



Amylase activity in culture filtrate of *Aspergillus chevalieri*

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ABSTRACT

This study was carried out to determine the growth and production of amylase by *Aspergillus chevalieri* in a defined medium. *A. chevalieri* was grown in a synthetic medium containing starch as the sole carbon source. Culture filtrate exhibited amylase activity. Optimum enzyme activity was observed on the tenth day of incubation. The presence of NaCl and MgCl₂ stimulated amylase activity while EDTA and HgCl₂ in the reaction mixture caused a reduction in the activity of the enzyme. The activity of the enzyme was optimum at 35°C and pH 6.5. The amylase of *Aspergillus chevalieri* was heat labile, losing its activity completely after twenty minutes of heating at 70 °C. The amylase produced by this fungus is of significance in the brewing industry and pharmaceuticals. The observed properties would aid in preserving the enzyme and knowing optimum conditions for activity to assist in maximizing industrial output.

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INTRODUCTION

Aspergillus mould is prevalent under a wide variety of moisture conditions as contaminants of almost indoor and outdoor surfaces (Ali and Abdel-Moneim, 1989). *Aspergillus chevalieri* is a storage mould affecting stored grains (Abu et al., 2005). It affects grains in terms of germination. Invasion of such stored grains is at hermetic and non-hermetic conditions (Christensen, 1979). It has been observed to cause mouldiness of maize (*Zea mays* L.) seeds (Crab and Mitchinson, 1997), stored cocoa, groundnut and palm kernels (Dixon and

Webb, 1977). It causes the spoilage of milk jam along with *Aspergillus fumigatus* (Doster et al., 1996). *A. chevalieri* has been associated with production of aflatoxins which are hepatotoxic, carcinogenic, heratogenic and mutagenic (Fersht, 1977). It is implicated in respiratory disorders of certain patients with suspected mycotic infections (Hinnikson et al., 2004). Limited research has been done on *A. chevalieri* in terms of enzyme production especially determining its properties under certain conditions of growth. This investigation was carried out to determine the growth and production of amylase by *A.*

chevallieri in a defined synthetic growth medium with starch as sole carbon source and to characterize the induced amylase.

MATERIALS AND METHODS

Organism and culture condition

The isolate of *Aspergillus chevallieri* was obtained from the culture collections of Professor Olutiola of the Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria. The organism was isolated from mouldy bread routinely grown and maintained on 1% malt yeast extract agar plates. It was subcultured on 1% malt yeast extract agar slant and incubated at 25 °C for 72 hours.

Ten millilitre of sterile distilled water was added to each tube. The spores on the surface of the agar medium were dislodged by carefully scrapping them with sterile inoculating loop, care being taken not to scrape the agar medium. The content of each tube was filtered into sterile conical flask through double layer of sterile muslin. The spore suspension was diluted to have a final concentration of approximately 10^5 spores per ml.

Growth medium

The growth medium contained starch (5g), K_2HPO_4 (1 g), $MgSO_4 \cdot 7H_2O$ (0.05), KH_2PO_4 (4.95 g), KNO_3 (0.05), L-cysteine (0.5 mg), $FeSO_4 \cdot 7H_2O$ (0.5mg), Biotin (0.0025 mg), Thiamine (0.0025 mg), and distilled water (500 ml). Each 250 ml flask contained 100ml of the growth medium. The growth medium was autoclaved at 121 °C for 15 minutes. Each flask was inoculated with 1ml of the spore suspension prepared as described above.

Enzyme extraction

On a daily basis of incubation at 27°C, the content of each flask was filtered using Whatman No 1 filter paper and the filtrate served as the enzyme preparation.

Enzyme assay

Amylase activity was determined by a modified dextrinogenic assay method of Pfueller and Elliot (1969) involving a direct spectrophotometric measurement of the changes of the blue starch-iodine complexes due to the decrease in the amount of starch.

