that of seawater (Gopinathan and Selvaraj, 1996). The submerged mangrove roots and trunk are surrounded by loose sediment, which attract epifaunal communities including bacteria, fungi, micro-algae and invertebrates (Kathiresan and Bingham, 2001).

The objectives of the present study were isolation of fungi from the mangrove soil, selection of a suitable strain for the production of \(\alpha\)-amylase, screening of different agricultural byproducts as substrates for the maximum enzyme production, optimization of growth conditions for production of \(\alpha\)-amylase, partial purification and characterization of the enzyme.

**MATERIALS AND METHODS**

**Sample Collection:**

The sediment was sampled from Muthupettai mangrove forest situated in Tamil Nadu, India (10°24′N, 79°30′E) using a sterile stainless steel corer sampler (inner diameter 32 mm) from the rhizosphere of *Avicennia marina*. Following sampling, the central portion of the 20 cm sediment sample was collected in zip-lock cover and transported to the laboratory.

**Isolation and Determination of Fungi Producing \(\alpha\)-Amylase:**

\(\alpha\)-amylase producing fungal forms were isolated from the sediment sample using serial dilution and spread plate technique. Primary screening was performed by growing the isolates as point inoculation on minimal agar medium containing (g/l): K\(_2\)HPO\(_4\), 7; KH\(_2\)PO\(_4\), 2; (NH\(_4\))\(_2\)SO\(_4\), 1; sodium citrate, 0.5; MgSO\(_4\), 0.1; agar, 20; and distilled water at pH 7, supplemented with 1% soluble starch at room temperature for 5 days. The amylolytic activities of the isolates were detected by flooding the plates with iodine solution (1% iodine and 2% potassium iodide in distilled water (w/v)). Comparison between the isolates for the amylolytic activity was made based on the zone of hydrolysis and comparing it to the colony diameter. The isolate demonstrating the best amylolytic activity was maintained on potato dextrose agar (PDA) slants in duplicates at 4°C until use.

**Identification of the Isolate:**

The isolate for the amylolytic activity was identified based on the growth pattern and the morphological characteristics on PDA plates and the structure of the conidiophores under light microscope (Pitt and Hocking, 1985).

**Inoculum Preparation:**

Spore suspension was prepared by mixing a loopful of fungal spores in 10 ml of sterile distilled water. A uniform spore suspension was obtained by mixing vigorously, which was measured for absorbance under white light. 1 ml of spore suspension (10\(^7\) spores/ml) was transferred to the 5 g of solid substrate.

**Substrates Used for \(\alpha\)-Amylase Production:**

Commercial quality molasses bran, maize meal, rice bran, sugarcane bagasse and wheat bran were procured from the local market of Bangalore city and used as solid substrates and their effect on the production of \(\alpha\)-amylase was determined. Prior to use all these agronomic wastes were treated with 1% NaOH and autoclaved at 121°C for 20 min to remove the core and noncore lignin fractions (Doran et al., 1994). Then, these solid substrates were thoroughly washed with tap water, subsequently with distilled water until neutrality and dried at 80°C (Doran et al., 1994). The best solid substrate was selected and used in subsequent experiments.

**Optimization of Cultural Conditions:**

The best solid substrate was supplemented with different carbon sources such as 1% (w/w) (maltose, lactose, soluble starch, sucrose, dextrose, fructose and glucose) and 1% (w/w) organic and inorganic nitrogen sources (peptide, tryptone, yeast extract, beef extract, ammonium sulphate, urea and sodium nitrate). Various physical parameters such as fermentation period (24, 48, 72, 96, 120, and 144 h), pH (4, 5, 6, 7 and 8), and effects of temperature (10, 25, 30, 35, 40, 50, 60, 70 and 80°C) were optimized by conventional methods for maximal enzyme production. All the experiments were conducted in triplicates.

