



ISSN Print: 2394-7500  
ISSN Online: 2394-5869  
Impact Factor: 5.2  
IJAR 2016; 2(6): 1006-1010  
www.allresearchjournal.com  
Received: 22-04-2016  
Accepted: 23-05-2016

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## A comparative study on fungal (*Aspergillus niger*) amylase and elephant foot yam (*Amorphophallus campanulatus*) amylase with yam starch as substrate

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### Abstract

Amylases are one of the most important and widely used enzymes whose application has widened in many sectors such as clinical, medicinal, and analytical chemistry. Amylase enzyme is widely used in bread making and to break down complex sugars such as starch into simple sugars. Amylase enzymes from *Amorphophallus campanulatus* and *Aspergillus niger* were isolated and purified by gel permeation chromatography using Sephadex G-75 column. The enzymes were assessed for activity, protein concentration and specific activity in different stages of purification. The specific activity was found to be increasing as the purity increases. The activities of both the enzymes were compared by taking commercial starch and yam starch as substrate. The activity of both the amylases increased when yam starch was taken as a substrate. The optimum pH, temperature and incubation time of  $\alpha$ -amylase from both the sources was found to be 7.5, 30 °C and 30 minutes respectively. The  $\alpha$ -amylase from both the sources are having the same optimum pH, temperature, incubation time, whereas only the activity was more in case of *Amorphophallus campanulatus* when compared to *Aspergillus niger*. This requires further study to find out the structural similarity among the two different sources of  $\alpha$ -amylase enzyme.

**Keywords:**  $\alpha$ - amylase, *Aspergillus niger*, *Amorphophallus campanulatus*

### 1. Introduction

Alpha-Amylase (EC 3.2.1.1) also named as 4- $\alpha$ -D-glucan glucanohydrolase, has found its application in a range of industries including food, brewing, distilling industry, textile, paper, pharmaceutical and bioconversion of solid waste etc. Large range of applications is the triggering factor for the industrialization of alpha amylase production. Amylases have been reported to be produced by plant, animal and microbial sources, although the microbial amylase production has been reported to be most effective (Pandey *et al.*, 2000; Gupta, 2003) [10, 6]. Amylase production comprises of about 10% of the world enzyme production.

The microbial sources for amylase production are *Bacillus spp* & *Aspergillus spp* (Deutch, 2002) [4]. Amylase of fungal origin was found to be more stable because of the non-fastidious nutritional requirement & ubiquitous nature. Generally, fungi secrete alpha amylase & other scarifying enzyme depending on the composition of growth medium & fermentation conditions. The production of fungal enzymes and secondary metabolites through fungal cultivation under submerged conditions is the other alternative method of fungal cultivation by solid state fermentation (SSF) where the fungus is grown on a moist solid substrate. *Aspergillus nigeris* a fungi belongs to the member of the genus *Aspergillus* that is generally ubiquitous in nature. They are geographically widely distributed and are commonly found as saprophytes growing on dead leaves, stored grain & other decaying vegetation and it appears as carbon black or very dark brown patches. *Aspergillus niger* is widely used in the food industry for the production of many enzymes such as  $\alpha$ - amylase, amyloglucosidase, cellulases, lactase & acid proteases.

*Amorphophallus campanulatus* plant belongs to the family of Dioscoreaceae that form edible tubers. These are perennial herbaceous vines cultivated for the consumption of their starchy tubers in Africa, Asia and Latin America.

The tuber has therapeutic properties like anti – inflammatory, antispasmodic, blood purifier & diaphoretic, hepatic, anti-rheumatic.

## 2. Materials and Methods

Elephant Foot Yam was obtained from the local market of Mysore. The Yam peel was used as enzyme source and the pulp was used as starch source. Sephadex G-75 was procured from Sigma and all the other chemicals of AR grade were procured from SRL and Merck.

