

## HYDROLYSIS OF CERTAIN NIGERIAN CEREAL STARCHES USING CRUDE FUNGAL AMYLASE.

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### ABSTRACT

Eight filamentous fungi were isolated from garden soil. The mesophilic isolates were *Aspergillus flavus* Link ex. Gray, *A. niger* van Thieghem, *Fusarium oxysporum* Schlect, *Penicillium citrinum* Thom, *Rhizopus oryzae* Went and *Trichoderma viride* (Persoon) Harz. *Aspergillus fumigatus* Fresenius and *Mucor pusillus* Lindt were the only thermotolerant and thermophilic species respectively. They all elaborated amylolytic enzymes in a defined liquid medium. These enzymes of *A. flavus*, *A. niger*, *M. pusillus* and *R. oryzae* showed appreciable hydrolytic quality and were, therefore, selected for subsequent studies. The temperature optima for the amylase activity of the culture filtrates (crude amylase preparations) of these selected isolates were 60°, 40°, 60° and 50°C respectively, while the pH optima were pH 6.0 4.0 6.0 and 5.0 respectively. They have optimum substrate concentration requirements of 5%, 3%, 5% and 3% respectively. There was no significant difference ( $p > 0.05$ ) in the amount of reducing sugars produced from starches obtained from maize, sorghum and millet using the different crude amylase preparations. The study showed that crude amylase preparations of *A. niger* can be used to hydrolyse sorghum and millet starches with an expectation of about 45% hydrolysis without prior concentration and purification. This represents a great improvement over the report malting efficiency of these test cereals. However, the practical application of the crude enzyme preparations on an industrial scale would depend on the toxicological evaluation of the crude enzymes since some of the test fungi are known to produce mycotoxins.

**Keywords:** Starch, Filamentous fungi, Amylase, Saccharification, reducing sugars.

### INTRODUCTION

Cereals are particularly noted for their starchy endosperms. Starches of these cereals can be converted to reducing sugars by either acid or enzymatic means (15).

In most of the starch-hydrolysate based industries in Nigeria, such as the breweries and

distilleries, enzymatic saccharification of cereal starches to fermentable sugars for subsequent alcoholic fermentation was very often carried out by using endogenous amylases produced in the grains during malting. However, studies (3, 13 and 15) have revealed that all the major cereals (maize, sorghum and millet) produced in Nigeria are of poor malting quality, this results in a low yield of amylases and consequently a low yield of fermentable sugars.

In the recent past, barely malt was widely employed as external sources of amylases (3, 13) for the saccharification of these cereal starches. But the cultivation of barley in Nigeria has met with little success. As such, a huge amount of foreign exchange was spent annually in importing barley malt before the ban. Following the ban, a fair amount of foreign exchange earnings that needs urgent conservation is still being spent on the important of brewing enzymes (amylases) of microbial origin.

Although the use of microbial amylase for the saccharification of starches has long been advocated (2,3,4,13,15) and practiced to a limited extent (5,15) in this country, no published work exists on the saccharification of starches from locally available cereal starches using culture filtrates of moulds locally produced in submerged cultures. The present investigation is, therefore, designed to isolate amylolytic moulds that can elaborate appreciable quantities of extracellular amylases in liquid medium for the saccharification of starches obtained from the major local cereals (maize, sorghum and millet) without prior concentration and purification.

## MATERIALS AND METHODS

### Isolation of Amylolytic Filamentous Fungi:

Soil samples were collected from the botanical garden of University of Jos. A small quantity (0.1ml) of 0.03% (w/v) soil suspension serving as the inoculum was plated out on each petri-plate (9cm) to which the basal medium was added aseptically. The basal medium consisted of 6% (w/v) gelatinized cassava flour sterilized by autoclaving. Fifteen petri-plates were plated out altogether and later sub-divided into three portions of five plates each. The first portion of five plates were incubated at 25°C for 7 days. The second portion of five plates were incubated at 37°C for 7 days and the last portion were incubated at 45°C for 4 days. A beaker of sterile distilled water was kept along side this plates in the incubator to prevent the medium from drying at this last temperature regime. These three temperature regimes were employed to find out the mesophilic, thermotolerant and thermophilic amylolytic microorganisms inhabiting the soil.