**Extraction of Amylase:**

Following the fermentation, the fermented substrate along with the fungal mycelia were mixed thoroughly with sterile distilled water (5 ml/g) and crushed in sterile pestle and mortar. The crushed substrate was pressed and then the extract was filtered through normal filter paper and then through Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, England) and the filtrate was collected. The filtrate was subjected to
centrifugation at 5000 rpm for 30 mins at 4°C. The clear supernatant was subjected to amylase assay and further purification.

**Assay of Amylase:**

\( \alpha \)-amylase activity was determined by taking a reaction mixture containing 1ml of 1% soluble starch, 0.5 ml acetate buffer (0.1 M, pH 5.0) and 0.5 ml of appropriately diluted crude enzyme extract. The reaction mixture was incubated at 37°C in water bath with shaking for 30 mins. Released reducing sugar was estimated by 3, 5-Dinitrosalicylic acid (DNS) method (Miller, 1959) and glucose as standard. The colour development was read at 540 nm using a UV-VIS spectrophotometer (SANYO Gallenkemp, Germany). Blank contained 1 ml of 0.1 M acetate buffer (pH 5.0) and 1ml of 1% soluble starch solution. One unit (U/gds) of amylase activity was defined as the amount of enzyme that releases 1 µg of reducing sugar as glucose per gm of dry substrate per minute, under standard assay conditions.

**Estimation of Protein Content:**
The soluble protein content of the enzyme sample was determined by Lowry’s method (Lowry et al., 1951) using crystalline bovine serum albumin as the standard.

**Characterization of \( \alpha \)-Amylase:**
For the purpose of amylase purification and molecular mass determination, the crude enzyme was purified by 40 % ammonium sulphate precipitation method. The precipitate obtained by centrifugation was dissolved in 0.1 M phosphate buffer (pH 7) and dialysed overnight against 0.01 M phosphate buffer at 4° C to prevent enzyme denaturation. Further, for the molecular weight determination, the dialysed enzyme sample was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using broad range pre-stained protein marker (NEW ENGLAND Biolabs, UK).

**Factors Affecting Enzyme Activity:**
Optimum temperature for enzyme activity was determined by incubating the enzyme substrate reaction mixture at different temperatures (25, 37, 45, 55, 65, 80 and 100°C) for 30 minutes and then carried out the assay for amylase activity. For the determination of the optimum concentration of substrate for maximum enzyme activity, starch solutions of various concentrations (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 %) were used for the assay and the enzyme concentration remained constant. The effect of pH on amylase activity was determined by incubating the reaction mixture with different buffers of 0.1 molarity (pH 3.5- Citrate buffer; pH 5.5, 6.8, and 7.5- phosphate buffer, pH 9.5- Tris –HCL buffer). The optimum enzyme concentration for maximum amylase activity was determined by taking 9, 18, 27, 36, 45, 54, 63, 72, 81 and 90 µg/ml of the enzyme extract when the substrate concentration remained constant. Effect of various metal ions like Ca\(^{2+}\), Mn\(^{2+}\) and Hg\(^{2+}\) and chelator like EDTA on enzyme activity was determined by using 0.5 ml of enzyme extract, 1ml of 1% starch solution and 0.25 ml of 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 mM concentrations of various metal ions and chelator at pH 5 and at 55°C.

**Statistical Analysis:**
All the optimization studies were conducted in triplicate and the data were analyzed using single factor analysis of variance (ANOVA). All the data are graphically presented as the mean ± S.D. of triplicates (n = 3). ANOVA was performed using Microsoft Excel 2007. \( P \) values < 0.05 were considered significant with a confidence limit of 95%.

**RESULTS AND DISCUSSION**

\( \alpha \)-Amylases (EC3. 2.1.1, endo- 1,4- \( \alpha \)-D- glucan glucohydrolase) are endo- acting enzymes which randomly hydrolyze \( \alpha \)-1,4-glycosidic bonds between adjacent glucose units in starch polymer leading to the formation of linear and branched oligosaccharides. They are usually used in synergy with glucoamylases or \( \beta \)-amylases or pullunases for the complete hydrolysis of the starch and are extensively used in sugar, textile, alcohol and paper industries. Further, they are employed in processed food industry such as baking, brewing, preparation of digestive aids, production of cakes, fruit juices and starch syrups (James and Simpson, 1996). In addition, they are used in sewage treatment for reducing the disposable solid content of sludge (Parmar et al., 2001) and as detergents (Kumar et al., 1998).