### 2.1 Extraction of starch from *Amorphophallus campanulatus* tuber

The *Amorphophallus campanulatus* (yam) tuber was cleaned with distilled water and made into small pieces. Then it was grinded in the mixer by adding distilled water to get fine slurry. Then the slurry was kept aside for an hour for the starch to settle down. The upper layer is discarded and the starch precipitated was washed with distilled water for 3-4 times. Then the starch was separated by filtering through muslin cloth and it is dried at room temperature. The dried starch was used as substrate for  $\alpha$  amylase in the further experiments.

### 2.2 Isolation and identification of *Aspergillus niger* strain

*Aspergillus niger* was isolated from soil by serial dilution method of Clark *et al.*, 1998 [3]. One g soil sample was dissolved in 100 ml sterilized distilled water. The soil suspension was diluted up to  $10^{-5}$  and 0.5 ml of diluted suspension was used to culture the microorganism. The *Aspergillus niger* producing starch digesting  $\alpha$  amylase was screened according to method described by Bergmann *et al.*, 1998 and Akpan *et al.* 1999 [2, 1]. *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 the method described by Raper 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.

### 2.3 Growth medium

The medium contained (% w/v): Starch (5%) to which a mineral salt composition of  $ZnSO_4 \cdot 7H_2O$  (6.2mg),  $FeSO_4 \cdot 7H_2O$  (6.8mg) and  $CuSO_4 \cdot 7H_2O$  (0.8mg) was added and volume was made upto one liter with distilled water. The pH of the medium was adjusted to 4.5 with 1molar HCl, the medium was sterilized by autoclaving at 121 °C and 15-17 psi for 30 minutes.

### 2.4 Inoculum and fermentation

A certain inoculum size of conidia (Each mL of cells suspension contained  $2.0 \times 10^5$  cells) was transferred from a stock culture in 250mL flask containing 100mL growth medium. The flask was incubated for 96 hrs at room temperature on a rotatory shaker at 150 rpm. On the last day of incubation period (96hr), the fungal mass was separated by centrifugation at 4500 rpm for 10 min. The clear supernatant (crude enzyme) was used for estimation of  $\alpha$ -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 (maltose) in one minute under assay conditions. The specific activity was defined as number of units per milligram protein.

### 2.5 Preparation of enzyme from spent media of *Aspergillus niger* and *Amorphophallus campanulatus* tuber peel

The enzyme was precipitated from the clear spent media at 4 °C by adding solid ammonium sulphate to achieve 45% saturation. The ammonium sulphate was added slowly, keeping the solution in ice and the protein was allowed to precipitate by keeping it overnight at 4 °C. The protein was separated by centrifugation at 2000 x g for 30 minutes at 4 °C, dissolved in minimum volume of 0.1M PBS and used immediately for activity determination (Gupta, 2008) [5]. 50gm of Yam peel was taken and grinded into fine paste. This was soaked in 0.1M NaCl and kept overnight (salting in). It was then centrifuged at 10,000 rpm for 15 min. The supernatant was estimated for protein concentration and  $\alpha$ -amylase activity. To the supernatant ammonium sulphate was added to precipitate the enzyme (45% saturation). The protein separated by salting out was separated by centrifugation at 10,000 rpm for 15 min and the pellet was dissolved in 0.1M PBS. The resulting protein precipitates were dialyzed and their protein concentration (Lowry *et al.*, 1951) [8] and  $\alpha$  amylase activity were determined.

### 2.6 Purification and partial characterization of $\alpha$ -amylase from *Aspergillus niger* and *Amorphophallus campanulatus* peel

The fraction obtained from ammonium sulphate precipitation of both Yam and *Aspergillus niger* was loaded on to Sephadex G-75 column (GPC), pre-equilibrated with 0.1M PBS buffer and eluted with the same buffer at a flow rate of 2ml/5min. Absorbance at 280 nm is taken using spectrophotometer, protein and  $\alpha$ -amylase activity of each fraction were determined. Peak showing high  $\alpha$ -amylase activity were pooled and used for further characterization.

