Amylase Production by *Aspergillus flavus* Associated with the Bio-deterioration of Starch-Based Fermented Foods

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Abstract: In this study, attempt was made to investigate the production of amylase using *Aspergillus flavus* implicated in the bio-deterioration of starch-based fermented foods. Temperature studies revealed that 30 ºC was optimum for amylase production by this isolate. The optimum pH supporting the highest amylase production was found to be 6.0 (stimulated amylase titre of 10.1 U/ml), closely followed by pH 5.0 that stimulated an amylase titre of 8.5 U/ml. Among the various carbon sources investigated for amylase production in, 2% (w/v) starch stimulated the highest amylase production of 30.1 U/ml closely followed by 2% (w/v) maltose concentration (22.0 U/ml). Studies involving the effects of Nitrogen sources on amylase production showed that NH_4NO_3 stimulated the highest amylase titre of 23.1 U/ml. Effect of incubation time (days) on amylase production revealed that an incubation period of 6 days was optimum for amylase production by this isolate.
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Key words: *Aspergillus flavus*, Amylase, Starch, NH_4NO_3, Temperature, Biodeterioration.

Introduction

Starchy substances which are synthesised naturally in a variety of plants such as corn, potatoes, rice, sorghum, wheat and barley, constitute the major part of human and animal diet in the world. Apart from serving as feed for humans and animals, they are also used as raw materials in biotechnological processes (Nwagu and Okolo, 2011). However, before the consumption of these starchy substances, they are usually processed. One of such processes is fermentation a practice as old as man.

Food biodeterioriation (Starch-based food inclusive) is a metabolic process that brings about change in texture, smell and appearance of food thereby making food undesirable and unacceptable for human consumption (Doyles, 2007). Such foods are usually referred to as spoilt foods. Foods are susceptible to spoilage because they are rich in carbohydrate, proteins and lipids which support the growth of microorganisms (Pitt and Hocking, 1997). The growth of these microorganisms leads to chemical reactions that cause offensive sensory changes in food as mediated by a variety of microbes that use food as carbon and energy sources.

Doyles (2007) described spoilage microbes as common inhabitants of soil, water or the intestinal tracts of animal which may be dispersed through the air, water, and by activities of small animals, particularly insects. Of all the microbes, the most commonly reported are the fungi belonging to the genera *Mucor*, *Rhizopus*, *Aspergillus* and *Penicillium* (Prescott *et al*., 1999) which are reported to dominate spoilage in temperate regions, but few species can spoil refrigerated and process foods such as Jams and Margarines. The invasion of food by these organisms may result in remarkable rapid quality deterioration (Sibanda *et al*., 1997; Boysen *et al*., 2000). Consequently the profitability and effectiveness of food utilization are considerably reduced.

Spoilage organisms while growing on food materials have been reported to produce some secondary metabolites including enzymes; amylase is one of such enzymes produced especially on starchy foods. Amylase are enzymes employed in processing industries for the hydrolysis of Starch into simple sugar constituents (Okolo *et al*., 2006) and they constitute a clan of enzymes having approximately 25% of the enzyme market (Sindhu *et al*., 1997; Rao *et al*., 1998). These enzymes although can be derived from various sources, Pandey *et al*. (2000) reported that enzymes from microbial sources usually meet industrial demands. This work was designed to screen for amylase-producing fungi associated with starchy-fermented foods and to investigate the production of amylase by the isolated organism. The amylase titre recorded in this study is among the highest recorded in literatures to the best of our knowledge.

Materials and Methods

Sample procurement

"Dokunu", "Eba", "Fufun" and "Lafun" samples undergoing bio-deterioration were collected from five geographical locations in south west Nigeria.

Isolation procedure and Identification

Isolation was carried out using the serial dilution of Harrigan and McCance (1966) on Potato Dextrose Agar (PDA) and plates were incubated at 30 °C for 5 days. Distinct colonies were sub-cultured repeatedly until pure cultures were obtained. Pure cultures were stored on PDA slants at 4 °C and sub-cultured fortnightly.

Screening of Isolates for Amylolytic activity

Pure isolates were screened for amylase production according to the method of Bergmann *et al*. (1988) and Akpan *et al*. (1999). Organism with the highest Amylolytic activity was selected for further studies. Identification was done as described by Brown (2005) and with reference to Compendium of Soil Fungi (Domschi *et al*., 1980).

Inoculum preparation

Spore suspension used as inoculum in this study was prepared as described by Nahar *et al*. (2008).