### Purification and Identification of Isolates:

All the filamentous fungal colonies were sub-cultured on to Malt-Extract Agar (MEA) for pure cultures. The identification of the filamentous fungal isolates was based on both their culture and morphological characteristics and appropriate references made to published identification keys (8) and relevant taxonomic descriptions in the publications of Commonwealth Mycological Institute, Kew, United Kingdom.

Slant cultures of the pure isolates maintained on MEA in McCartney bottles were referred to the Crop Protection Department of the Institute of Agricultural Research (I.A.R) Zaria, for further Identification and authentication.



### Estimation of Percentage Frequency of occurrence

The percentage frequency of occurrence of the filamentous fungal isolates was calculated with the aid of the following formula:

$$X/5 \times 100/1 = 20 \times \%$$

Where X is the number of plates in which a particular organism appeared out of the five plates used at a particular temperature regime.

### Production of Crude Amylase

The isolates were grown in a defined medium as described by Adams and Deploey (1). The medium was composed of starch - 5g; KNO<sub>3</sub> - 3g; KH<sub>2</sub>PO<sub>4</sub> - 1g; and MgSO<sub>4</sub> · 7H<sub>2</sub>O - 0.5g all dissolved in 1 litre of distilled water and sterilized by autoclaving at 120°C and 1.05kg/cm<sup>2</sup> for 15 minutes. The pH of the medium was subsequently adjusted to pH 6.9.

Immediately after cooling, 100ml of the liquid medium was aseptically dispensed into each sterile Erlenmeyer flask (250ml). The inoculum for each flask consists of one agar disc bearing mycelium and spores of each isolate. The agar disc was obtained from the edge of 7-day old purified cultures of the selected isolates (maintained on MEA) with sterile cork borer (4mm). The flask now containing both the liquid medium and inoculum were then incubated at the respective temperature of isolation.

At the end of the incubation period (7-days), the flask were removed from the incubator and the mycelial mats carefully and aseptically removed. Further separation of spores and mycelia fragments was achieved by suction filtration of the culture broth through whatman No. 1 filter paper (9cm) contained a Buchner funnel using Compton Vacuum pump Model D/351 Vm. The resulting culture filtrates then served as the crude amylase preparations for subsequent studies.

### Qualitative assessment of the Crude Amylase Preparations

The quality of the elaborated amylase of each of the fungal isolate was assessed by assaying for their dextrinizing activity. This involves monitoring the change in iodine staining property of a 1% starch solution. To 2ml of a starch solution (containing 1% (w/v) starch and 0.006M NaCl at pH 6.9) was added 1ml of the crude amylase and incubated at room temperature (25±2°C) for one hour. Time course for dextrinization was followed by withdrawing samples of the enzymatic digest (starch + enzyme) after reaction periods of 0, 5, 10, 20, 30, 40, 50 and 60 minutes and then tested with two drops of Lugol's iodine. The resultant colour formations were photorecorded as visually seen. Complete dextrinization was assumed when there is no more colour formation on addition of iodine (i.e. retain the yellow colour of iodine).

### Determination of Amylase Acidity of the Crude Amylase Preparations

Amylase activity was determined using a modification of Bernfield (6) method. One ml of crude amylase was added to 1ml of standard starch solution (containing 1% soluble starch and 0.006M NaCl in 0.2M phosphate buffer at pH 6.9) and incubated at 30°C for 30 minutes. The reducing sugars produced after the period of incubation were determined by adding 2ml of dinitrosalicylic acid (DNSA) reagent, boiled for 5 minutes and then cooled under running tap water. Twenty milliliter of sterilized distilled water was then added and allowed to stabilize. The absorbance of the resulting solution was determined at 540nm with a Ciba - Corning Colorimeter model 254. One milliliter of the starch solution similarly treated

but without enzyme was used to set transmittance at zero.

The amounts of reducing sugars produced were read off from a calibration curve established by recording absorbance of increasing aqueous solution of glucose (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8. and 2.0mg/ml). The results are expressed as amylase units.

One unit of amylase activity being the amount of enzyme in 1ml of crude amylase that produced 1.0mg of reducing sugars under the assay conditions. The amount of reducing sugars in the crude amylase preparations were earlier determined and appropriate corrections were made for the amylase activities of the different crude amylase (culture filtrates) preparations.