### 2.7 Determination of $\alpha$ – Amylase activity in Elephant foot Yam peel and *Aspergillus niger*

Enzyme activity is defined as the amount of enzyme that liberated micromole of maltose per 1 min under standard assay. The reducing sugars released from starch are measured by reduction of 3, 5- dinitrosalicylic acid. One unit releases from soluble starch one micromole of reducing groups (calculated as maltose) per minute at 25 °C and pH 6.8 under the specified conditions; absorbance is read at 540 nm (Miller, 1959) [9].

### 2.8 Effect of pH on $\alpha$ -amylase activity

The pH of the reaction medium affects the enzyme activity; hydrogen ions influence the enzyme activity by altering the ionic charge on the amino acids at the active site of the enzyme this affects the enzyme substrate complex formation. Hence each enzyme has an optimum pH at which the reaction velocity is maximum. Above or below the optimum pH the enzyme activity decreases and at an extreme pH the enzyme become totally inactive.

$\alpha$ -Amylase activity was determined by pre incubating enzyme with 0.8 ml of various buffers of pH 4.5, 6.0, 7.5, 9 followed by the addition of substrate for 30 minutes. The reaction was stopped by adding DNS and kept in boiling water bath for 15 minutes, then diluted with distilled water and the absorbance was read at 540nm. The optimum pH was determined by using maltose standard.

### 2.9 Effect of temperature on $\alpha$ -amylase activity

The temperature at which the enzyme activity is maximum is known as optimum temperature. Beyond this temperature the

enzyme becomes inactive and the rate of the reaction decreases. In order to determine the optimum temperature of  $\alpha$ -amylase, the enzyme was incubated with substrate at different temperature. The temperature between 5° to 50°C was taken for the determination of the enzyme activity.

### 2.10 Effect of time kinetics on $\alpha$ -amylase activity

The amount of product formed at different time intervals determines the efficiency of the enzyme. The optimum incubation time was calculated by keeping the temperature, pH, substrate and enzyme concentration constant.

The activity of Alpha- Amylase were determined by separately incubating amylases with 1% soluble starch, in phosphate buffer with pH 7.5 and incubated at their optimum temperature for 30 min. The reaction was stopped at regular time interval of 5, 10, 20, 30, 40, & 45 minutes by adding DNS. The tubes were kept in boiling water bath for 15 minutes, cooled and diluted with distilled water and absorbance was read at 540nm.

### 2.11 Effect of activators and inhibitor on $\alpha$ - amylase enzyme activity

Activators are the molecules which enhance the enzyme activity. Activators are often involved in allosteric regulation of the enzymes. Enzyme inhibitors are those which alter the catalytic action of the enzyme and consequently slow down or in some cases stop the reaction. The effect of the inorganic salts such as sodium chloride, potassium iodide and copper sulphate in different concentration (10mM, 50 mM and 100mM) on the enzyme activity was studied.

### 2.12 Determination of molecular weight by SDS-PAGE

Polyacrylamide gel electrophoresis with 7.5% gel under native basic conditions and SDS-PAGE was carried out to determine the molecular weight of the amylases. The gels were stained for protein by Coomassie Brilliant Blue (Walker, 1996) [14].

## 3. Results and Discussion

The Yam was taken from the local market (Fig.1) and the fungi *Aspergillus niger* was isolated from the soil sample (Fig.2). A strain of *Aspergillus niger* showing the maximum starch hydrolyzing property was isolated, purified and taken as a source of fungal  $\alpha$ - amylase for further studies.

Amylase enzymes from Yam and *Aspergillus niger* were purified by gel permeation chromatography using Sephadex G-75 column. The  $\alpha$ - amylase activity was found in third peak in Yam and in second peak in *Aspergillus niger* (Figure 3 &4). The enzymes were assessed for activity, protein concentration and specific activity in different stages of purification. The specific activity was found to be increasing as the purity increases which are compared in the table 1 and 2 given below. Much higher  $\alpha$ - amylase enzyme activity has been reported from the mutated strains of *Aspergillus spp.* (K N Varalakshmi *et al.*, 2009) [13] where as the activity of the Yam amylase with Yam starch as substrate is comparable with the activity mentioned earlier (Preethi & Veerabasappa Gowda, 2013) [11].

