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Amylase Production by *Aspergillus Niger* Cultured on *Adansonia Digitata* Seed Powder

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Abstract

Aspergillus niger, Aspergillus flavus and *Aspergillus fumigatus* isolated from soil were screened for their ability to produce amylase enzyme. *Aspergillus niger*, showed the highest amylase activity by exhibiting the widest halo on starch agar medium. The fermentation process was optimized using the following parameters pH, temperature, inoculum size, incubation period and the optimum conditions for the production of amylase. Amylase activity was determined using Dinitrosalicyclic acid (DNS) method. It was found that the amylase enzyme was produced at pH 7.9, temperature 75^0C, and 10% inoculum size for 9 days incubation period gives a maximum yield. This study shows that *Aspergillus niger* strain was able to produce thermostable alpha-amylase with characteristics suitable for application in starch processing and other food industries.

Keywords: *Adansonia digitata*, amylase enzymes, *Aspergillu*s spp., fermentation, Dinitrosalicyclic acid.

Introduction

In recent years the potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms (Akpan et al., 1999; Pandey et al., 2000; Abu et al., 2005). Microbial enzymes are enzymes which are derived from microorganisms such as fungi and bacteria through the process of fermentation. An example of such enzymes are amylases which are important enzymes employed in the starch processing industries for the hydrolysis of polysaccharides such as starch to

simple sugar constituents (Akpan et al., 1999; Pederson and Nielsen, 2000) by degrading 1 – 4 linkage of starch. Amylases can also be derived from plants and animals. Animal amylase is mainly α-amylase, while β-amylase occurs in plants, but due to their short growth period, the enzymes from microbial sources generally meet industrial demands (Miner et al., 1997; Jensen and Olsen, 1992). They are preferred to both plants and animals sources because they are cheaper to produce, their enzyme contents are more predictable, controllable, reliable and the supplies of raw materials are of constant compositions (Burhan et al., 2003). Production of amylase is dependent on the substrates (cereal and tuber starches) that are heavily competed for as staple food, especially in the developing countries like Nigeria. These countries depend on these starches as major source of energy and other nutrients. Exploration for alternative substrate such as pomace for the production of this enzyme could immensely reduce the level of competition for theses starches in the developing countries. Amylase production is also dependent on the strains of the organism, composition of the media to be used, the methods of cultivation and environmental conditions. Abe et al., (1988) and Oyeleke and Oduwole, (2009) reported that very few microorganisms possess the ability to produce starch degrading amylase this include *Bacillus* species, *Rhizopus* spp, and *Aspergillus* spp. The new potential of using micro-organisms as biotechnological sources for industrial production of enzymes in recent years has stimulated renewed interest in the exploration of extracellular enzymatic activity in several micro-organisms (Akpan et al., 1999; Buzzini and Martini, 2002).

Aspergillus niger is cultured for the industrial production of many substances. Its primary uses are for the production of enzymes such as amylases and organic acids by the process of fermentation. These amylases are examples of hydrolases and function in the hydrolysis of molecules. They are important enzymes used in biotechnology (Burhan et al., 2003).

This study is aimed at: isolating *Aspergillus* species with amylolytic activity; exploiting *Adansonia digitata* seed powder as an alternative substrate for amylase production; and optimization of the production process.

Materials and Methods

Sample collection

Dried fruits of *Adansonia digitata* were collected from the crown of standing tree by using a hook to cut the hanging fruits. The tree is located behind Nana Asma'u Hostel (females' hostel) in Usmanu Danfodiyo University Sokoto (main campus). The seeds were then extracted by hitting the fruits on a hard surface in order to break it. The mixture of seeds and pulp was then soaked in water for about 6 hours to remove the pulp by gently squashing and floating in water. Floatation helped separate dead seeds, with those that float being discarded. The seeds were then dried in the sun for 2 days to reduce the moisture content to make them more susceptible for crushing. The crushing was done by pounding the seeds using a cleaned pestle and motar. The crushed substrate was sieved using sieve pore size of 0.2mm to get fine powder. The fine powder was then stored at room temperature until when required.