### Physico-chemical Studies on the Crude Amylase Preparations of the Selected Test Fungi

#### Effect of Temperature on Amylase Activity

The effect of temperature was assayed between 20°C and 80°C at pH 6.9 for 30 minutes. The starch solution for each assay was earlier pre-incubated at the desired temperature for 10 minutes. The amylase activity was then determined for each temperature regime as earlier described. One unit of amylase activity is, however, defined here as the amount of enzyme in 1ml of the crude enzyme preparation catalyzing the liberation of 1.0mg of reducing sugars from 1% starch solution in 30 minutes at pH 6.9 at the respective temperature of incubation. Triplicate readings were recorded.

#### Effect of pH on Amylase Activity

The effect of pH on amylase activity was determined on starch solutions (1% starch and 0.006M NaCl) of various pH values using buffers between pH 3.0 - 9.0. The buffers used were 0.2M Citrate-phosphate buffer (pH 3.0 - 6.0); 0.2M phosphate buffer (pH 7.0 - 8.0) and Boric - Borax buffer (pH 9.0).

The amylase activity was determined as earlier outlined. One unit of amylase activity in this case is defined as the amount of enzyme present in 1ml of crude amylase preparation catalyzing the formation of 1.0mg reducing sugars from a 1% solution in 30 minutes at 30°C at the respective pH value. Triplicate readings were also recorded.

#### Effect of Substrate Concentration on Amylase Activity

The amylase activities of the various crude amylase preparations were determined at various substrate concentration of 1%, 2%, 3% 4% and 5% starch solutions containing 0.006M NaCl in 0.2M phosphate buffer at pH 6.9 by the method earlier described (2,3).

One unit of amylase activity is define here as the amount of enzyme present in 1ml of crude amylase preparation catalyzing the formation of 1.0mg reducing sugars from the various starch substrate concentration in 30 minutes at 30°C and pH 6.9. Triplicate readings were also recorded.

#### Extraction of Starches from the Cereals

Starches were obtained from the following test cereals - *Zea mays* Linn. (maize), *Sorghum bicolor* (L). Moench (Sorghum) and *Pennisetum typhoides* stapf and Hubbard (Millet).

Following steeping period for six hours, grains were wet-milled to form a starch slurry. The slurry was then sived and filtered through fine muslin cloth to remove fibre, hulls and a portion of the germ. The filtrate was then washed in five changes of water through the screen



(muslin cloth) and then allowed to settle. After settling, the supernatant was decanted and the starch air-dried.

### Hydrolysis of the Extracted Starches

Hydrolysis was carried out by incubating 1ml of the crude amylase preparation with 1ml of the respective cereal starch solution (containing 1% starch and 0.006M NaCl). The starch solution was prepared in buffer solutions of optimum pH for the respective test fungal crude amylase. This solution was autoclaved at 121°C for 15 minutes to sterilize, gelatinize and inactivate any endogenous amylase that might accompany the native starch granules. The reaction mixture was then incubated in a thermostatted water-bath (Techne-Temperor model WB-2) for 30 minutes at the pre-determined optimum temperature for the crude amylase activity of each fungus.

At the end of the incubation period, the reducing sugars produced were determined by the DNSA (3.2.2.) method.

## RESULTS

### Isolation of Test Fungi

Eight species of azyotic filamentous fungi belonging to six different genera were isolated. The isolates include *Aspergillus flavus* Link ex. Gray, *A. fumigatus* Fresenius, and *A. niger* Van Tieghem. The others were *Fusarium oxysporum* Schlecht, *Mucor pusillus* Lindt, *Penicillium citrinum* Thom, *Rhizopus oryzae* Went and *Trichoderma viride* (Persoon) Harz.

The isolates were mainly mesophiles except *A. fumigatus* which was a thermotolerant and *M. pusillus* which was the only thermophile. The frequency of occurrence of these isolates at the various temperature regimes is shown in Table 1.

### Production and Assessment of the Crude Amylase Preparations

All the isolates elaborated extracellular amylase in the define liquid medium. Table 2 shows the qualitative assessment of the elaborated amylase based on the dextrinization power. The amylase produced by *A. flavus* effected complete dextrinization in 20 minutes. This was closely followed by that of *M. pusillus* at 30 minutes. Those of *A. niger* and *R. oryzae* achieved the same feat at 40 and 50 minutes respectively. The amylase from the other isolates failed to effect complete dextrinization of the starch solution after 60 minutes. *A. flavus*, *A. niger*, *M. pusillus* and *R. oryzae* were therefore selected for the subsequent investigations.