The optimum pH for  $\alpha$ - amylase in Yam peel & *A. niger* was found to be 7.5. At this pH enzyme activity was found to be 37.03  $\mu$ M/ml/min at 37 °C in yam peel & 13.888  $\mu$ M/ml/min at 37 °C in *A. niger* (fig.5).The optimum temperature for  $\alpha$ -amylase in Yam peel & *A. niger* was found to be 30 °C. At this temperature enzyme activity was found to be 8.33

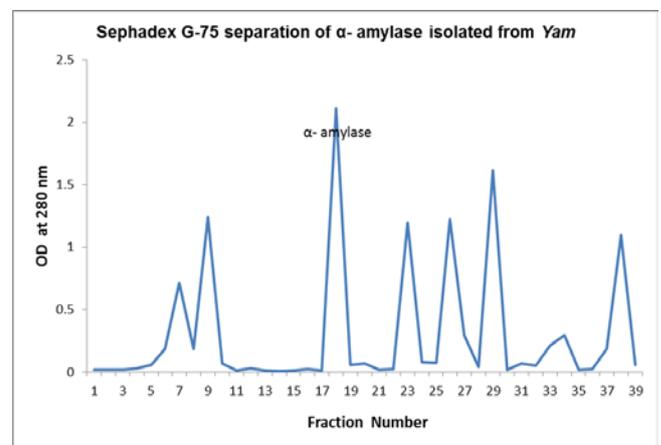
$\mu$ M/ml/min at 37 °C in yam peel & 3.70  $\mu$ M/ml/min at 37 °C in *A. niger* (fig.6). The optimum incubation time for  $\alpha$ -amylase enzyme in Yam peel & *A. niger* was found to be 30minutes. At this time interval enzyme activity was found to be 9.25  $\mu$ M/ml/min at 37 °C in yam peel & 2.777 $\mu$ M/ml/min at 37 °C in *Aspergillus niger* (fig.7). Copper sulphate acts as inhibitor for  $\alpha$ - amylase from both yam and *A. niger*. Copper sulphate at 50mM concentration the enzyme activity was increased by 20% in Yam and 25% in *A. niger* (fig. 8). Sodium chloride acts as activator for  $\alpha$ - amylase for both yam and *A. niger*. Sodium chloride at 100 mM concentration the activity of the enzyme is increased to 90% in *Amorphophallus campanulatus* and 70% in *Aspergillus niger* (fig. 9). The approximate molecular weight of alpha amylase enzymes isolated from *Amorphophallus campanulatus* and *Aspergillus niger* were found to be  $\approx$  48 Kda by SDS-PAGE (fig.10). Both amylases seem to be of same molecular weight but the structural similarity has to be verified by further studies. In the present study Yam starch was used as a substrate and there was a report on using tapioca starch as a substrate for *Aspergillus flavus* which produced approximately 50 U/mL of amylolytic activity (Khoo, 1994) [7].

**Table 1:** Activity, protein concentration and specific activity of  $\alpha$ -amylase isolated from *Aspergillus niger*.

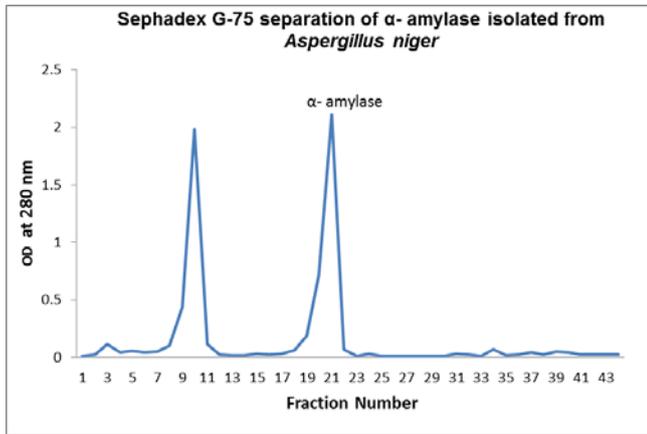
Sample	$\alpha$ - amylase activity per ml	Protein concentration mg/ ml	Specific activity
Crude	0.37	1.31	0.28
Ammonium sulphate precipitated	0.74	0.93	0.78
Sephadex G-75 fraction	3.70	0.50	7.40