With the advent of Solid State Fermentation (SSF), the production of industrial enzymes received a lot of importance. Both fungal and bacterial systems are widely used in the production of \( \alpha \)-amylases under SSF. Production of enzymes by SSF using the moulds turned a cost-effective production technique (Pandey et al., 2000). Filamentous fungi have been widely used for the production of amylases for centuries. As these moulds are known to be prolific producers of extracellular proteins, they are widely exploited for the production of
different enzymes including α-amylase. Fungi belonging to the genus *Aspergillus* have been most commonly employed for the production of α-amylase.

**Isolation and Identification of Fungal Forms:**

31 fungal isolates were obtained from the mangrove soil. Upon colony morphology study and microscopic analysis, the isolates were found to be *Aspergillus niger*, *A. flavus*, *A. oryzae* and *Penicillium* sp. Following the primary screening (Starch hydrolysis test forming clear halo of the zone of starch hydrolysis), further work was carried out with *A. flavus*, which showed the highest zone of clearance (11 cm) as compared to *A. niger* (9 cm), *A. oryzae* (6 cm) and *Penicillium* (5 cm), respectively. Pure culture of this amylolytic *A. flavus* strain was preserved on starch agar slants in triplicates at 4°C.

**Substrates for α-Amylase Production:**

In recent years, research on the selection of suitable substrates for SSF has mainly centered around more efficient utilization of different agro-industrial residues, including spent brewing grain, sugarcane bagasse, wheat bran, wheat straw, sunflower meal, rice husk, cottonseed meal, soybean meal, rye straw and corn cob leaf and oil cakes, for amylases production (Ramachandran et al., 2004; Bhargav et al., 2008) and also due to the potential advantages for filamentous fungi, which are capable of penetrating into the hardest of these solid substrates aided by the presence of turgor pressure at the tip of the mycelium (Ramachandran et al., 2004) and, on the other, helps in solving pollution problems, which otherwise may cause their disposal.

All the substrates used in the study supported the growth and enzyme production by the isolate, while sugarcane bagasse proved to be the best solid substratum with the highest titer of amylase activity (3.128 U/gds) (Fig. 1). This might be because of the high moisture content in the sugarcane fibers and also presence of satisfactory amount of residual sugars. Some of the results of the earlier studies on amylase production by *Aspergillus niger* strain UO-01 indicated that sugarcane bagasse acted as the best solid substratum when compared with the other solid substrates used (Pandey et al., 2000; Roses and Guerra, 2009). Other solid substrates had less amount of water content in them which might have resulted in the poor penetration of the fungal hyphae leading to the less utilization of the nutrients and resulting in production of lesser amount of the enzyme. To our knowledge, there is less literature available on the use of sugarcane bagasse as a solid substratum for the production of amylases by *Aspergillus* strains. Thus considering the substantial availability of sugarcane bagasse at very low prices from the sugar cane factories, and the ability to produce high titre of the amylase favours its use as a support material which in turn could offer an attractive possibility for a low cost production of amylases by *A. flavus* strain in SSF.

![Fig. 1: Effect of different solid substrates on α-amylase production. Data represent mean ± S.D. (n=3); P< 0.05.](image)

α-amylase catalysing the D-glucose production from the non-reducing starch end is synthesized in the presence of an inducer. According to the most accepted mechanism of synthesis, the regulator gene codifies the production of a repressing protein, which may bind to the operator gene and block the transcription of the structural gene (Jacob and Monod, 1961). In the presence of the inducer, the repressing protein binds to it and does not interact with the operator gene, therefore allowing both the transcription process and enzymatic synthesis to occur. Thereby, the induced synthesis of the enzyme is due to its de-repression (Pazur et al., 1980).

