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Partial Purification of Amylase from the Culture Filtrates of *Aspergillus flavus* Grown on Cassava Peels

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Abstract

Amylase from a Cassava peel culture of *Aspergillus flavus* was partially purified by Ammonium Sulphate precipitation as well as dialysis. The dialysed 60% $(NH_4)_2$ SO_4 precipitated enzyme had an activity of 1.087 mg T.R.S released/ min/mg protein which was two folds of the activity of the crude culture filtrate. Hydrogen ion concentration as well as temperature had profound influence on enzyme activity of the partially purified enzyme while Amylase activity increased progressively as pH was increased from 3 to 7 reaching a maximum of 1.68 mg T.R.S released/min/mg/protein at P^H 7.0. A rapid decrease in amylase activity was observed as pH was increased from 7 to 9 while the amylase activity increased with increase in temperature from 30^0C to 45^0C and reached a maximum of 1.15 mg T.R.S. released/min/mg/protein at 45^0C. Subsequent increase in temperature resulted into decrease in activity of the amylase enzyme.

Keywords: *Aspergillus flavus*; dialysis; pH; temperature; partial purification; cassava peel

Introduction

Up to the early 1970's it was considered that plant and animal materials were the best sources of enzymes. Nowadays, however microbial enzymes are becoming increasingly important for their technical and economic advantages, (Kelly and Fogarthy, 1976; Mohammed *et al.*, 2007).

In recent years the capability of some fungi to degrade starch has aroused the interest of several researchers who recognized the potential values of various fungi for certain biotechnological applications such as single cell proteins, or ethanol from starchy biomass (Demot and Verachtert, 1987; Ettalibi and Baratti: 1988). Amylase belong to the group of hydrolyzing enzyme, with a wide range of utilization in various industrial sectors like brewing, bread making, textiles, paper, food processing and canning (Sanjeer Kumar and Satyanarayana, 2005; Rai and Deshmukh, 2005). Recently, I reported that *Aspergillus flavus* produces high activities of amylase when grown on low cost carbon sources such as Cassava peel and Sorghum which are alternative carbon substrates for large scale cultivation of *Aspergillus flavus* for amylase production (Oyewale, 2012). The objective of this paper was to compare activities of crude and partially purified amylase and also characterize the partially purified enzyme.

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Materials and Method

Preparation of Inoculums

With aid of sterile corkborer 5 mm disc of an advancing edge of a 4-day old fungal culture was inoculated on to an agar slant prepared with Czapekdox agar containing 2% (W/V) soluble starch. The cultures were then incubated at 30^0C for 48 hours before use.

Culture Methods

Spores of 48 hours old cultures of *Aspergillus flavus* was harvested using the methods described by Akinyosoye and Akinyanju (1989). The 48 hour old cultures of *Aspergillus flavus* was washed with 10 mL of sterile distilled water, by shaking to obtain a suspension of spores. An aliquot (0.5 ml) of spore suspensions was aseptically used to inoculate 50mls of liquid medium, containing any of the carbon sources, and then incubated at room temperatures (29 ± 1^0C) on a rotary shaker at 80 rev per minute. Culture was then suction filtrated through a pre-weighed No. 1 Whatmann filter paper and the filtrate obtained was used as crude extract. At intervals of 24 hours the protein content as well s the amylase activity in the culture filtrate were determined. The mycelium retained on the pre-weighed No. 1 Whatman filter paper was used for growth measurements.

Determination of Fungal Growth

Growth of fungus was determined at intervals of 24 hours over a period of seven days by the dry mycelia weight method. The filter pads used to harvest the mycelia were first oven dried at 80^0C to constant weight. After filtration the mycelia on the filter paper were oven dried at 80^0C. The weight was expressed in mg/50 mL of culture.

Determination of Amylase Activity

The amylase activity in the culture filtrate of *Aspergillus flavus*wa determined by the D.N.S.A. methods (Bernfield, 1955; Ogundero, 1979, 1982 a & b; Ettalibi and Barratti, 1988). 1 mL of culture filtrates was added to 3 mL of standard soluble starch in 0.002 M Na_2HPO_4, and 0.006M NaCl (PH 6.9) and incubated at 45^0C for one hour (Bernfield, 1955; Ogundero, 1979). The reducing sugars produced were determined by addition of 3 mL of Dinitrosalicylic acid D.N.S.A. reagent which contained D.N.S.A. (1.0 g); 2M NaOH, (20 mL); Potassium sodium tartarate (30 g); in 100 mL of diluted water (Fergus 1969); boiled for five minutes to complete the reactions. The absorbance of cooled solution was then read at 540 nm with a W.P.A. S106 spectrophotometer. The reaction mixture of the uninoculated control was used to set absorbance readings at zero. The amylase activity of the culture filtrates was expressed as total reducing sugars released/min/mg protein.

