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CHARACTERIZATION AND OPTIMIZATION OF CRUDE AMYLASE PRODUCED BY *ASPERGILLUS NIGER* RESPONSIBLE FOR CASSAVA (*MANIHOT ESCULENTUM*) TUBER ROT

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Abstract: The production of amylase by *Aspergillus niger*, isolated from mould deteriorating cassava (*Manihot esculentum*) was studied and characterized using different conditions of substrate (starch) concentrations, pH, temperature and heating duration. Enzyme activity generally increased from 29.7 units/ml on the fourth day to 37.3 units/ml on the sixth day. The optimal enzyme activity for starch concentration, pH and temperature were 50mg/ml, 5.0 (140 units/ml), and 35^{0}C (43 units/ml) respectively. Enzyme activity was inhibited at 70^{0}C as the enzyme was subjected to periodic heating over time. Approximately, about 68.8%, 15.9%, 13% and 6.7% of activity was lost within one, five, ten and fifteen minutes of heating at 70^{0}C. The synthesis of amylase is of special importance in the phyto-pathogenicity of *Aspergillus niger* during infection of hosts rich in starchy products.

Key Words: Cassava, *A. niger,* amylase, enzyme activity.

INTRODUCTION

Cassava (*Manihot esculenta*) tubers are the third largest source of food carbohydrates in the tropics, after rice and maize (Fauquet and Fargette, 1990; Tewe, 2004). Cassava is a major staple food for millions of people all over the world (Akinwunmi and Andoh, 2013) and plays a particularly important role in agriculture in developing countries, especially in sub-Saharan Africa, because it does well on poor soils and with low rainfall, and because it is a perennial that can be harvested as required (Onyenwoke and Simonya, 2014). Cassava's wide harvesting window allows it to act as a famine reserve and is invaluable in managing labour schedules, offering flexibility to resource-poor farmers because it serves as either subsistence or a cash crop (Stone, 2002; Daramola and Osanyinlusi, 2006). Cassava root has been promoted as a treatment for bladder and prostate cancer (Abeygunasekera and Palliyaguruge, 2013). However, there is yet no convincing scientific evidence that cassava is effective in preventing or treating cancer.

The primary uses of *A. niger* are for the production of enzymes and organic acids by fermentation (Finkelstein *et al*., 1989). The history of safe use of *A. niger* comes primarily from its use in the food industry for the production of many enzymes such as α-amylase, amyloglucosidase, cellulases, lactase, invertase, pectinases, and acid proteases (Bennett, 1985; Ward, 1989).

Amylases are enzymes capable of hydrolysing starch and related saccharides (Horváthová *et al*., 2001; Reddy *et al.,* 2003). There are more than about 30 different amylolytic and related enzymes (Janeček, 1997). Degradation of starch is essentially performed by the four groups of enzymes: endo-amylases acting primarily on α- 1, 4-linkages; exo-amylases acting primarily on α- 1, 4-linkages; debranching enzymes attacking mainly the α- 1,6-linkages and cyclodextringlycosyl transferases that degrade starch by catalyzing mainly cyclisation and disproportionate reactions (Antranikian, 1992; Guzmán- Maldonado and Paredes-López, 1995; Ayansina and Owoseni, 2010).

The best known amylolytic enzymes are α-amylase, β-amylase and glucoamylase, and these three amylases are quite distinct from both functional and structural points of view (Horváthová *et al*., 2001). This perhaps implies that there exists a rather long evolutionary distance between them (Janeček, 1994). They constitute their own independent families with no sequence similarities (Pujadas *et al*., 1996; Coutinho and Reilly, 2000; Janeček, 1997).

In this study we investigated some characteristics controlling the action of crude amylase enzyme produced by *Aspergillus niger* responsible for cassava (*Manihot esculentum*) tuber rot.

MATERIALS AND METHODS

Fungal Isolation and Culturing

Cassava tubers were collected from the Bowen University, Iwo farm house, transported to the laboratory and cut into pieces. Each piece was placed in transparent polythene bags that had been moistened with sterile de-ionised water. The bags were observed daily for fungal out-growth. On the 4th day, filamentous fungal growth with black spores on some of the tuber pieces were observed and isolated by appropriate pure culture techniques on Sabouraud dextrose agar. Pure cultures of the *Aspergillus niger* were kept on the agar slant in the refrigerator for storage and subsequent uses later.