The reaction mixture contained 2 ml of 0.15% starch (w/v) in 0.2 M citrate phosphate buffer pH (6.0) and 0.4 ml of the enzyme for experimental tubes. The control tubes contained only 2 ml of 0.15% starch. Both the experimental and control tubes were incubated in a water bath at 35 °C for 30 minutes. After 30 minutes, the reaction was terminated by adding 3 ml of 1N HCl to each of the experimental and control tubes. 0.4 ml of the enzyme was added to each of the control tubes. Two milliliters of the mixture from each test tube were dispensed into another set of test tubes. Three milliliters of 0.1 N HCl were added. Each of the test tubes was shaken and 0.1 ml of iodine solution was added. Optical density readings were taken at 600 nm.

One unit of amylase activity was defined in arbitrary units as the amount of enzyme in 1ml of reaction mixture which produced 0.1% reduction per ml in the intensity of the blue colour of the starch-iodine complex under the assay conditions.

Characterization of enzyme

Effect of pH

Starch solution (0.15% w/v) was prepared at different pH values of 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 respectively and used as substrate. Reaction mixture consisted of 2 ml of substrate and 0.4 ml of enzyme. Incubation was at 35 °C for 30 minutes. Amylase activity was determined as described above under enzyme activity.

Effect of heat

Samples of enzyme preparation were heated at 70 °C for 0, 2, 5, 10, 15 and 20 minutes respectively. Appropriate quantity (0.4 ml) of the heated enzyme was added to 2

ml of 0.15% starch solution and incubated at 35 °C for 30 min. Amylase activity was determined as described under enzyme assay.

Effect of temperature

The effect of temperature of the activity of the enzyme was examined. The reaction mixture constituted 2 ml substrate (0.15% starch) in citrate phosphate buffer, pH (6.0) and 0.4 ml of enzyme. The reaction mixture were incubated at 20, 25, 30, 35, 40 and 45 °C respectively for 30 minutes and then analyzed for amylase activity as described above under enzyme assay.

Effect of chemicals

Different concentrations, 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 mM of HgCl₂ and EDTA were dissolved separately in 0.15% (w/v) starch solution (pH 6.0) and employed as substrate; 0.4 ml enzyme was added and incubated at 35 °C for 30 minutes. Amylase activity was analyzed as described above under enzyme assay.

Effect of cations

Different concentration (0.5, 10, 15, 20, 25, and 30 mM) of MgCl₂ and NaCl dissolved in citrate phosphate buffer (pH 6.0) containing 0.15% starch were used as substrate. Reaction mixture consisted of 2 ml of 0.15% starch solution and 0.4 ml of the enzyme. Reaction mixtures were incubated at 35 °C for 30 minutes. Amylase activity was determined as described above.

Effect of substrate concentrations

Different concentrations (0.8, 1.0, 1.2, 1.4, 1.6, and 1.8 mg/ml) of starch solution were used as substrate. 0.4 ml enzyme was added to 2 ml of substrate, mixed and incubated at 35 °C for 30 minutes. Amylase activity was determined, as described under enzyme assay.

RESULTS

Aspergillus chevalieri grew in a synthetic growth medium containing starch as the sole carbon source. Culture filtrate exhibiting amylase activity was observed on the 10th day of incubation.

Effect of pH

In the present study, amylase activity increased slightly from pH 3.5 to 4.5. There was a drastic increase from pH 4.5 to pH 5.0 and a gradual increase to pH 6.5 after which there was a decline in activity (Figure 1).

Effect of Temperature

The amylase activity was affected by the temperature of the reaction medium. Optimum activity of enzyme was observed at 35 °C after which there was a gradual decline (Figure 2).

Effect of Heat

Enzyme activity gradually decreased with increase in the time of heating the enzyme at 70 °C. The reaction mixture at 0 minute exhibited the highest enzyme activity after which there was a gradual decline. Enzyme activity was completely lost after 20 minutes of heat (Figure 3).

Effect of Cations

Amylase activity steadily increased with increasing concentrations of NaCl and MgCl₂ (Figure 4).

Effects of chemicals

Enzymes activity decreased gradually with increasing concentrations of HgCl₂ and EDTA. A more pronounced decline was observed with HgCl₂ (Figure 5).