Enzyme substrates are typical inducers with different potentials for induction, as in case of glucoamylase synthesis. Although lactose, glycerol and sucrose have been reported to produce significant induction of enzyme in bacteria (Tanyildizi et al., 2005), no such effect was observed in α-amylase yield by *A. oryzae* (Shivaramakrishnan et al., 2007). In our study involving α-amylase synthesis by *A. flavus*, soluble starch gave higher enzyme titres (3.866 U/gds) when compared to other carbon supplements (Fig. 2) and hence selected as inducer for the rest of the media optimization. The findings of our study is in perfect correlation with previous studies involving α-amylase synthesis by *A. oryzae*, which was reported to produce higher titres of α-amylase in the presence of maltose and starch as inducers (Shivaramakrishnan et al., 2007). *Bacillus thermooleovorans* is
reported to prefer starch, glucose, lactose, maltose and maltodextrins as carbon sources for \( \alpha \)-amylase secretion (Narang and Satyanarayana, 2001). Starch acted as the best carbon supplement possibly because the expression of \( \alpha \)-amylase is controlled by catabolic repression, or by repression mediated by the product that result from enzyme action (glucose, for instance). In the case of catabolic repression the cell blocks the enzyme synthesis when substrates of easier assimilation are available (Wang et al., 1979). Glucose has been known to induce only a minimal level of \( \alpha \)-amylase by \( A. \) oryzae (Arst and Bailey, 1977). Still the role of glucose in production of amylase is controversial. In contrast to our result, carbon sources such as starch, maltose did not enhance \( \alpha \)-amylase production by \( B. \) coagulans in solid state fermentation using wheat bran (Babu and Satyanarayana, 1995).

![Graph showing effect of different carbon sources on \( \alpha \)-amylase production.](image)

**Fig. 2:** Effect of different carbon sources on \( \alpha \)-amylase production. Data represent mean ± S.D. (n=3); P < 0.05.

Similarly, enzyme production was more efficient in medium containing organic nitrogen sources, especially yeast extract (3.442 U/gds) as compared with inorganic nitrogen sources (Fig. 3). Earlier studies also indicate that organic nitrogen sources are preferred for the production of \( \alpha \)-amylase, where a maximum \( \alpha \)-amylase production was supported by yeast extract, peptone or beef extract (Krishnan and Chandra 1982; Emanuilova and Toda, 1984; Hayashida et al., 1988).

![Graph showing effect of different nitrogen sources on \( \alpha \)-amylase production.](image)

**Fig. 3:** Effect of different nitrogen sources on \( \alpha \)-amylase production. Data represent mean±S.D. (n=3); P< 0.05.

**Optimization of Physical Parameters:**

Among the physical parameters, pH of the growth medium plays an important role by inducing morphological changes in microbes and in the enzyme secretion. Microorganisms are sensitive to the concentration of hydrogen ions present in the medium. Thus pH change observed during the growth of microbes also affects product stability in the solid state fermentation medium (Kunamneni et al., 2005). Different organisms have different pH optima and decrease or increase in pH on either side of the optimum value results in poor microbial growth (Lehninger, 1982). The obtained results demonstrated that even if \( \alpha \)-amylase production was good at acidic pH values, it was maximum at pH 6.0 (4.104 U/gds). On the other hand, relatively low values for \( \alpha \)-amylase production were recorded at neutral and alkaline conditions (Fig. 4). The pH of the fermentation media may change since organic acids are produced during fermentation. Earlier studies have revealed that fungi required slightly acidic pH and bacteria required neutral pH for optimum growth. pH is
known to affect the synthesis and secretion of α-amylase just like its stability (Ertan et al., 2006). Similar results were obtained for other fungi such as A. fumigatus, Thermomyces lanuginosus, Penicillium janthinellum NCIM 4960 where they gave significant yields of α-amylase at pH 5.0-6.0 (Goto et al., 1998; Kunamneni et al., 2005; Sindhu et al., 2009). However, A. oryzae released amylase only in alkaline pH above 7.2 (Yabuki et al., 1977).