Amylase production

This was done using the medium of Abe *et al*. (1988). The pH of the medium was adjusted to 6.5 and dispensed in 100 ml aliquots into 150 ml Erlenmeyer's flasks plugged with non absorbent cotton wool and sterilized at 120 °C for 15 minutes. After sterilization, the flasks were allowed to cool and inoculated with a 1 ml of spore suspension (containing about $1.3X10^7$ spores) and incubated at 30 ºC for 5 days. After incubation, the whole content of the flask was filtered using Whatman No1 filter paper and the filtrate was used as the crude enzyme.

Amylase assay

Amylase activity was assayed for by incubating 1 ml of the crude enzyme with 1 ml of 1% soluble starch in 0.02 M phosphate buffer (pH 6.5) at 30 ºC for 1 h. After incubation, liberated reducing sugar (glucose) was estimated by 3, 5-dinitrosalicylic acid method of Miller (1959). The colour developed was read by measuring its optical density using a Jenway Spectrophotometer at 540 nm. A control containing water instead of crude enzyme was also set up as the blank. One unit of enzyme activity was defined as the amount of amylase liberating 1 µmol per minute under the assay conditions.

Effect of temperature on amylase production

This was done using the production medium above. Temperatures investigated were 25 ºC, 30 ºC, 40 ºC, 50 ºC and 60 ºC and incubation was for 5 days. After incubation, crude enzyme was obtained as previously described and amylolytic activity determined as previously described.

Effect of pH on amylase production

This was studied using the method of Alva *et al*. (2007) and pH levels investigated were 4, 5, 6, 7, 8 and 9. Incubation was done at 30 ºC for 5 days and amylolytic activity assayed for thereafter as previously described.

Effect of Carbon sources on amylase production

The effect of various carbon sources on amylase production was done as described by Olama and Sabry (1989) using the previously described production medium. The carbon sources studied are xylose, galactose, fructose, sucrose, soluble starch, maltose and glucose. The pH of the medium was adjusted to 6.5 and cultures were incubated at 30 ºC for 5 days. Amylase activity was determined thereafter.

Effect of different Nitrogen sources

The effect of various nitrogen sources on amylase production was investigated by supplementing NH_4N0_3 in the basal medium with each of urea, yeast extract, casein, NH_4SO_4, NH_4Cl. The pH of the medium was adjusted to 6.5 and cultures were incubated at 30 ºC for 5 days amylase activity was determined as previously described.

Effect of incubation time on amylase production

The effect incubation time on amylase production was studied by assaying for the amount of amylase produced at 3, 4, 5, 6, 7, 8, 9 and 10 day of incubation respectively as described by Gupta *et al*. (2008).

Statistical Analysis

Data were analysed using Analysis of Variance (ANOVA) using SPSS version 17.0 at level of significance of $P \leq 0.05$.

Results

Fifteen different Fungi were isolated from the various fermented starch-based food undergoing bio-deterioration. These were then screened for amylolytic activities on starch agar plates and the result is shown in Table 1. Isolate AE2 with the highest clearance zone was identified to be *Aspergillus flavus* based on the observed cultural and microscopic characteristics as described by Domschi *et al*., (1980) and Brown (2005) and this was used for further studies. This *Aspergillus flavus* was found to produce 10.2 U/ml of amylase in the amylase production medium used in this study.

Investigation into the effect of incubation temperature on amylase production by the *Aspergillus flavus* as presented in Figure 1 revealed that 30 ºC was the optimum temperature for amylase production stimulating an amylase titre of 12.2 U/ml. Above the optimum temperature, amylase production was found to decrease as the temperature increases; 8.10 U/ml at 40 ºC, 7.10 U/ml 50 ºC, 3.80 at 60 ºC and finally an amylase titre of 1.20 U/ml at 70 ºC (Figure 1).

The results of pH studies as its affects amylase production by this *Aspergillus flavus* showed that there was an increase in amylase production as the initial pH of the medium increases with an amylase activity of 6.0 U/ml at pH 4, 8.50 U/ml at pH 5.0 reaching an optimum of 10.1 U/ml at 6.0. Thereafter an increase in pH led to a decrease in amylase production (Figure 2).

Investigation into the effects of various carbon sources on amylase production is presented in Figure. 3. Starch at 2% concentration was found to support the highest amylase production of 30.10 U/ml closely followed 2% maltose (22.0 U/ml) and 2% glucose (15.7 U/ml). The lowest amylase titre (2.0 U/ml) was however recorded in a medium Xylose as the sole carbon source (Figure 3).

The result of the effect of various nitrogen sources on amylase production is presented in Figure 4. The highest amylase titre of 23.10 U/ml was recorded in a medium containing 0.14% NH_4NO_3 closely followed by urea (23.0 U/ml) and casein (13.0U/ml) while the lowest enzyme titre (5.0 U/ml) was recorded in yeast extract-containing medium (Figure 4).