Effect of substrate concentrations

There was a gradual increase in amylase activity as the concentrations of substrate (starch) increased. Enzyme activity reached an optimum at 1.4 mg/ml starch concentrations after which there was a decline (Figure 6).

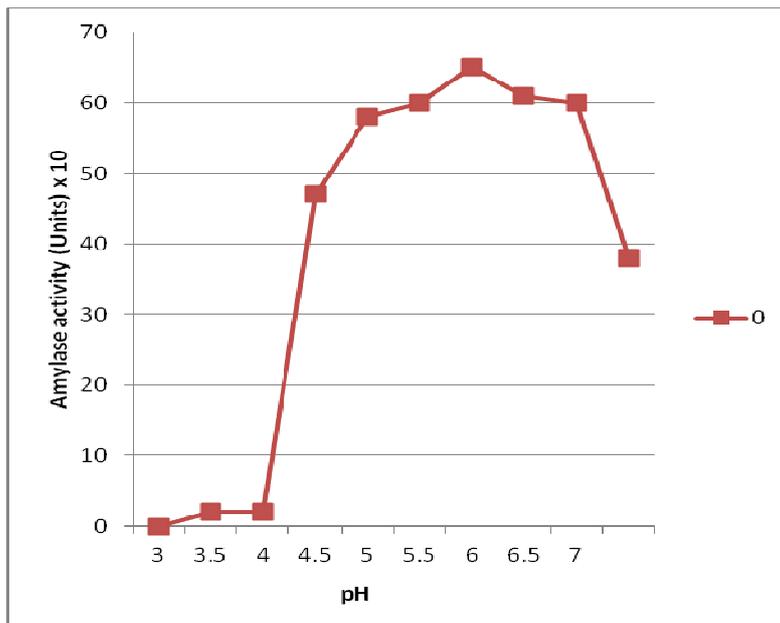


Figure 1: Effect of pH on the activity of amylase produced by *Aspergillus chevalieri*.

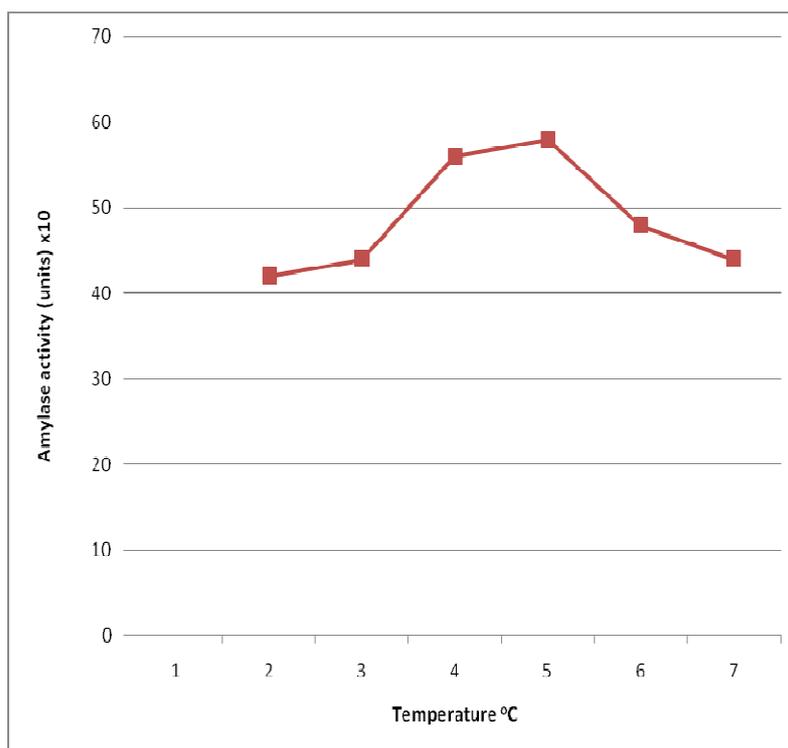


Figure 2: Effect of Temperature on the activity of amylase produced by *Aspergillus chevalieri*.

