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Studies on Amylolytic Enzyme Synthesized by *Aspergillus flavus* Associated with Mouldy Bread

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Abstract: Studies were carried out on amylolytic enzymes produced by *Aspergillus flavus* isolated from mouldy bread with the aim of establishing some factors that affect its activity. *Aspergillus flavus* grew in synthetic medium containing starch as the sole carbon source and synthesize enzymes which exhibited amylolytic activities. Production of the enzyme increased with increase in days of incubation with optimum activity occurring on the tenth day of incubation. Activity of the enzyme increased at 4mg/ml starch concentration. Activity also increased with increase in temperature reaching a maximum at 40°C. The pH of the reaction mixture influenced the activity of the enzyme, optimum activity being at pH 7.0.

Key words: *Aspergillus flavus*, amylolytic activity, amylase, bread

INTRODUCTION

Moulding is the most common and hence the most important cause of spoilage of bread and most bakery products (Bharat and Hoondal, 1998). However, the temperature attained in the baking procedure is usually high enough to kill all mould spores in and on the loaf, hence it is most probable that moulds reach the outer surface or penetrate the loaves after baking, usually from the air during cooling and thereafter from handling or wrappers (Miller, 2001). Prominent moulds involved in the spoilage of bread are usually referred to as bread-moulds and they include *Rhizopus stolonifer, Penicillium expansum, Penicillium stolonifer, Aspergillus niger* and *Aspergillus flavus* (Duke *et al.*, 1996).

Aspergillus flavus is a phytopathogenic fungus able to grow on different crops, but most commonly on corn, cotton and peanuts contaminating them with mycotoxins (William and Dennis, 1998). *A. flavus* can also be pathogenic in animal species, including humans and some domestic animals (Collier *et al.*, 1998). The mycotoxins produced by *A. flavus* cause damage during metabolism and this induces vital changes in the chemical constituent of the associated substrate (Anjana and Sinha, 1983). Aflatoxins that are primarily produced by the mould *Aspergillus flavus* and *Aspergillus parasiticus* are among the most toxic and carcinogenic compounds occurring naturally (Lillehoj and Ciegler, 2003).

Amylases are enzymes that hydrolyse starch molecules into polymers composed of glucose units (Reddy *et al.*, 2003). Amylases are important enzymes employed in the starch processing industries for the hydrolysis of starch into simple sugar constituents (Mitchell and Lousane, 1990; Akpon *et al.*, 1996 El-Saadany *et al.*, 2006).

Amylases from plants, animals and microorganisms have been studied since enzymes were discovered (Horikoshi, 1996). Amylases have received a great deal of attention because of their significance especially in biotechnology (Reddy *et al.*, 2003). Amylase constitutes a class of industrial enzymes having approximately 25% of the enzyme market world-wide (Sindhu *et al.*, 1997). Many *Bacillus* species and thermostable *Actinomycetes* like *Actinomycetes thermomonospora* and *Actinomycetes thermoactinomyces* are versatile producers of amylase (Buzzini and Martini, 2002). The genus *Bacillus* produces a large range of extracellular enzymes of which amylases and proteases are of industrial importance (Bajpai and Bajpai, 1999; Demiorijan *et al.*, 2001). Currently two types of amylases, glucoamylase and alpha-glucosidase are important for starch hydrolysis. Glucoamylase attacks α-1, 4-bonds, releasing D-glucose molecules (Guzman-Maldonato and Paredes-Lopes, 1995). This enzyme also attacks α-1, 6 bonds at branching points in the amylopectin molecule but much more slowly than α-1, 4 linkages (James and Lee, 1997). Alpha-glucosidase catalyzes the splitting of alpha-D-glucosyl residues from the non-reducing end of substrates to release alpha-glucose (Pandey *et al.*, 2002). In this study we investigated some factors affecting amylase produced by *Aspergillus flavus* isolated from mouldy bread.

MATERIALS AND METHODS

Organism and culture condition: The strain (MIC/OAU/038) of *Aspergillus flavus* used in this work was isolated from mouldy bread. It was obtained from the culture collection of Prof. P.O. Olutiola of the Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria. The stock culture was routinely

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grown and maintained on 1% malt-yeast extract agar slants in test-tubes.

Preparation of assay medium: Sterile 50 ml of basal medium was aseptically added to 50 ml of the starch medium and final pH was adjusted to 6.7. Thereafter 1 ml of 10^6 spore suspension was added to each of the 100 ml growth medium.
The content of each flask was monitored daily for amylase activity. Five milliliters were aseptically obtained from each flask and filtered with small disc of Whatman filter-paper.