Table 1: Amylase screening profile (depicted by clearance zone (mm) on starch agar plates) of fungi isolated from biodeteriorating starch-based fermented food.

Isolate code	Clear zone (mm)
AE1	3.0±0.00[a]
AE2	13.0±0.08[b]
AE3	3.1±0.05[a]
AE4	2.0±0.01[a]
AD1	2.6±0.19[c]
AD2	2.8±0.05[a]
AF1	2.7±0.00[b]
AF2	2.6±0.04[d]
AL1	3.4±0.01[e]
AL2	2.9±0.02[c]
AL3	1.8±0.24[a]
AO1	3.3±0.02[c]
AO2	3.0±0.31[d]
AO3	3.5±0.09[e]
AO4	1.9±0.07[c]

Data are means of three replicates ± SEM. Values followed by the same letters are not significantly different by Duncan's multiple range test ($P < 0.01$).

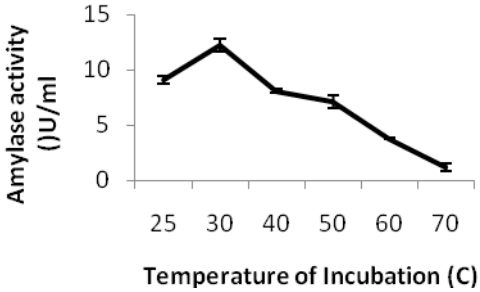

Fig. 1; Effect of Temperature of incubation on Amylase production by *Aspergillus flavus*

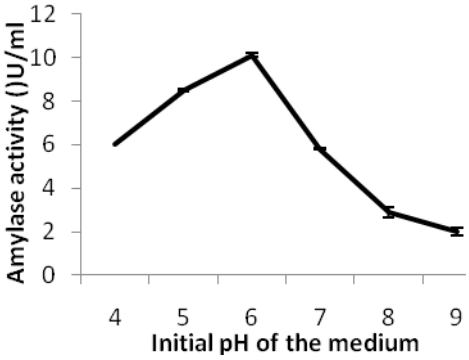

Fig. 2; Effect of initial pH of the medium on Amylase production by *Aspergillus flavus*

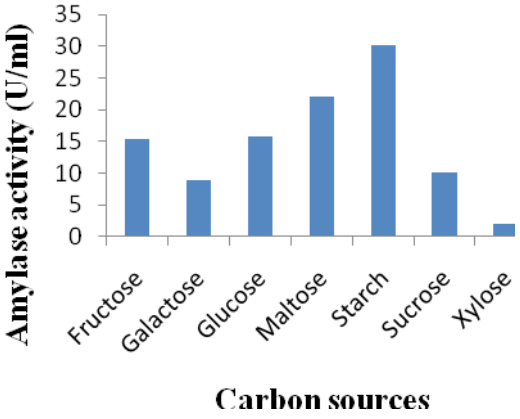

Fig. 3; Effect of different carbon sources on Amylase production by *Aspergillus flavus*

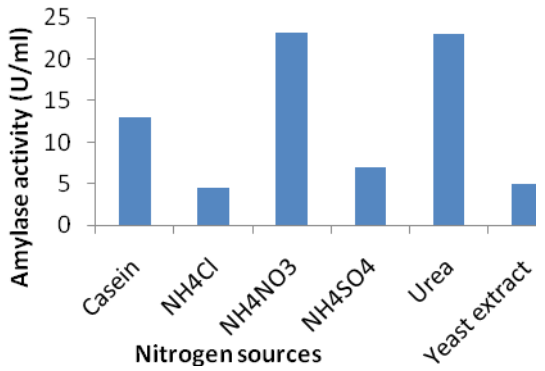

Fig. 4; Effect of different Nitrogen sources on Amylase production by *Aspergillus flavus*

Results on the studies of effect of time of incubation (days) on amylase production by the *Aspergillus flavus* presented in Figure 5 revealed that the highest amylase titre of 13.0U/ml was recorded at day 6. This was followed by 11.9 U/ml and 11.8 U/ml at 7 and 5 days of incubation respectively, while the lowest amylase (4.5U/ml) was recorded at 10 days of incubation (Figure 5).