Isolation and Identification of *Aspergillus* spp

The *Aspergillus species* used in this study were isolated from soil by serial dilution method of Cheesbrough. (2003). The soil sample was aseptically collected by sweeping off the debris from the top soil and using a hand trowel to collect about 100g of the top soil into a plastic bag. About 1g of the soil sample was suspended in 9 ml of sterile distilled water and was properly mixed. 1ml of the soil suspension was pipetted from the above and then transferred into another 9ml of sterile distilled water. The soil suspension was further diluted in two more 9ml of sterile distilled water (blanks). An aliquot of 0.1ml from the dilution was spread on potato dextrose agar plates using a sterile glass spreader. The plates were incubated at room temperature for 3 days. The growth of fungal colonies was observed. The individual colonies re-streaked on molten potato dextrose agar plates. Then the cultures were identified on the basis of macroscopic and microscopic characteristics of their conidiophores and conidial heads as described by Robert and Ellen (1988).

Preparation of fermentation medium used for amylase production

The fermentation medium contained the following in grams per litre; KH_2PO_4 1.4; NH_2NO_3 10; KCL 0.5; $MgSO_4.7H_2O$ 0.1; $FeSO_4.7H_2O$ 0.01, *A. digitata* seed powder 20g. The above medium composition were dissolved in 1000ml of distilled water after which 100ml of the medium was measured into conical flask (250 ml capacity each) heated on hot plate to homogenized and then sterilized in an autoclave at 121^0C for 15 minutes after which they were removed and allowed to cool.

Screening for amylolytic activity of the Identified *Aspergillus* spp

The amylolytic activity of the test isolates was determined by using the starch agar plate method as described by Bertrand et al. (2004), by inoculating the identified organisms which include *A. niger, A. flavus* and *A. fumigatus,* respectively into Sabourad Dextrose Agar medium which was supplemented with 1g of starch. The agar plates were then incubated at 30^0C for 3 days, after which they were flooded with Lugol's iodine solution. The diameter of clearing formed was measured to represent the amylolytic activity. The species with the widest diameter was chosen for further investigation.

Inoculum preparation

This was prepared as described by Negi and Banerjee. (2006). For inoculums preparation, 25 ml of sterile distilled water was added to the 5-day-old slant grown on malt- extract agar plate and scraped aseptically with inoculating loop. This suspension, having spore concentration of approx. 13·12 cells/ml, was used as inoculums for the subsequent fermentation.

Optimization of amylase production process

The effect of some fermentation parameters on amylase production by *Aspergillus niger* was evaluated. The different temperature values used were 30^0C, 45^0C, 55^0C, 65^0C and 75^0C; pH values were 3.6, 4.2, 5, 5.8 and 7.9; various inoculums size were

2%, 4%, 6%, 8% and 10%; at different incubation period such as 5 days, 7 days [30^0C], 7 days [55^0C], 8 days, and 9 days.

Amylase assay

Fungal mycelia were removed from the fermentation broth by pouring through sterile funnel fitted with No: 1 Whatman filter paper. The filtrate was used as crude amylase enzyme.

Amylase activity was measured as described by Bertrand et al. (2004). A reaction mixture containing 0.5 ml of 1 % (mass per volume ratio) soluble starch solution prepared in 0. 2 M citrate buffer and 0.5 ml of appropriately diluted enzyme solution was incubated at 50°C. After 10 min of incubation the reaction was terminated by adding 1.0 ml of DNS (Dinitrosalicyclic acid) solution (1 g of DNS dissolved in 20 ml of 1NaOH, to which 30 g of sodium potassium tartarate were added and filled with water to 100 ml). Reaction mixture was boiled for 15 min and after cooling, 18 ml of water were added. Absorbance was measured by the use of spectrophotometer set at 540 nm.

One unit of enzyme activity was defined as the amount of enzyme that releases 1 mmol of reducing sugar (glucose) in 1 min under the assay conditions (Bertrand et al. 2004).

Results

Production of amylase by *Aspergillus* species cultured on *Adansonia digitata* seed powder revealed that *Aspergillus niger* had the highest zone of amylase activity (Table 1). The effect of pH on the production of amylase enzyme by *Aspergillus niger* reveal that enzyme production reduced significantly as the pH level increased from 3.6 to 5.8, but at pH 7.9 there was an optimum production i.e 0.062, 0.048, 0.047 and 0.031 concentrations (mg/ml) there was a decrease in the production, and at 0.100 concentration (mg/ml) there was a maximum enzyme production which was the peak (Figure 2).