### Effect of Temperature on Amylase Activity

The amylase activity of the crude enzyme preparations of all the test fungi increased progressively with increase in temperature from 20°C to 40°C (Fig. 1). The amylase preparations of *A. flavus* and *M. pusillus* showed their optimum temperature for amylase activity at 60°C. *R. oryzae* displayed its peak amylase activity at 50°C while in case of *A. niger*, optimum amylase activity was recorded at 40°C.

Apart from *A. niger* amylase that showed appreciable activity at 70°C, the others recorded only residual activities at 70°C. Above 70°C, all the amylase activities were rapidly inactivated.

**Effect of pH on Amylase Activity**

All the crude amylase preparations showed similar responses to pH with respect to their maximum amylase activity. Their optimum amylase activities were in the acid region between pH 4.0 and 6.0 (Fig. 2). *A. flavus* and *M. pusillus* exhibited their peak amylase activities at pH. 6.0. *A. niger* had its optimum amylase activity at pH 4.0 while *R. oryzea* recorded an optimum amylase activity at pH 5.0. *A. flavus* had the widest tolerance of pH in the range of pH 3.0 to 9.0. The others showed only residual amylase activities at pH 8.0.

**Effect of Substrate Concentration on Amylase Activity**

Increase in substrate concentration from 1% up to 3% led to progressive increase in amylase activities of all the test fungi crude amylase preparations. Beyond this point (3%), the amylase activities of both *A. flavus* and *M. pusillus* showed minimal increase whilst those of *A. niger* and *R. oryzea* actually declined (Fig. 3).

**Hydrolysis of the Extracted Starches**

Fig. 4 is a histogram showing the total reducing sugars produced from 1ml of 1% starch solutions of the various test cereals after 30 minutes using the various crude amylase preparations. The highest amount of reducing sugars was obtained when amylase of *A. niger* was used to hydrolyse both millet and sorghum starches (4.5 and 4.4mg/ml respectively). These values were more than double the amount obtained from maize starch by the same enzyme. *A. flavus* amylase produced more reducing sugars from maize starch than from millet and sorghum. *M. pusillus* amylase produced a considerably higher amount of reducing sugars from maize starch than the other starches. The amount of reducing sugars produced was about twice those obtained from millet and sorghum starches. *R. oryzea* produced a relatively higher amount of reducing sugars from sorghum starch than from both maize and millet which were almost the same. That from millet represented the least amount of reducing sugars obtained from the entire tests carried out (Table 3).

Statistical analysis of variance (Table 3a) indicated that there is no significant difference ( $P>0.05$ ) in the total amount of reducing sugars produced from the test cereal starches by the amylase of the various test fungi.

**DISCUSSION**

All the isolates elaborated detectable quantities of extracellular amylase in the defined liquid medium. This result is in agreement with earlier reports (1,2,5,8,10 12, 14). As such the procedure employed represents a simple, rapid and inexpensive method of screening for amylolytic fungi. Based on the dextrinizing activity of the amylase produced, those of *A. flavus*, *A. niger*, *M. pusillus* and *R. oryzea* were thought to be the best. However, this study has confirmed that the dextrinizing activity of an amylase complex does not necessarily give an indication of the saccharifying power as was observed by earlier workers (10, 14).

From the result of the present study, it is apparent that the effectiveness of the crude amylase preparations for the saccharification of starches from the test cereals will require prior gelatinization of the starches since they cannot be used during the heating of the raw starches considering their thermal stability and the determined gelatinization temperatures of the starches (68°, 71°, and 74° for maize, sorghum and millet respectively). Even the thermophilic nature of *M. pusillus* did not confer any superiority in terms of temperature stability on its amylase. This latter observation is in agreement with the observations of Ogundero (12) but



at variance with the assertion of Stark and Tetrault (16) that enzymes elaborated at thermophilic temperatures were more heat stable.

For optimal utilization of resources, the use of the amylase from these fungi should be correlated with the starch to be hydrolysed at 3% substrate concentration level.

The result of the hydrolysis of the extracted starches using crude amylase preparations represent a great improvement on the malting efficiency of these cereal starches (3, 13,15) and also eliminates the additional cost of using isolated and purified enzymes. More importantly, the result implies that the different cereal starches could be utilized interchangeably with any of the crude amylase. This would eliminate the cost of changing specialized plants when one source of starch runs short and there is need to shift to other sources among the test cereals.

However, practical application of these crude amylase preparations for starch saccharification on an industrial scale would depend on the toxicologically evaluation of the crude enzymes since some of the test fungi are known to produce mycotoxins.