Enzyme assay: The activity of the enzyme (amylase) was determined by a modification of the dextrinogenic assay of Pfueller and Elliot (1999). It involved the measurement of changes in the blue values of starch-iodine complex due to the decrease in the amount of the starch in the reaction mixture. The reaction mixture consisted of 2ml of 0.4% starch (w/v) and 0.5 ml of enzyme preparation. The control tubes contained only 2 ml of 0.4% starch (w/v). Experimental and control tubes were incubated at 40°C for 30 min.
After incubation, 2 ml of 1 N HCl was added to the experimental tubes to terminate the reaction. To each of the control tube was added 2 ml of 1 N HCl followed by 0.5 ml of the enzyme. The contents of experimental and control tubes were further diluted by adding 3 ml of 0.1 N HCl to each tube. Iodine mixture (0.1 ml) was added to each tube. The content of each tube was mixed thoroughly by using a test-tube mixer (Gallenkemp). Optical density readings were made at 600 nm using colorimeter (Jensway, Essex, UK).
One unit of enzyme activity was arbitrarily defined as the amount of enzyme in 1 ml of reaction mixture which produced 0.01% reduction in the intensity of the blue colour of the starch-iodine complex under the assay conditions.

Characterization of amylase: Effects of growth period i.e incubation period (4-10 days), various concentrations of starch (0.5-4 mg/ml), temperature (10-45°C) and time of heating (0-30 min) at 80°C were tested to characterize the amylase produced.

RESULTS AND DISCUSSION

Aspergillus flavus grew in a synthetic medium containing starch as sole carbon source. During the period of growth, the culture filtrate exhibited amylase activity. Optimum activity was observed on the tenth day of incubation (Fig. 1). *Aspergillus flavus* was able to degrade starch when used as substrate within a concentration range of 0.5-4 mg/ml. Optimum activity was observed at 4 mg/ml starch concentration (Fig. 2). *Aspergillus flavus* exhibited amylase activity within a temperature range of 10-45°C. Activity of the enzyme

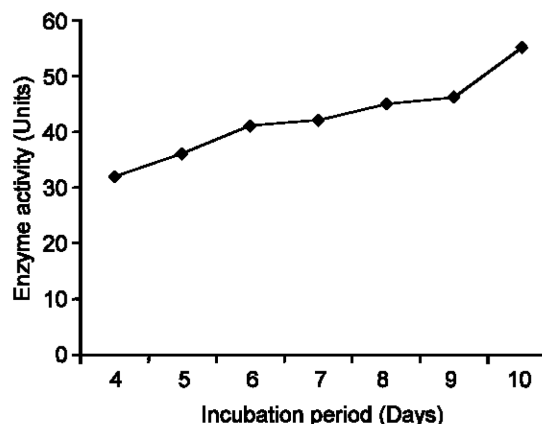

Fig. 1: Effect of growth period on the activity of amylase produced by *Aspergillus flavus*

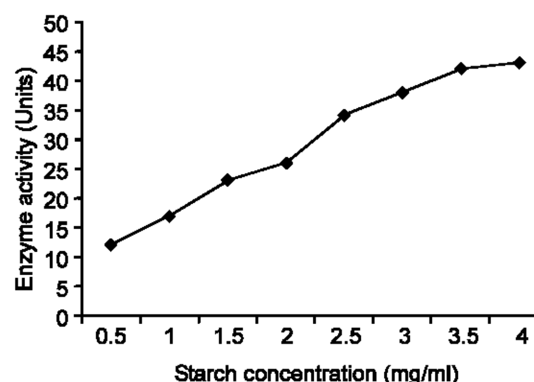

Fig. 2: Effect of starch concentration on the activity of amylase produced by *Aspergillus flavus*

increased as the temperature increased. Maximum activity occurred at 40°C, beyond which no further increase in activity was obtained (Fig. 3). When amylase produced by *Aspergillus flavus* was heated at 80°C, there was a gradual decrease in the activity of the enzyme with increase in period of heating (Fig. 4). When heated for 5 min, approximately 33% loss in enzyme activity was obtained. Heating for 30 min caused a decrease of about 63% in enzyme activity (Fig. 4).
The results of this study showed that *A. flavus* grew in a medium containing starch as the sole carbon source and produced the enzyme amylase required for the hydrolysis of starch. Malankar (2005) reported the production of a raw starch-degrading alpha amylase by a strain of *Aspergillus flavus*.
The results of this work showed that amylase activity increased as the substrate (starch) concentration in the medium increased until a substrate concentration of 4 mg/ml was reached, when no further increase in enzyme activity occurred. Pederson and Nielson (2001) reported similar observations for amylase activity. Dixon and Webb (1997) reported that at low substrate