Partial purification of amylase from the culture filterate of *aspergillus flavus* grown on cassava peel

Ten milliliters of spore suspension of *Aspergillus flavus* was used to inoculate 1000 cm^3 of sterile basal salts medium containing 2% (w/v) cassava peel as sole carbon source. The medium was incubated at room temperature (29 ± 1^0C) for six days after which it was suction filtrated through No. 1 Whatman filter paper. The crude culture filtrate obtained was centrifuged at room temperature using a Gallenkamp laboratory centrifuge at 5,000 rpm.

To the supernatant, ammonium sulphate was added to 2% saturation. The suspension was stored and kept in the refrigerator at 5^0C for 12 hours. This was later centrifuged at 5000 rpm and the ammonium sulphate concentration of the supernatant increased to 60% saturation and left in the refrigerator. After 12 hours the suspension was centrifuged at 5000 rpm at room temperature (29 ± 1^0C). The precipitate was resuspended in 16 mL of Trizma HCl buffer (pH 7.0) and then centrifuged at 5000 rpm and at room temperature. The supernatant was collected and dialyzed against Trizma HCl (pH 7.0), for 12hr in the refrigerator at 5^0C.

Effects of temperature on activity of partially purified amylase from the culture filtrate of *Aspergillus Flavus* grown on cassava peel

The effect of temperature on activity of partially purified amylase was determined by assaying for amylase activity at 30^0C, 35^0C, 40^0C,

45^0C, 50^0C, 55^0C and 60^0C respectively. Amylase activity was expressed as amount of T.R.S. released/min/mg protein.

Effects of pH activity of partially purified amylase from the culture filtrate of *Aspergillus flavus* grown on cassava peel

The effect of pH on activity of partially purified amylase was determined by using phosphate buffer solutions of PH values 3, 4, 5, 6, 7, 8 and 9. The amylase activity and protein contents of each buffered starch solution were determined and expressed as T.R.S. released/min/mg protein.

Results

Amylase enzyme in the culture filtrate of *Aspergillus Flavus* grown on cassava peel was partially purified with $(NH_4)_2SO_4$ precipitation (Table 1). The crude culture filtrate had the lowest amylase activity of 0.52 mg T.R.S released/min/mg protein, which subsequently increased to 0.573 mg T.R.S. released/min/mg protein with 20% $(NH_4)_2SO_4$. After precipitation with 60% $(NH_4)_2SO_4$ amylase activity increased to 0.723 mg T.R.S. released/ min/mg protein, before dialysis.

The dialysed 60% $(NH_4)_2SO_4$ precipitated enzyme had an activity of 1.087 mg T.R.S. released/min/mg protein, which was two folds that of the crude culture filtrates (Table 1).

Effects of pH on activity of partially purified amylase from *Aspergillus flavus*

Buffered starch solutions at pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 were used to determine the effects of pH on activity of partially purified amylase from *Aspergillus flavus* (Figure 1).

Amylase activity increased progressively as pH was raised from 3.0 to 7.0 reaching a maximum of 1.68 mg T.R.S. released/min/mg protein at pH 7.0 (Figure 1). A rapid decrease in amylase activity was observed as pH was increased from 7 to 9 (Figure 1).

Effects of temperature on activity of partially purified amylase from *Aspergillus flavus*

Starch solutions and enzymes were incubated for 1 hour at temperature of 30, 35, 40, 45, 50, 55 and 60^0C and amylase activity at each temperature was determined.

Amylase activity increased steadily with increase in temperature from 30^0C to 45^0C and reached a maximum of 1.15 mg T.R.S. released/min/mg protein at 45^0C. Subsequent increases in temperature resulted into decreases in activity of amylase enzyme (Figure 2).

Table 1: Partial Purification of Amylase from culture filtrate of *Aspergillus Flavus* grown on 2% W/V Cassava peel.protein

Purification Step	Total Volume (mL)	Amylase activity	Protein content	Specific amylase activity
Culture Crude Filtrate	1000	0.0262	0.050	0.524
20% $(NH_4)_2\ SO_4$	800	0.0258	0.045	0.573
60% $(NH_4)_2\ SO_4$	400	0.0252	0.035	0.723
After Dialysis	16	0.0250	0.023	1.087

Amylase activity, protein content and specific amylase activity are expressed in mg/mL/min, mg/mL and mg/mL/min/mg protein.