The effect of temperature on amylase enzyme production by *A. niger* revealed that amylase production by *A. niger* was low at temperature 30^0c (0.044 concentration mg/ml). It then increased at 45^0c (0.058 mg/ml) and then the production become very low at 65^0c (0.018 concentration mg/ml). But the optimum production was at 75^0c (0.159 concentration mg/ml). (Figure 3). The effect of inoculums size on amylase enzyme production by *A. niger* show that at 4% and 10% inoculums size (0.068 and 0.142 concentrations) but the production significantly reduced with decrease in the inoculum size, that is 8%, 6%, 2% at 0.050, 0.020 and 0.020 concentrations (mg/ml) respectively. But the peak of the amylase production was at 10% (Figure 4).

The effect of incubation periods on the production of amylase enzyme by *Aspergillus niger* reveals that at 5 days and 9 days of incubation period the production was 0.065 and 0.159 concentrations (mg/ml) but the peak was at 9 days of incubation period (Figure 5).

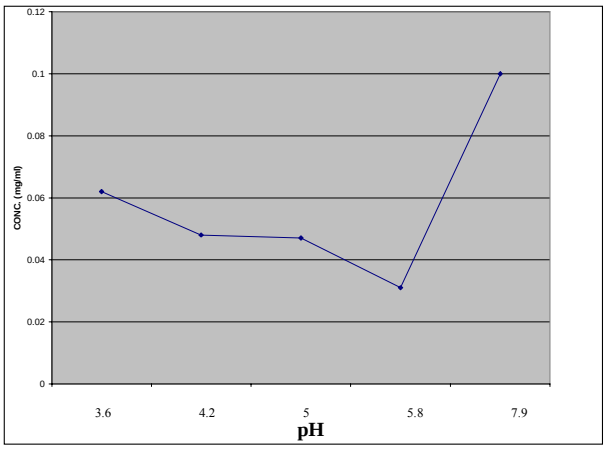

Figure 1: The effect of pH on amylase production by *A. niger.*

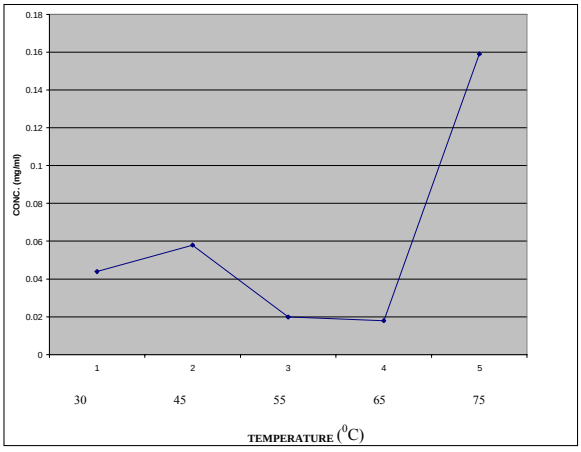

Figure 2: The effect of Temperature on amylase production by *A. niger.*

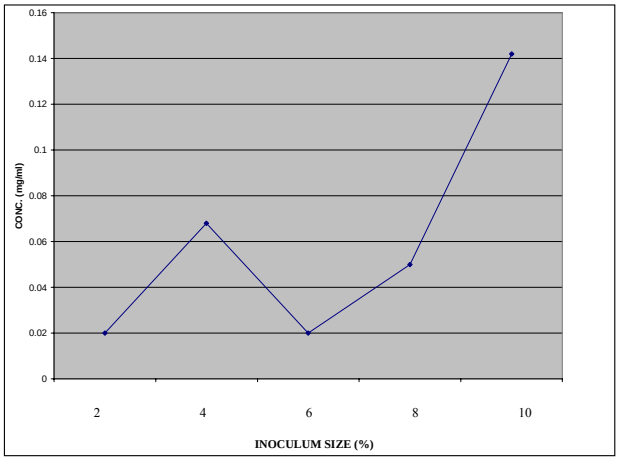

Figure 3: The effect of inoculum size on amylase production by *A. niger.*

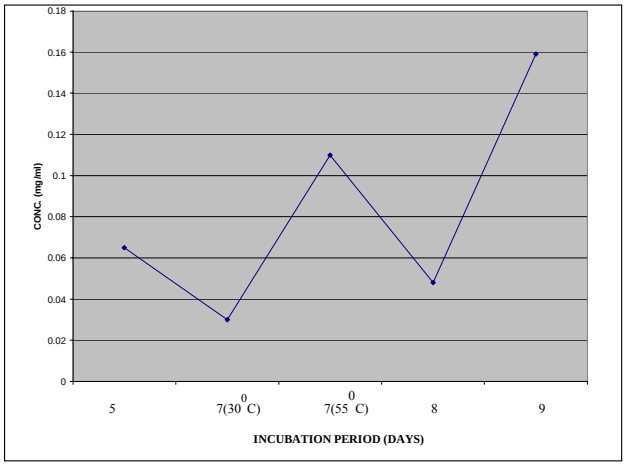

Figure 4: The effect of incubation period (Days) on amylase production by *A. niger.*

Table 1: Amylolytic activity of *Aspergillus* species screened.