The influence of temperature on amylase production is related to the growth of the organism. Since temperature influences protein denaturation, enzyme inhibition and cell growth, it plays a significant role in the development of the biological process. The optimum temperature depends on whether the culture is mesophilic or thermophilic. A. flavus being a mesophilic fungus, the optimum temperature for maximum amylase production was found to be 30°C (4.668 U/gds), when the effect of temperature on enzyme production was studied (Fig. 5). At this temperature the membrane was highly permeable and the enzyme synthetic machinery of the organism was highly functional with the maximum conversion rate of the starch polymer into reducing sugars. A decrease in enzyme titres was observed when temperature range fell outside the mesophilic range, because of the reduction of the metabolic activity of the culture. Among the fungi, most amylase production studies have been done with mesophilic fungi within the temperature range of 25-37°C (Ramachandran et al., 2004). Our result is in perfect accordance with the results obtained by other workers, where raw starch degrading α-amylase was produced respectively by A. niger UO-01 at 30°C (Mazutti et al., 2006).

The incubation time for achieving the maximum enzyme level is governed by the characteristics of the culture and is based on growth rate and enzyme production. The incubation time varies with enzyme productions (Smith and Onions, 1994). After optimization of all the process parameters, the time course of maximal enzyme production was studied. The results elucidated that the incubation period influences the enzyme production, wherein, the amylase activity increased steadily and reached maximum (5.466 U/gds) at 120 h of incubation (Fig. 6), as against a short duration of 24 h in the case of bacteria (Dharani Aiyer, 2004). Further increase in the incubation period led to a reduction in α-amylase production. This might be due to the depletion of nutrients in the medium. Similarly, the maximal productivity of amylase, was achieved in 120 h at 30°C on a wheat bran substrate using starch and urea as supplement for A. oryzae and Thermomyces lanuginosus respectively (Ellaiah et al., 2002; Kunamneni et al., 2005).
Characterization of α-Amylase:

The SDS-PAGE analysis of the enzyme showed that the band appeared approximate to 55 kDa, thus confirming it to be of α-amylase (Fig. 12). In previous studies α-amylase from Aspergillus flavus was purified by ammonium sulfate precipitation and ion-exchange chromatography and was found to be homogeneous on sodium dodecyl sulphate polyacrylamide gel electrophoresis. The purified enzyme had a molar mass of 52.5±2.5 kDa with an isoelectric point at pH 3.5 (Khoo et al., 1994). But the molecular weight of the partially purified amylase from A. oryzae was found to be approximately 68 kDa (Ramachandran et al., 2004).

Temperature and pH are the most important factors, which markedly influence enzyme activity. Maximum amylase activity (7.336 U/gds) was recorded at 55°C. Further increase or decrease in temperature resulted in decrease in the amylase activity (Fig. 7). The effect of temperature on the activity of amylase obtained from Penicillium rugulosum was measured and it was found that the enzyme activity increased from 27°C to 57°C and decreased thereafter. 57°C was found to be the optimum temperature for amylase activity (Tiwari et al., 2007). Similarly, for Bacillus sp the optimal temperature was found to be 60°C at pH 6.5 (Thippeswamy et al., 2006). The effect of pH on the enzyme activity indicates that the amylase is active in the pH range 4-8 with the highest activity (8.333 U/gds) at pH 5 (Fig. 8). This suggests that the enzyme would be useful in processes that require wide range of pH change from acidic range to slightly alkaline range. Previous investigations concluded that the optimum pH values for MM-α-amylase enzyme ranged between 5.8 and 6.4 for RH-α-amylase (El-Safey, 1994). The α-amylase enzyme obtained from A. flavus was found to have maximum activity at pH 6.0 (Khoo et al., 1994). Similarly other workers have reported acidic pH optima for amylases from A. niger (Uguru et al., 1997).

