Tropentag 2011
University of Bonn, October 5 - 7, 2011
Conference on International Research on Food Security, Natural Resource Management and Rural Development

Amylase from *Aspergillus fumigatus* associated with deterioration of rice (*Oryza sativa*)

Ladokun O.A and Adejuwon A.O

Department of Biochemistry Lead City University, Ibadan, Nigeria

In recent years the new potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated renewed interest in the exploration of extracellular enzymatic activity in several microorganisms. Starch degrading enzymes like amylase have received great deal of attention because of their perceived technological significance and economic benefits. In the work reported here *Aspergillus fumigatus* was subcultured on potato dextrose agar slants incubated at 25^{o}C for 72hours. The spores on the surface of the agar medium were dislodged by carefully scraping them with sterile inoculating loop. *Aspergillus fumigatus* grew in a medium containing rice. Cultures of the filtrate exhibited amylase activity. Amylase activity was determined using the modified method of Pfueller and Elliot (1959). Maximum enzyme activity was observed on the 5th day of incubation. Amylase activity was determined by measurement of dextrinized power which is a measure of the change in the blue colour of starch-iodine complex due to decrease in the amount of starch. The effect of temperature on the enzyme was examined. The activity of the enzyme was optimum at 35^{o}C and pH 6.0. The enzyme was heat labile, with a gradual decline in activity as period of heating increased, losing its activity completely after thirty minutes of heating at 80^{o}C. The cations Al^{3+} and Fe^{3+} stimulated the activity of the amylase. P-chloromercuric benzoate and iodoacetamide inhibited enzyme activity. There was a gradual increase in the amylase activity as substrate concentration increased. Optimum activity was observed at 0.1% starch concentration.

INTRODUCTION

Enzymes are proteins that speed up biochemical reactions. Amylases are important enzymes employed in the starch processing industries for hydrolysis of polysaccharides such as starch into simple sugar constituents (Akpan *et al*., 1999). Starch degrading enzymes like amylase have received a great deal of attention because of their perceived technological significance and economic benefits. Evidences of amylase in yeast, bacteria and moulds have been reported and their properties documented (Akpan *et a*l., 1999; Adebiyi and Akinyanju, 1998; Buzzini and

Martini, 2002). Among the microorganisms, many fungi have been found to be good sources of amylolytic enzymes.

Solid state fermentation has been used in the production of industrial enzymes like amylase and it has great potentials in the developing countries due to its simplicity of operations, low capital cost and high volume productivity (Akpan *et al*., 1999).

Several microorganisms are known to produce raw starch digesting amylase, however most of these microorganisms were effective for cereal starches but root or tuber starches were more resistant to the enzyme reaction (Okolo *et al.*, 1995). Although the use of microbial amylase for the hydrolysis of raw starches has long been advocated and practiced to a limited extent, there is paucity of information on the hydrolysis of raw starches of locally available tubers in the tropics. Most screening procedures used for the detection of amylase producing microorganisms involve growing the organism on solid media containing soluble starch and testing for starch hydrolysis by flooding the plates with either iodine solution or 95% ethanol.

Aspergillus species are highly aerobic and are found in almost all oxygen-rich environments, where they commonly grow as molds on the surface of a substrate as a result of the high oxygen tension. Commonly, fungi grow on carbon-rich substrates such as monosaccharides (for instance glucose) and polysaccharides (such as amylase). Aspergillus species are common contaminants of starchy foods (such as bread and potatoes), and grow in or on many plants and trees. In addition to growth on carbon sources, many species of Aspergillus demonstrate oligotrophy where they are capable of growing in nutrient-depleted environments, or environments in which there is a complete lack of key nutrients. *Aspergillus niger* is a prime example of this, it can be found growing on damp walls, as a major component of mildew.

Species of *Aspergillus* are important in commercial microbial fermentations. For example, alcoholic beverages such as Japanese sake are often made from rice or other starchy ingredients (like manioc), rather than from grapes or malted barley. Typical microorganisms used to make alcohol, such as yeasts of the genius Saccharomyces cannot ferment these starches, and so *koji* mould such as *Aspergillus oryzae* is used to break down the starches into simpler sugars.

Almost all processes in a biological cell need enzymes to occur at significant rates. Since enzymes are selective for their substrates and speed up only a few reactions from among many possibilities, the set of enzymes made in a cell determines which metabolic pathways occur in that cell. Some enzymes are used commercially, for example, in the synthesis of antibiotics. In addition, some household products use enzymes to speed up biochemical reactions (for example, enzymes in biological washing powders break down protein or fat stains on clothes, enzymes in meat tenderizers break down proteins, making the meat easier to chew).

Amylase is an enzyme that breaks starch down into sugar. Amylase is present in human saliva, where it begins the chemical process of digestion. The pancreas also makes amylase (alpha amylase) to hydrolyse dietary starch into disaccharides which are converted by other enzymes to glucose to supply the body with energy. Plants and some bacteria also produce amylase. As

diastase, amylase was the first enzyme to be discovered and isolated (Hill *et al*., 1970). All amylases are glycoside hydrolases and act on α-1, 4-glucosidic bonds.

Alpha amylase can be used in improving anaerobic solid waste treatment (Higuchi *et al*., 2005). Carbohydrate-hydrolysing enzymes have long been used by industrial product markers as major catalysts to transform raw materials into end products in such areas as food processing, beverage production, animal nutrition, leather and textiles (Uhlig, 1998). With the advent of new frontiers in biotechnology, the spectrum of amylase application has widened in many fields such as clinical, medicinal and fine-chemical industries, as well as a widespread application of starch saccharification in the textile, food, brewing and distilling industries (Pandey *et al*., 2000).