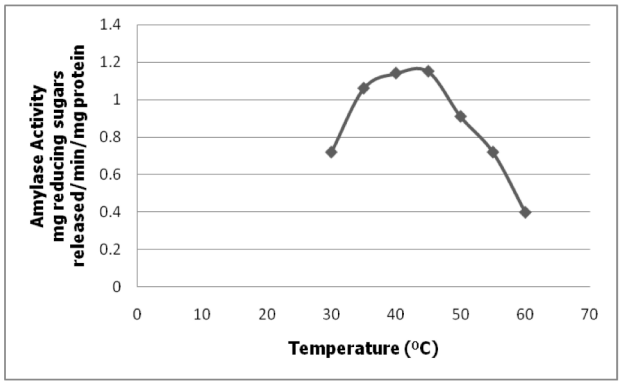

Figure 1: Effects of pH on the activity of partially purified amylase from the culture filtrates of *Aspergillus flavus grown on cassava peel*

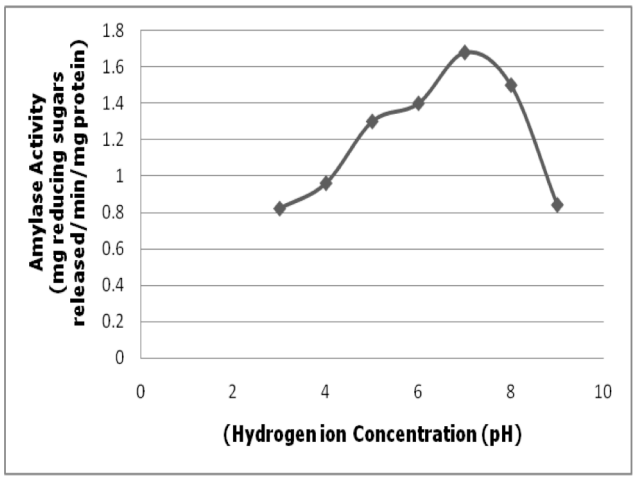

Figure 2: Effects of temperature on the activity of partially purified amylase from the culture filterates of *Aspergillus flavus grown on cassava peel*

Discussion

Partial purification of amylase from the culture filtrates of *A. flavus* grown on cassava peel by $(NH_4)_2SO_4$ precipitation method gave a two fold increase in enzyme activity (Table 1). Similar results were reported by Pestana and Castillo (1985), during the purification of Glucoamylase from rice flour medium. The increase in activity from 0.524 to 1.087 mg T.R.S. released/min/mg protein after dialysis, (Table 1), may suggest that dialysis may have removed some inhibitors, which may be present in the $(NH_4)_2SO_4$ precipitated enzyme.

The partially purified amylase from *A. flavus* had an optimum pH of 7.0 (figure 1). This value falls within the range of maximum pH values for amylase activity from several organisms. However, this result is different from that reported for *A. terreus* which was shown to have optimum pH in the range of 3.0 - 4.5 (Ueda *et al.,* 1984). The low level of amylase activity observed at pH 3, 4, 5, 6, 8 and 9 (Figure 1), may be due to enzyme denaturation or significant alteration in active site configuration which may not only have affected the effective binding of enzymes to the substrate.

This investigation also showed that the partially purified amylase from *A. flavus* had a broad peak of temperature, but with optimum at 45^0C (Figure 2). The optimum temperature of 45^0C recorded in this investigation is similar to that reported for amylase produced by *Papulospora thermophile* (Chapman *et al.*, 1975), *Aspergillus awamori* (Pestana and Castillo 1985), and *Rhizopus oryzae* (Rochi-Chui-Yu and Hang, 1990). Amylase activity from *A. clavatus* had an optimum temperature of 30^0C (Ogundero and Osunlaja 1986). The variation in optimum temperature for amylase activity from different organisms, probably suggests a wide range of enzymes. This ability may depend on the source, nature and physiological activities of the organism concerned.

The low level of amylase activity observed at low temperatures of 30, 35 and 40^0C may be due to retardation of amylase activity while the low level of amylase activity at higher temperatures of 50, 55 and 60^0C may be as a result of denaturation of enzyme or significant alteration in the active sites configuration which affect the effective binding of enzymes to substrates. The Partially purify amylase from cassava peel produced activities, which is twice that of the crude enzyme, hence cassava peel has proven to be a good carbon source for large-scale production of Amylase enzyme. With an ever increase number of Garri processing plants and cassava flour mills in Nigeria, there is an increase in the volume of raw materials readily to produce Amylase enzymes at cheaper prices.

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