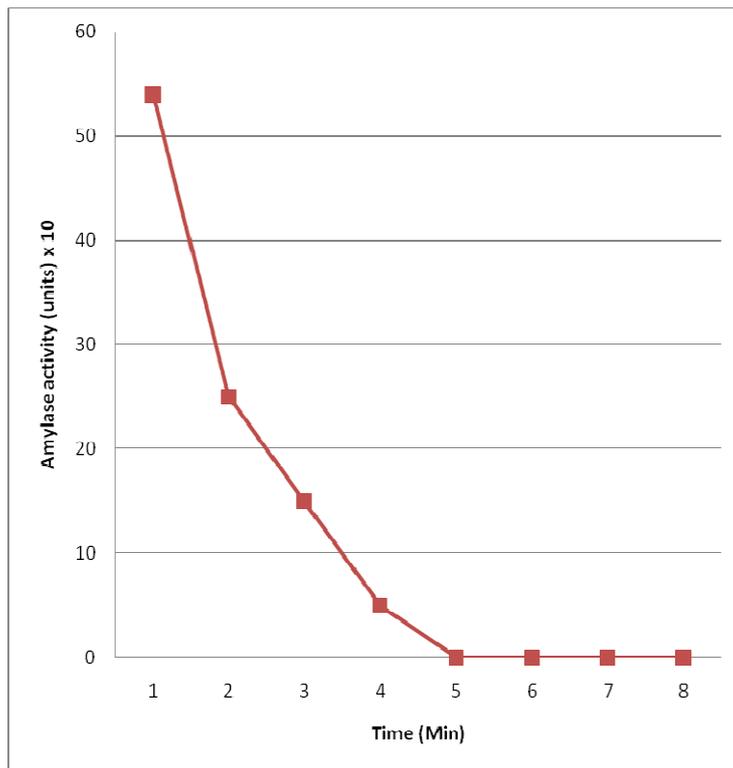


Figure 3: Effect of Time of Heating on the activity of amylase produced by *Aspergillus chevalieri*.

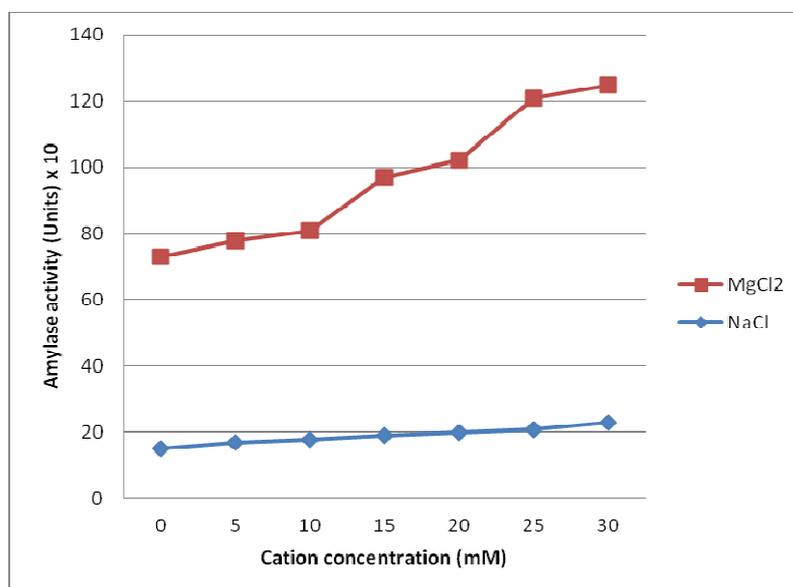


Figure 4: Effect of Cations on the activity of amylase produced by *Aspergillus chevalieri*.