**Table 1: A checklist of the isolated filamentous fungi at the different temperature regime with their percentage frequency of occurrence.**

Isolates	Percentage incidence at various temperature regime		
	25°C	37°C	45°C
<i>Aspergillus flavus</i>	100	40	-
<i>A. fumigatus</i>	20	60	40
<i>A. niger</i>	100	100	-
<i>Fusarium oxysporum</i>	60	-	60
<i>Mucor pusillus</i>	-	-	60
<i>Penicillium citrinum</i>	60	-	-
<i>Rhizopus oryzae</i>	60	60	-
<i>Trichoderma viride</i>	40	-	-
Mean	55	32.5	12.5

All the isolates elaborated detectable quantities of amylase activity in the defined liquid medium. This result is in agreement with other reports (1, 2, 3, 10, 12, 14). As such, the procedure employed (simple, rapid and inexpensive method) of screening for amylolytic fungi. Based on the deactivating activity of the amylase produced, Stark & Tetrault (16) reported that amylase activity was highest at 37°C. However, this study has confirmed that the deactivating activity of an amylase enzyme does not necessarily give an indication of the saccharifying power as was observed by other workers (10, 14). From the result of the present study, it is apparent that the effectiveness of the crude amylase preparations for the saccharification of starches from the test cereals will depend on the gelatinization of the starches and a likely cause may be the heating of the starches considering their different sources and the determined gelatinization temperatures of the starches (68, 71, and 74 °C for maize, sorghum and millet respectively). Even the thermal stability of *M. pusillus* did not confer any superiority in terms of temperature stability on the amylase. This latter observation is in agreement with the observations of Odeh & El-Bel

**Table 2:** Qualitative assessment of the extracellular amylase elaborated by the isolates

	Time Course for dextrinization (minutes)							
	0	5	10	20	30	40	50	60
<i>A. flavus</i> *	Blue	Purple	Red	Yellow				
<i>A. fumigatus</i> *	Blue	Blue	Purple	Purple	Purple	Red	Red	Red
<i>A. niger</i> *	Blue	blue	Purple	Purple	Red	Yellow	Purple	Purple
<i>F. oxysporum</i> *	Blue	Blue	Blue	Blue	Purple	Purple	Red	Red
<i>M. pusillus</i> *	Blue	Blue	Purple	Red	Yellow	Red	Yellow	Purple
<i>P. citrinum</i> *	Blue	Blue	Blue	Purple	Purple	Red	Purple	
<i>R. oryzae</i> *	Blue	Blue	Purple	Red	Red	Purple		
<i>T. viride</i> *	Blue	Blue	Blue	Blue	Purple			

\* = Incomplete dextrinization after 60 minutes  
 + = Selected test Isolates

**Table 3a:** Statistical Analysis of Variance of total reducing sugars produced from the test cereal starch solutions by the crude amylase preparation of the isolates

Sources of Variations	Sum of Square	Degree of Freedom	Mean Square	F-Ratio
Crude Amylase Preparations	58.9335	3	19.6444	1.5324 N.S.
Starches Solutions	3.35	2	1.6750	0.1306 N.S.
Residual	76.91667	6	12.8195	
Total	139.2	11		

L.S.D. 0.05 = 3.5116 P<0.05  
 N.S. Not significant

**Table 3b:** Hydrolysis of the test cereal starches using crude amylase preparations (culture filtrates) from selected fungi

Culture filtrates	Incubation condition		Total mg/ml	reducing sugars		
	Temperature	PH		Maize	Sorghum	Millet
<i>Aspergillus flavus</i>	60°	6	2.80	2.20	1.80	
<i>A. niger</i>	40°	4	1.70	4.40	4.50	
<i>Mucor pusillus</i>	60°	6	4.10	2.70	2.00	
<i>Rhizopus oryzae</i>	50°	5	1.60	2.70	1.40	

All values are average of 3 readings.