Enzyme Preparation

A sterile cork (3mm) was aseptically used to pick the fungal inoculum on Sabouraud dextrose agar plate. Three discs were inoculated into separate conical flasks containing 100ml of the growth medium. The flasks were then incubated at room temperature. After 4th, 5th and 6th days of incubation, the contents of each flask were filtered through Whattman 1 filter paper. The filtrate (crude enzyme) was collected in separate conical flasks and analysed for amylase activity.

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Enzyme Assay

Enzyme activity was determined each day immediately after filtration. Citrate phosphate buffer solution (0.02M) at pH 6.0 was prepared. Starch (0.5g) was added to 10ml sterile de-ionised water and warmed to dissolve. The dissolved starch solution was made up to 100ml with the citrate phosphate buffer solution which serves as the substrate. From the substrate 2ml was pipette into separate test tubes labelled 'experimental' and 'control'. To each of the experimental tubes only was added 0.5ml of the enzyme. All the experimental and control tubes were incubated for 1hr at 35^{0}C. After incubation, termination of the reaction was done by the addition of 2ml of 1N HCl into each test tube and shaken properly. Thereafter, 2ml of the content of each test tube were pipette into each new tube and appropriately labelled. Three millilitres of 0.1N HCl were then added to each tube. Potassium iodine solution was added to each of the tubes, shaken properly and thereafter optical density was taken using the spectrophotometer at 670nm. One unit of activity is defined as the amount of enzyme in the reaction mixture that produced 0.01 reduction in the intensity of the blue colour of the starch-iodine complex under the assay condition (Reddy *et al.,* 2003).

Enzyme Characterization

Effect of substrate (starch) concentration: Starch concentrations of 0.1%, 0.2%, 0.3%, 0.4%, 0.5% & 0.6% were separately dissolved in citrate phosphate buffer solutions were used as substrates in test tubes. Amylase activity was determined as described under enzyme assay.

Effect of hydrogen ion concentration (pH): Citrate phosphate buffer solution containing 0.5% starch were adjusted to pH 4.0, 4.5, 5.0, 6.0 & 6.5 respectively and these served as substrate. Amylase activity was determined as described under enzyme assay.

Effect of temperature: Starch (0.5%) in citrate phosphate buffer solution served as substrate. Substrate-enzyme mixture was incubated at different temperatures of 20^0, 25^0, 30^0, 35^0, 40^0 & 45^{0}C. Amylase activity was determined as described under enzyme assay.

Effect of heating duration (Thermo stability at 70^{0}C): Enzyme preparation (0.5ml) and 1ml of citrate phosphate buffer solution (pH 6.0) was pipette into each tube. All the tubes were placed in water bath (70^{0}C) and withdrawn at intervals of 0min, 1min, 5min, 10min &15min respectively and placed in ice-bar to prevent further heating. One millilitre of the buffer solution and 0.5% starch were added to each of the experimental tubes. All the tubes were incubated at 35^{0}C for 1 hour. Amylase activity was determined as indicated under enzyme assay.

RESULTS AND DISCUSSION

There was a general increase in enzyme activity each day from 4th day (29.7 units/ml) to the 6th day (37.3 units/l) as shown in Table 1. A gradual increase in enzyme activity was also observed as substrate concentration increased (Fig. 1). This is shown by an increase in enzyme activity as starch concentration increased from 10.0mg/ml (25units/ml) to 50.0 units/ml). Optimal enzyme activity was obtained at 50mg/ml and further substrate increase to 60.0mg/ml of starch resulted in decrease in enzyme activity. This observation is similar to that of Nyamful (2013) who had explained this by assuming that so many substrate molecules were competing for the active sites on the enzyme surfaces such that they block the sites and tend to prevent any other substrate molecule from occupying them. Roskoski (2007), had reported that the reaction velocity of enzymes decreases after its maximum velocity has been attained.

Table 1: Effect of days of incubation on production of amylase by *Aspergillus niger*

Day	Enzyme activity (units/ml)
4	29.7
5	36.0
6	37.3

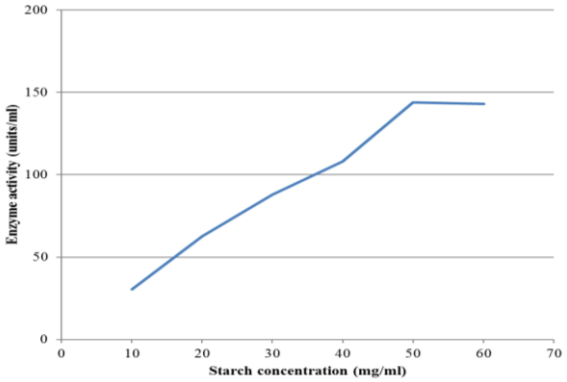

Figure 1: Effect of substrate (starch) concentration on amylase activity synthesized by *Aspergillus niger*

There was a gradual increase in enzyme activity from pH 4.0 reaching an optimum point (140 units /ml) at pH 5.0. Further increases in pH resulted in a decrease in enzyme activity as shown in Figure 2. This result suggests a slightly acidic nature of the amylase produced by the *A. niger* (Oyewole and Agboola, 2011).