**Table 2:** Activity, protein concentration and specific activity of  $\alpha$ -amylase isolated from *Yam*.

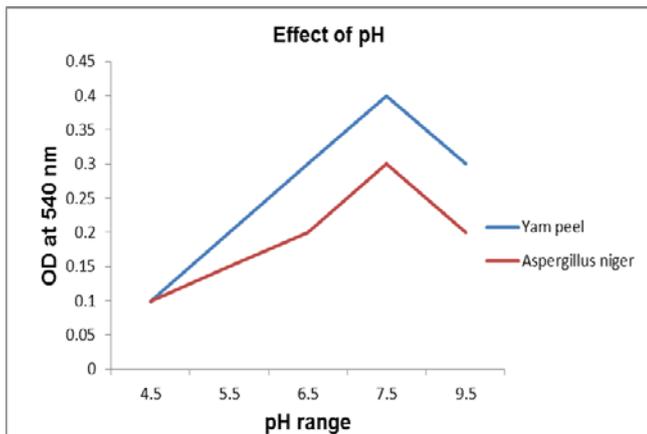
Sample	$\alpha$ - amylase activity per ml	Protein concentration mg/ ml	Specific activity
Crude	1.85	1.12	1.64
Ammonium sulphate precipitated	2.92	0.56	5.20
Sephadex G-75 fraction	4.6	0.50	9.20



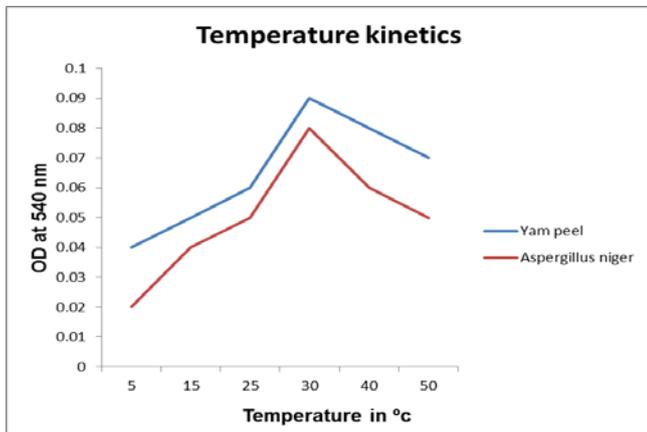
**Fig 3:** Gel permeation chromatographic (Sephadex G-75) separation of  $\alpha$ - amylase isolated from *Yam*.



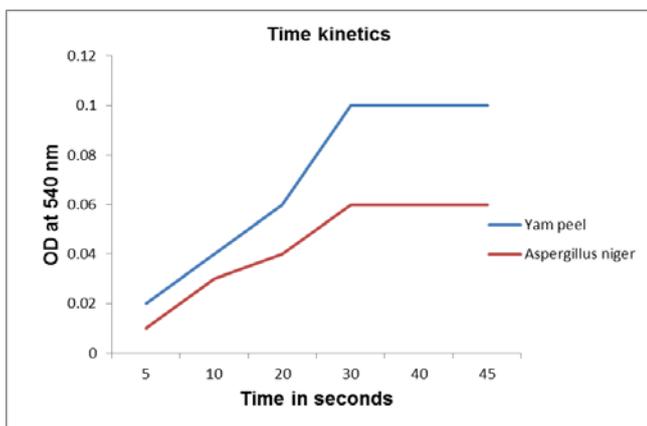
**Fig 4:** Gel permeation chromatographic (Sephadex G-75) separation of  $\alpha$ -amylase isolated from *Aspergillus niger*.