Table 1 Quantitative assessment of the catalytic activity of the extracellular amylase elaborated by the selected fungal isolates

Isolate	20°C	30°C	40°C	50°C	60°C	70°C	80°C
<i>R. oryzae</i>	1.0	2.8	3.2	3.1	2.6	2.5	0.2
<i>M. pusillus</i>	2.2	3.8	3.9	3.9	3.7	0.5	0.1
<i>A. niger</i>	1.8	3.8	3.8	3.8	3.8	0.5	0.1
<i>A. flavus</i>	3.1	4.4	4.5	4.6	4.8	0.2	0.1

**KEY**  
 --- *R. oryzae*  
 - - - *M. pusillus*  
 - - - *A. niger*  
 — *A. flavus*

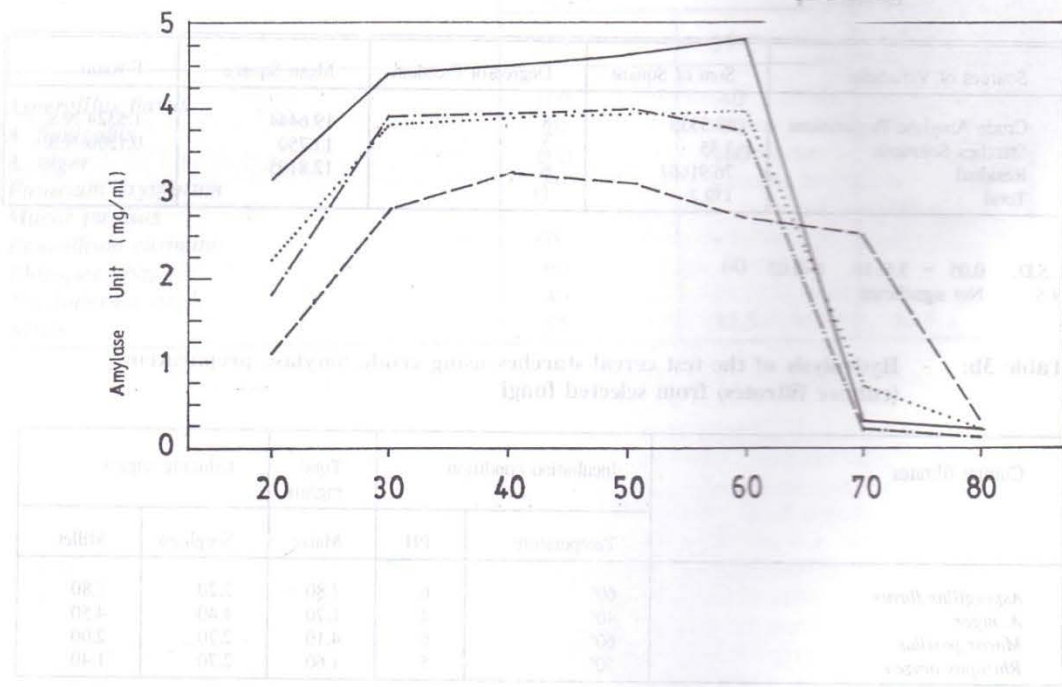


Fig. 1 Effect of Temperature on Amylase activity of culture filtrates of selected of fungal isolates