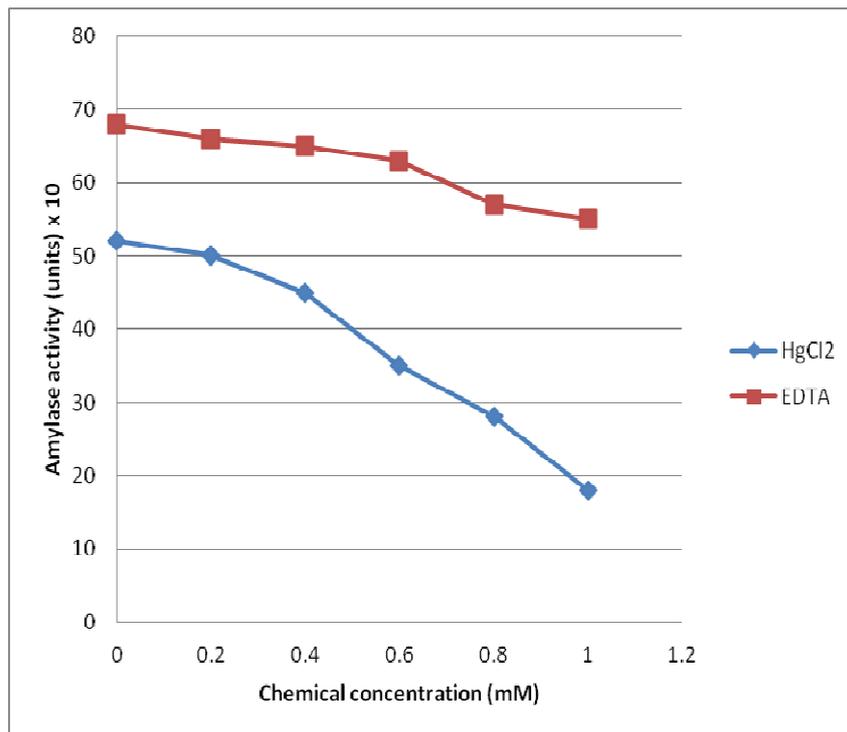


Figure 5: Effect of Chemicals on the activity of amylase produced by *Aspergillus chevalieri*.

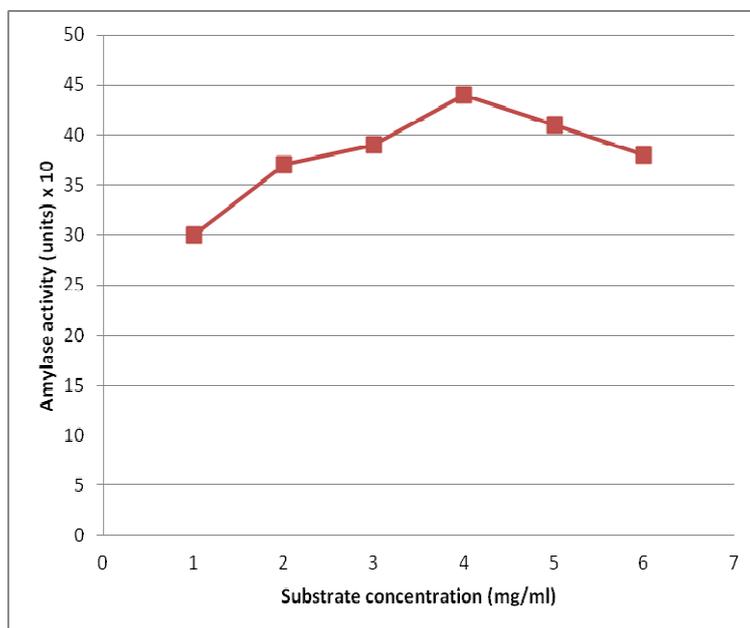


Figure 6: Effect of Substrate Concentration on the activity of amylase produced by *Aspergillus chevalieri*.

DISCUSSION

The results of this study showed that *Aspergillus chevalieri* grew in a medium which contained starch as its sole carbon source, producing amylase during the process. This result is in support of earlier reports (Lehninger, 1982; Moreno Martinez and Mario, 2000) which showed that *Aspergillus chevalieri* and some other fungi synthesize appreciable quantities of amylase.

The activity of the amylase in this research was influenced by the pH of the reaction mixture with optimum enzyme activity at pH 6.5. Similar results were obtained by Mahmoud et al. (2005) while researching on amylase produced by *Aspergillus fumigatus* isolated from poultry feeds. Olutiola (1982) also obtained similar result on *Aspergillus niger* associated with the black mould rot of tomato fruits. Sanni et al. (1992) had similar results on amylase from *Aspergillus flavus*.