Evaluation of enzyme activity with different concentrations of soluble starch revealed that a maximum activity (4.806 U/gds) was obtained with 1% starch as the substrate (Fig. 9). Similarly, previous workers allowed the crude amylase extract to react with different substrate concentrations and found that 1% of soluble starch gave the maximum enzyme activity (Alva et al., 2007). Our results are in accordance with these workers who found that with the increase in the concentration of soluble starch beyond a certain concentration there is a decrease in the enzyme activity. This can be attributed to the fact that with increasing substrate concentration, the rate of its conversion becomes greater until it approaches its optimum value (Nelson and Cox, 2002).
For a defined substrate concentration, the initial velocity of the reaction is dependent on enzyme concentration and increases with the enzyme concentration until a certain point from which the total amount of substrate is found in the form of enzyme-substrate complex and the initial reaction velocity is constant and maximal. For kinetic studies it is thus necessary to provide the enzyme at a lower concentration. Thus the amount of enzyme must be extremely low when compared to the substrate concentration to ensure that the enzyme-substrate complex formation do not modify or only slightly modify the substrate concentration (Scriban, 1984). The results obtained in our study support the above observation, as the maximum amylase activity (7.273 U/gds) was obtained at 81 µg/ml concentration (Fig. 10).

**Fig. 8:** Effect of pH on α- amylase activity. Data represent mean ± S.D. (n=3); P < 0.05.

**Fig. 9:** Effect of substrate concentration on α- amylase activity. Data represent mean ± S.D. (n=3); P < 0.05.

**Fig. 10:** Effect of enzyme concentration on α- amylase activity. Data represent mean ± S.D. (n=3); P < 0.05.
Most of amylases are known to be metal ion-dependent enzymes, namely divalent ions like Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, Fe$^{2+}$, etc. (Pandey et al., 2000). Study of the different metal ions and chelator, that influence amylase activity, indicated that the enzyme was considerably activated by Mn$^{2+}$ and Ca$^{2+}$ ions and inhibited by HgCl$_2$ and EDTA (Selvakumar et al., 1996) (Fig. 11). Our result contradicts the previous observations, where calcium slightly inhibited the amylase activity (Reyed, 2007) and that Ca$^{2+}$ had no effect on enzyme activity (Asgher et al., 2007). The fact that α-amylase is not inhibited by Ca$^{2+}$ ions makes it suitable for use in the starch industry. Previously, the stimulatory effects of Mn$^{2+}$ and Ca$^{2+}$ ions were also reported in the case of amylase produced by A. niger, alkaliphilic Bacillus sp. ANT-6 (Burhan et al., 2003). The inhibitory effect of Hg$^{2+}$ has also been reported on glucoamylase produced by A. awamori (Abe et al., 1988). The inhibition by mercuric ions may indicate the involvement of indole amino acid residues on enzyme function (Inokuchi, 1999). EDTA, an ion chelate, was not effective on amylase activity suggesting that this enzyme does not require such metallic ion in its active site for activity.

![Fig. 11](image1.png)

**Fig. 11:** Effect of metal ions on α- amylase activity. Data represent mean ± S.D. (n=3); P < 0.05.

![Fig. 12](image2.png)

**Fig. 12:** Protein profile of the partially purified amylase by SDS-PAGE. Lane M, standard protein marker; Lane 1, amylase from A. flavus. Molecular sizes of the marker proteins (in kDa) are shown on the left.

**Conclusion:**

The results obtained in the present study suggest that the mangrove isolate of A. flavus may act as a potent strain for α- amylase production using SSF with sugarcane bagasse as the substrate. Optimization of the fermentation parameters and the use of suitable carbon and nitrogen supplements resulted in 5 folds increase in the enzyme yield. The enzyme was significantly active at higher temperatures and the optimum temperature for the activity was found to be 55ºC. The enzyme was found to be active over a wide range of pH and showed the optimum activity at pH 5. This isolate can thus be industrially exploited for the synthesis of α- amylase which can have several industrial applications.

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