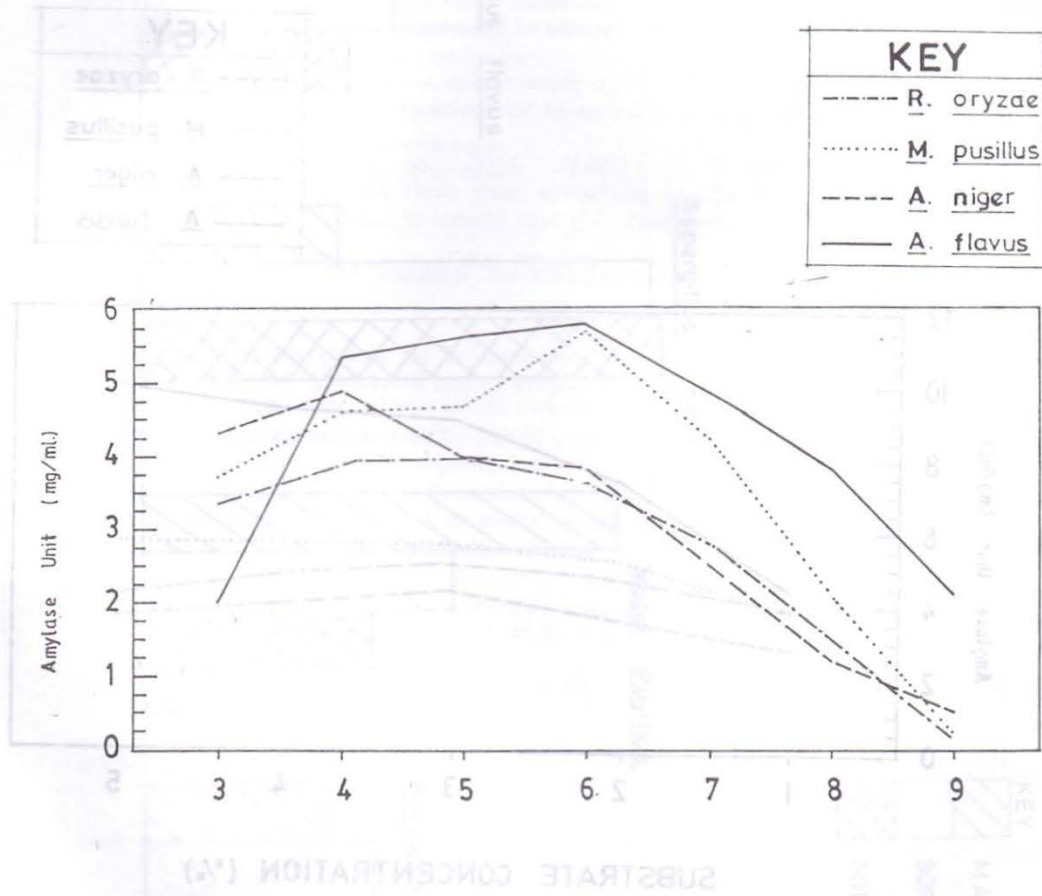


Fig. 2 Effect of pH on Amylase activity of culture filtrates of selected of fungal isolates



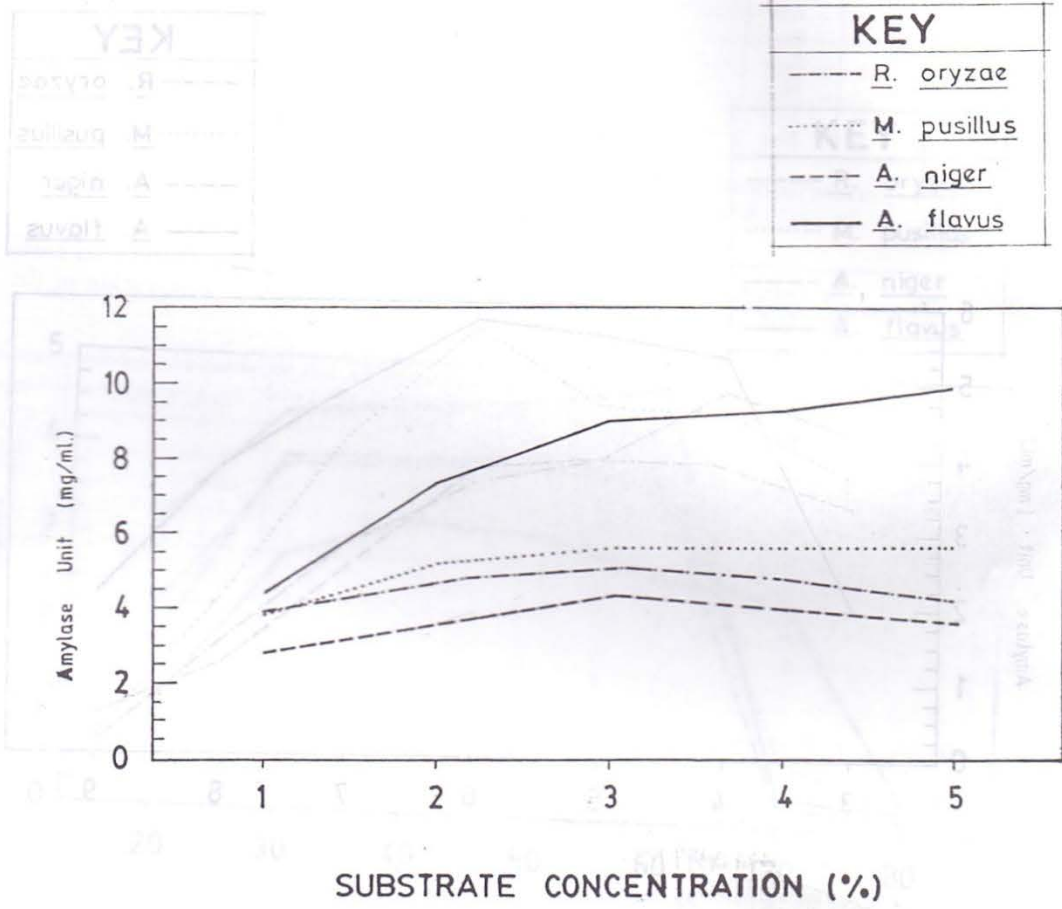


Fig. 3 Effect of Substrate Concentration on Amylase activity of culture filtrates of selected fungal isolates.