Identified isolates	Zones of amylase activity (mm)
Aspergillus niger	3.98
Aspergillus flavus	2.90
Aspergillus fumigatus	1.24

Discussion

Three species of *Aspergillus* were characterized and identified on the basis of macroscopic and microscopic. The macroscopic and microscopic characterization revealed the presence of *Aspergillus niger, Aspergillus flavus and Aspergillus fumigatus*. All the *Aspergillus* isolates were hydrolyzers of starch. The amylolytic activity of the *Aspergillus* isolates following their zone diameter of clearing on starch agar (Table 2). *Aspergillus niger* had the highest halo (3.98mm) while *Aspergillus fumigatus* had the least (1.24mm). *Aspergillus niger* which showed the highest amylolytic activity (3.98mm), was selected for further analysis. Variation in amylolytic halo produced by the different species of *Aspergillus* may probably was as a result of differences in their genetic make-up. Similar observations were made by Gupta et al. (2008). The result of effect of pH on amylase enzyme production by *Aspergillus niger* revealed that the enzyme production was significantly reduced as the pH level increased from 3.6 to 5.8, but at pH 7.9 there was an optimum production of the amylase enzyme by *Aspergillus niger* (figure 2) i.e 0.062, 0.048, 0.047 and 0.031 concentrations (mg/ml). There was a decrease in the production, and at 0.10 concentrations (mg/ml) there was a maximum enzyme production which was the peak. The result obtained in this study agrees with the earlier investigations by Gupta et al. (2008), who worked on production and characterization of α-amylase from *Aspergillus niger* and who reported optimum pH of 7.3 at 0.16(mg/ml). However,

Fabiana et al. (1999), who worked on production of amylases by *Aspergillus tamari* reported a lower optimum pH of 6.8 at 0.192 (mg/ml).

The effect of temperature on amylase enzyme production by *Aspergillus niger* reveals that the amylase enzyme production by *Aspergillus niger* was low at temperature 30^0C at 0.04 concentration (mg/ml) and then the production became very less at 65^0C at 0.018 concentration (mg/ml), but the optimum amylase enzyme production was at 75^0C at 0.16 concentration (mg/ml). The result obtained in this study is similar to that obtained by Goto et al. (1998) who worked on production of amylase enzyme production at 70^0C at 0.189 (mg/ml). The result obtained in this research work also agrees with that reported by Mukherjee and Majomdar (1993) who worked on Fermentative production of α-amylase by *Aspergillus flavus* and who reported optimum α-amylase enzyme production at 73^0C at 0.230 mg/ml.

The effect of inoculum size on amylase enzyme production by *Aspergillus niger* shows peak of the amylase enzymes production was at 10%. This agrees with the report made by Ellaiah et al. (2002) who worked on optimization of process parameters for glucoamylase production under solid-state fermentation by a newly isolated *Aspergillus* species and who reported maximum production of gluco-amylase at 9% inoculum size at 0.189 concentrations mg/ml. The result is also similar to that obtained by Murado et al. (1997) who worked on amylase production by solid-state culture of *Aspergillus oryzae* on polyurethane foams and reported optimum amylase enzyme production at 8% inoculum level. This is contrary to the result obtained in this study.

The effect of incubation periods (days) on the production of amylase enzyme by *Aspergillus niger* reveals an agreement with the result obtained by Fadel. (2000) who reported optimum amylase enzyme production of incubation period ranges from 5 to 8 days in their production of thermostable amylolytic enzyme by *Aspergillus* F – 909. However, Abu et al. (2005) reported maximum amylase enzyme production at incubation period range of 7 to 10 days by *Aspergillus niger* and *Sacchoromyces cerevisae* grown on Sorghum pomace. Omemu et al. (2005) in their research also reported similar result in which the maximum amylase enzyme production was at incubation period range of 4 to 7 days by *Aspergillus niger* AMO7 isolated from soil. This is contrary to the result obtained in this study.

Conclusion

The present study shows that *Adansonia digitata* seed powder can be exploited as a substrate for industrial production of amylase enzyme using *Aspergillus niger* which is at no cost.

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