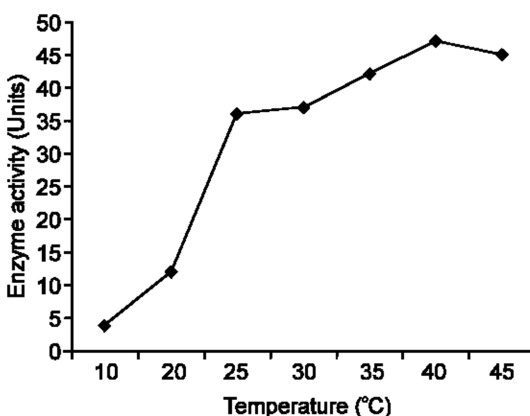

Fig. 3: Effect of temperature on the activity of amylase produced by *Aspergillus flavus*

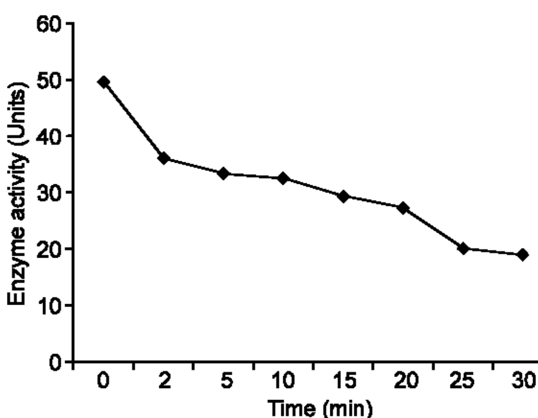

Fig. 4: Effect of time of heating (80°C) on the activity of amylase produced by *Aspergillus flavus*

concentrations the active sites of enzymes are not saturated and hence the activities of such enzymes increased with increase in substrate concentration. Yang and Wang (1999) reported an increase in amylase activity of germinated barley as starch concentration increase. Kuiper *et al.* (1998) reported that maximum activity of alpha amylase enzyme was obtained at 1.67% of substrate (starch) concentration. In addition, Abdel-Rahman (2006) concluded that maximum alpha amylase activity was between 2-3% concentrations.

In this study, the activity of the enzyme was greatly influenced by the pH of the reaction mixture, with optimum activity *occurring* at pH 7.0. Haruyuki-Efugi (1996) reported that amylase activity was affected by the substrate type, temperature, substrate concentration and pH. Kamekura (1997) obtained an optimum pH of 5-6 for amylase from *Micrococcus* sp. However some amylases act best at acidic pH 5.0 and 5.5 (Do and Kin, 1995; Gupta *et al.*, 2008). In the present work, there was a slight decrease in activity of the enzyme beyond pH 7.0. Eke and Oguntimehin (1992) reported that a drastic decline in the activity of amylase at pH above 7.0 indicated a loss of activity at alkaline region.

The effect of pH on the activity of an enzyme has been attributed to a change in the ionic character of the amino and carboxylic acid components of the enzyme, which will in turn affect both the catalytic site and conformational status of the enzyme protein (Rice and Stephen, 2002; Prakash *et al.*, 2009). Extremes of low and high pH values have been protein (Russel and Jacobsen, 1997).

The results of this study showed that temperature greatly affected the activity of amylase synthesized by *A. flavus*. Optimum activity of the enzyme occurred 40°C. This agrees with the work of Khoo *et al.* (1994) who reported an optimum temperature 40-45°C for amylase from *Aspergillus niger*. However, Chakraborty *et al.* (2000) obtained an optimum activity at 50°C for a thermostable alpha amylase.

In this study, enzyme activity was lost with increase in the time of heating at 80°C and within 5 min of heating (80°C), approximately 33% of the activity of enzyme was lost. This agrees with the result of Mulimani and Rudrappa (2002) who reported the inhibition of amylase from pea when subjected to heat and Guerra *et al.* (2005) who showed that amylase activity was inhibited at temperatures above 70°C.

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