The optimum pH for a particular enzyme is the pH at which the activity of the enzyme is at a maximum and this depends on substrate used, temperature, ionic strength and other factors including substrate concentrations (Olutiola et al., 1982). A change in the pH of the reaction mixture will affect the concentration and character of the amino acid and carboxylic acid of the enzyme protein, which will in turn affect the catalytic site and concentration of the enzyme (Prescott et al., 2000). Low and high pH values will also cause a considerable denaturation and hence inactivation of the enzyme protein (Prescott et al., 2005).

The activity of amylase obtained in this study was influenced by the concentration of the substrate with optimal activity recorded at 1.4mg/ml starch beyond which the activity of the enzyme gradually decreased. Prescott et al. (2005) reported that with fixed enzyme concentration, an increase in the concentration of the substrate will result in an increase in enzyme activity until it reaches a saturation point beyond which enzyme activity declines. Thus, at low concentration of substrate, the active sites of the enzymes are not saturated

by substrate and hence, the activity of the enzyme increases with increase in substrate concentration (Sanni et al., 1992). As the number of substrate molecules increases, the active site of the enzyme is covered to a higher degree until a saturation is attained and no more active sites are available and hence no further increase in enzyme activity (Reed, 1995). Also, at higher substrate concentration there is a tendency for ineffective complexes to be formed between enzyme and substrate. Since the substrate molecules are too many around the enzyme, they may bind to regions on the enzyme that are not active sites or may sometimes crowd on the active sites (Sanni et al., 1992).

The result obtained from this study showed that temperature affected the activity of amylase. Optimum activity of enzyme was obtained at 35 °C for amylase from other fungi (Olutiola, 1982). The rate of enzyme-catalyzed reactions generally increases with the temperature within a temperature range at which the enzyme is stable and retains full activity (Prescott et al., 2000). The effect of temperature on enzyme activity may be due to several causes which include an influence on the stability of the enzyme-substrate affinity or the effect of increasing rate of thermal denaturation of the enzyme above the critical temperature (Reed, 1995).

The amylase activity of *Aspergillus chevalieri* was completely lost after heating at 70 °C for 20 minutes. It has been suggested that the loss of enzyme activity beyond the optimum temperature level could be attributed to the effect of temperature on the velocity of the enzyme reaction (Streets, 1969). Enzymes are heat labile and denaturation of the enzyme protein by heat results in gradual loss of its catalytic properties (Satyanarayana, 2009).

The results obtained from this study showed that the activity of amylase was stimulated by concentrations of Mg²⁺ and Na⁺ employed. The site of the action may determine the activation of the enzyme and the metal may form an essential part of the enzyme active centre, acting as a binding link between the enzyme and substrate (Prescott et

al., 2000). Metal activated enzymes which require added metals for activity may specifically depend on whether the metal is monovalent, divalent, or trivalent. In this study, the observations show that the divalent nature of Mg compared to the monovalent Na in the active site of the amylase allows a more rapid speed up of converting the starch to maltose and glucose, which are the products of enzymatic hydrolysis. Activity can be enhanced based on the interaction of such metals with the active centers of the enzyme (Sanni et al., 1992). Karlson (1965) also reported that the metals also help to remove enzyme inhibitors that may be present in the enzyme preparation by forming a complex or precipitates with such inhibitors.

Concentrations of HgCl₂ and EDTA employed in this research had an effect on amylase activity. They compete with the starch substrate for the active site of the amylase and rapidly modify the enzyme structurally. With increasing concentrations, the enzyme becomes structurally modified (Prescott et al., 2005). Similar results have been reported by other researchers. HgCl₂ and EDTA have been reported to cause a rapid loss in the activity of microbial amylase (Moreno-Martinez and Mario, 2000).

The amylase produced by *Aspergillus chevalieri* can be purified and molecular weight determined. Purification techniques must be carefully so as not to lose the enzyme or activity. The results of this investigation add to existing reports on this fungus. Amylases are of utmost importance industrially especially in brewing and in textiles where the properties for optimum activity will be useful.

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