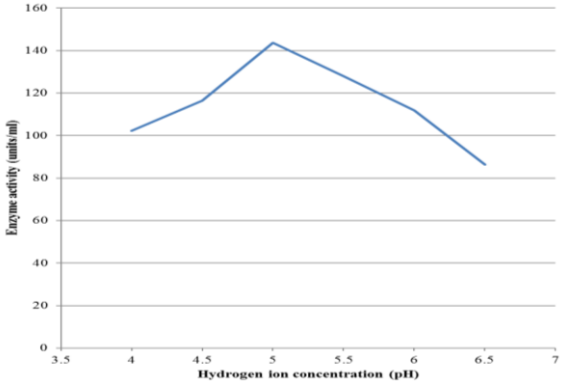

Figure 2: Effect of hydrogen ion concentration (pH) on amylase activity of synthesized by *Aspergillus niger*

Enzyme activity increased gradually with incubation temperature from 20^{0}C (21.0 units/ml) to 35^{0}C (43units/ml); at which point also enzyme activity was optimal. Further increases in temperature resulted in

decreases in enzyme activity (Fig. 3). This is slightly different from the report of Nyamful (2013) who had shown that culture filtrates of *A. niger* produced optimum activity at 40^{0}C.

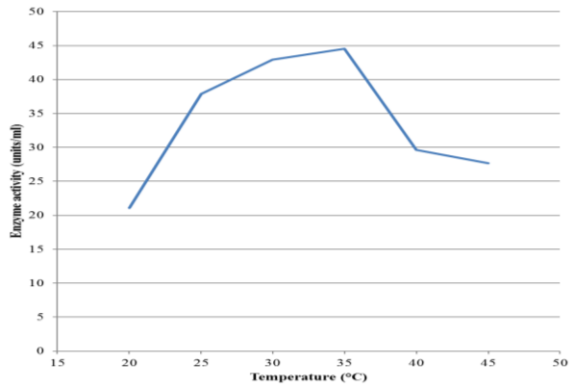

Figure 3: Effect of Temperature on amylase activity synthesized by *Aspergillus niger*

There was a sharp decrease in enzyme activity with heating duration (70^{0}C) from 80 units/ml at 0min to 25 units/ml after 1min of exposure to heat (Fig. 4). Further increases in heating time to 5, 10, and 15 minutes resulted in 15.9%. 13.0% and 6.7% losses respectively in the enzyme activity. This indicates that heating at 70^{0}C with increase in time remarkably inhibited enzyme activity. This agrees with the report of Dutta *et al.,* (2006) which showed that amylase activity was inhibited by heat as a result of denaturing of the active sites.

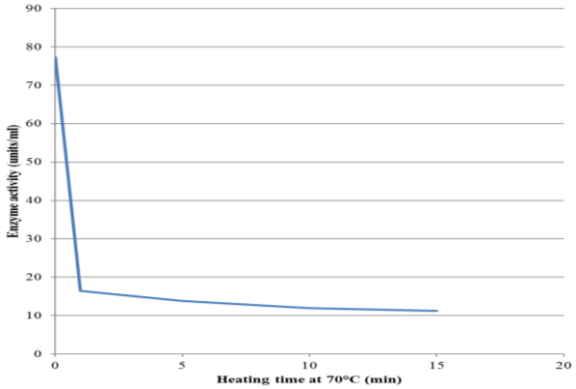

Figure 4: Effect of heat duration (thermo stability at 70°C) on amylase activity synthesized by *Aspergillus niger*

Conclusively, this report shows that amylase produced by *A. niger* growing on cassava tubers could be well exploited for their ability to degrade starchy substances. Cassava tubers are known to be rich in starchy components. Therefore, the ability of strains of *A. niger* to synthesize amylase would be of importance in the pathogenicity of the organism during infection of hosts rich in starchy components. Many workers have implicated amylase in microbial phyto-pathogenicity (Kumar *et al.,* 2014; Avwioroko *et al.,* 2015).

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