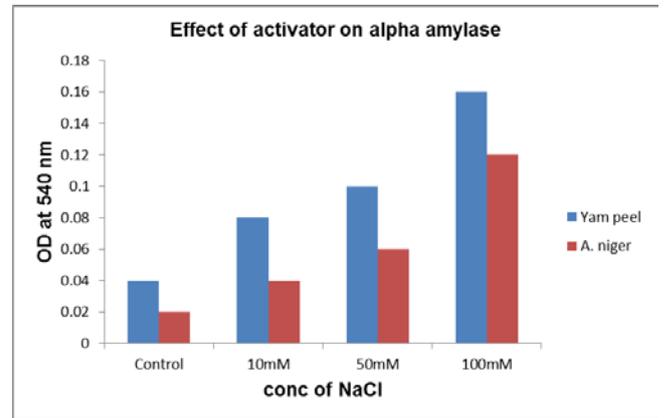
**Fig 5:** Effect of pH on alpha amylase



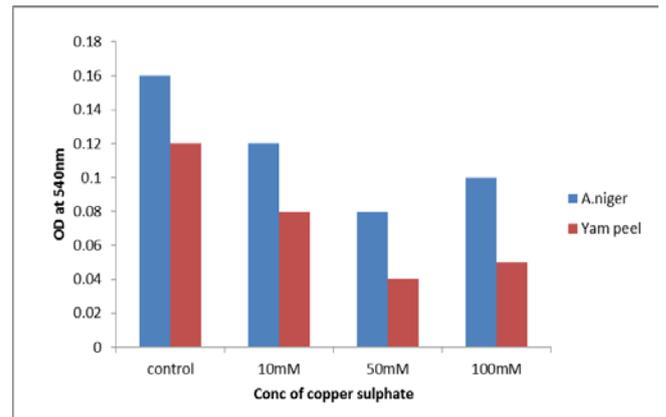
**Fig 6:** Effect of temperature on alpha amylase



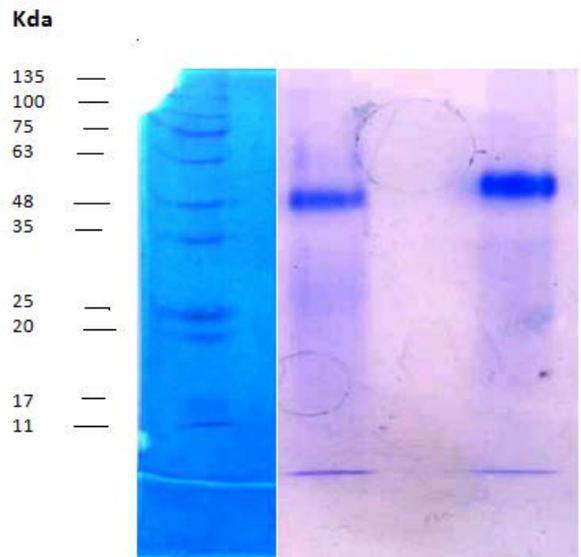
**Fig 7:** Time kinetics on alpha amylase



**Fig 8:** Effect of activator on alpha amylase activity



**Fig 9:** Effect of inhibitor on alpha amylase activity



**Fig 10:** SDS-PAGE of amylase Lane 1-molecular weight ladder, Lane 2 - amylase from *Aspergillus spp.*, Lane 3-amylase from Yam peel

**4. Conclusion**

Rapid screening for the fungi producing alpha amylase in soil has led to the isolation of the *Aspergillus* strain having maximum starch hydrolyzing activity. The fungal and the *Amorphophallus campanulatus*  $\alpha$ -amylase enzymes were purified, partially characterized and compared to understand the differential behavior upon Yam starch as substrate. Since the Yam starch is endogenous its degradation products are expected to serve as an energy source for the growing plant. So the  $\alpha$ -amylase activity is more in Yam peel when compared to that of *Aspergillus niger* with Yam starch as a

carbon source. However amylases from microbial sources, especially fungi (*Aspergillus spp.*) have gained much attention because it is ubiquitous in nature and are amenable to genetic manipulation. The present investigation reveals the regulation of enzyme activity by evolving enzyme substrate combination to meet their metabolic requirement.

## 5. Acknowledgement

Authors are grateful to UGC-SWRO for the financial assistance and the JSS College of Arts, Commerce and Science for providing the facilities to carry out this work.

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