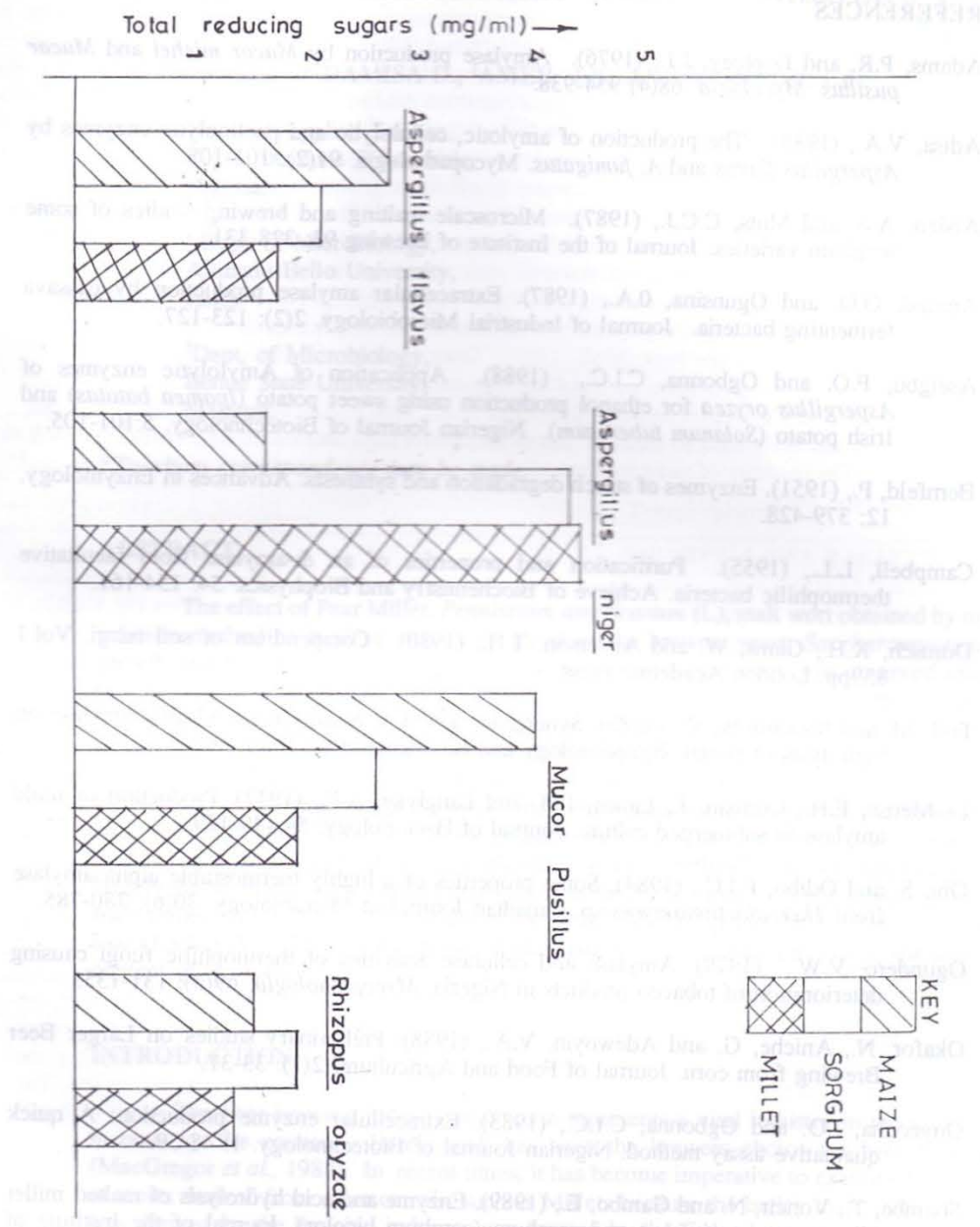


Fig. 4 Hydrolyses of the test cereal starches using the crude amylases of the selected fungal isolates



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