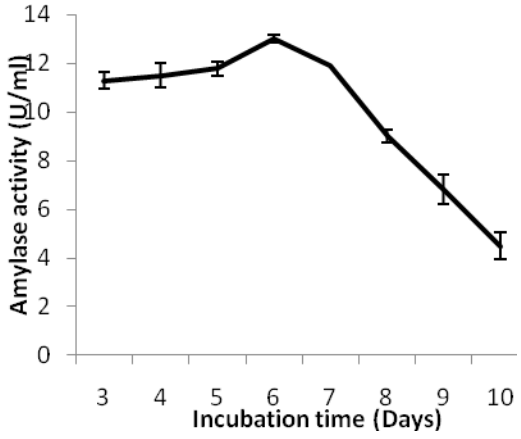

Fig. 5; Effect Time of incubation on Amylase production by *Aspergillus flavus*

Discussion

Various amylase-producing organisms have been reported in literatures (Ogundare and Adetuyi, 2003; Njobeh *et al*., 2009; Grillo and Lawal, 2010). The Amylolytic *Aspergillus flavus* associated with spoilt starch-based fermented food reported in this study is among the previously reported amylolytic organism (Abu *et al*., 2005; Gomes *et al*., 2005).

Okolo *et al*. (2006) and Nagamine *et al*. (2003) reported that temperature is one of the environmental conditions affecting amylase production. This study is also a pointer to this fact. The optimum temperature of 30 ºC reported for amylase production by this *A.flavus* is in agreement with the report of Alva *et al*. (2007). The authors reported that *Aspergillus* sp. JG12 showed highest amylase yield at 30 ºC. Also, Nwagwu and Okolo (2011) observed that a particular strain of *Aspergillus fumigatus* showed maximum mycelial yield and amylase titre at 30 ºC. Low enzyme titre at temperature values higher than the optimum may be as a result of the inactivation amylase enzyme or suppression of cell viability while low amylase titre at low temperature values may be as a result of reduction in the metabolic activities of the microorganism and consequently, the enzyme synthesis (Mazutti *et al*., 2006).

The initial pH of the production medium has been reported to play a significant role in the growth and metabolite production by various organisms (Olutiola, 1976). Amylase production in this study was also found to be affected by the initial pH of the enzyme production medium. However, the highest amylase titre was recorded at pH 6.0 in this study. Maximum amylase production at pH 6.0 by various species of *Aspergillus* has been earlier reported (Sinha and Charkrabarty, 1978; Sasi *et al*., 2009). However, the pH results of this study is a shift from the earlier reports of Olama and Sabry, (1989) who reported maximum amylase production by *Aspergillus flavus* at pH 7.0. This shift may be due to environmental influences on the growth and enzyme production by the organisms. Variation in amylase production as pH of the medium varies could be as a result of the various morphological changes induced in microbes due to the changing pH.

Starch at a concentration of 2% stimulating the highest production of starch in this study is in agreement with earlier reports (Yabuki *et al*., 1977; Lachmund *et al*., 1993; Morkeberg *et al*., 1995). All the authors suggested that amylase production is inducible and it is produced in the presence of starch, its hydrolytic product, or maltose. Xylose in this present study was found to stimulate the minimal amylase production. This is in agreement with the reports of Kathiresan and Manivannan (2006) who reported that xylose strongly repress amylase production.

Among the various nitrogen sources investigated in this study, NH_4NO_3 appears to stimulate the highest amylase titre. Similar results have been reported in literatures (El-Safey and Ammar, 2002; Sasi *et al*., 2009; Nizolek, 1998). Some workers have however reported organic nitrogen sources such as Urea, Casein and Peptone to support amylase production by various *Aspergillus* species (Hamilton *et al*., 1999; Gupta *et al*., 2008; Monga *et al*., 2011). Generally, there seem to exist variations in the type of nitrogen compound required for maximum amylase production by various species of *Aspergillus*. Maximum amylase

production by this *Aspergillus flavus* in a medium containing NH_4NO_3 could be as a result of the ease with which the compound was absorbed into the cells of the organism.

The effect of time of incubation (days) on amylase production revealed that an incubation period of 6 days was optimum for amylase production, after which amylase production was found to decrease with a further increase in the days of incubation. This is very similar to reports of Olama and Sabry, (1989) and Mukherjee and Majumdar (1973). The authors both reported maximum amylase production by *Aspergillus flavus* on the 7th day incubation period. These two reports however are in contrast to the report of Sasi *et al*. (2010) who reported maximum amylase production on the 18th day of incubation. Decrease in amylase production beyond the optimum incubation period could be attributed to catabolite repression caused by glucose released into the medium. It could also be as a result of the secretion of proteolytic proteins which are known to cause the denaturation of proteins (Gupta *et al*., 1994). Short incubation period offers potential for inexpensive production of enzyme (Somjoy *et al.,* 1995).

Conclusively, this paper presented amylase production by *Aspergillus flavus* associated with bio-deterioration of starch-based fermented food in Nigeria as it is affected by various physico-chemical parameters. The amylolytic activity reported is also among the highest recorded in literatures however, the amylase produced will need to be further characterised in order to determine its suitability for industrial purposes.

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