Rice (*Oryza sativa*) is a monocotyledonous cereal which belongs to the Grass family Gramineae or Poaceae (Stern, 2003). With over 7,000 varieties of rice, its pericarp and embryo contain 70-80% starch, 7% proteins, 1.5% oils, some vitamins (mostly A, B and C) and some essential minerals (Dutta, 2007). According to Sizer and Whitney (2000), rice contains fibre and the vitamin folate and provides 80% of the calories consumed by humans worldwide (Stern, 2003). The discovery of Gibberellins arose from infected rice (Stern, 2003).

This study was designed to examine the production and activity of amylase by *Aspergillus fumigatus* associated with deterioration of rice. Attempts were also made to characterize the enzyme.

MATERIALS AND METHODS

Aspergillus fumigatus was subcultured on potato dextrose agar slants, incubated at 25^{o}C for 72 hours. Ten millimeter of sterile distilled water was added to each tube. The spores on the surface of the agar medium were dislodged by carefully scraping them with sterile inoculating loop, care being taken not to scrap the agar medium. The content of each tube was filtered into sterile flask through double layered sterile muslin. The spore suspension was diluted to have a final concentration of approximately 10^5 spores per ml.

Rice in distilled water (1% w/v), was autoclaved at 121^{o}C for 15mins. This was the growth medium. One hundred millimeter of the growth medium in 250ml flasks was inoculated with spore suspension of the isolate, prepared as previously described. The content of each flask was analysed for amylase activity on a daily basis.

On the 5th day of incubation the content of each flask was filtered using Whatman No. 1 filter paper. The filtrate served as the enzyme preparation. Amylase activity was determined using the modified method of Pfueller and Elliot (1959). Two milliliter of 0.1% starch (w/v) in citrate phosphate buffer pH 6.0 was pipette to test tubes. The enzyme (0.5ml) was added to experimental tubes. Experimental and control tubes were incubated at 35^{o}C for 20minutes. Three milliliter of 1N HCL was added to terminate enzyme activity. Thereafter 0.5 ml of enzyme was added to the control tubes. Two milliliter of the mixture was transferred to a new set of clean test tubes. Three milliliter of 0.1N HCL was added to each tube. Iodine solution (0.1 ml) was then added to each

test tube. Optical density readings were taken at 620nm using an optima microprocessor controlled digital colorimeter. One unit of amylase activity was defined in arbitrary units as the amount of enzyme in the reaction mixture which produced 0.01% reduction in the intensity of the blue color of the starch-iodine complex under the assay conditions.

EFFECT OF TEMPERATURE

The effect of temperature on the enzyme was examined. The reaction mixture consisted of two milliliter substrate (0.1% starch in citrate phosphate buffer, pH 6.0 and 0.5ml of enzyme preparation). The incubation of the reaction mixture was done at 20^{o}C, 30^{o}C, 35^{o}C, 40^{o}C and 45^{o}C respectively for 20 minutes and then analyzed for enzyme activity.

EFFECT OF HEAT

Samples of the enzyme preparation were heated at 80^{o}C for 0, 5, 10, 15, 20, 25 and 30 minutes respectively. Appropriate quantity (0.5ml) of the heated enzyme preparation was added to two milliliter of 0.1% starch solution. After incubation of the reaction mixture, amylase activity was determined as described above.

EFFECT OF pH

Starch solution (0.1% w/v) in citrate phosphate buffer was prepared at different pH values of 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 respectively. They were used as substrate. The reaction mixture was two milliliter of substrate and 0.5 milliliter of enzyme. Incubation was at 35^{o}C for 20 minutes. Thereafter the enzyme activity determined.

EFFECT OF CATIONS

Different concentrations (0mM, 5mM, 10mM, 15mM, 20mM and 30mM) each of Al^{3++} and Fe^{3+} were prepared in citrate phosphate buffer pH 6.0 containing 0.1% starch used as substrate. Incubation was at 35^{o}C for 20 minutes. Amylase activity was then determined.

EFFECT OF CHEMICALS

Different concentrations (0mM, 2mM, 4mM, 6mM, 8mM and 10mM) each of iodoacetamide and p-chloromercuric benzoate were prepared in citrate phosphate buffer pH 6.0 containing 0.1% starch used as substrate. Incubation was at 35^{o}C for 20 minutes. Amylase activity was then determined.

EFFECT OF SUBSTRATE CONCENTRATION

Different concentrations (0.02%, 0.04%, 0.06%, 0.08%, 0.1% and 0.12%) of starch solution were used as substrate. Two milliliter of each substrate concentration was incubated with 0.5ml of the enzyme preparation at 35oC for 20 minutes. Amylase activity was then determined.

RESULTS

Aspergillus fumigatus grew in the growth medium expressing amylase activity. Activity gradually increased with an optimum on the 9th day as shown in Table 1. Result of the effect of substrate concentration is presented in Table 2. There was a gradual increase in the amylase activity as substrate concentration increased. Optimum activity was observed at 0.1% starch concentration. Table 3 shows the effect of temperature. Within the temperature range of 20-45^{o}C, there was a gradual increase in amylase activity. Optimum activity was observed at 35^{o}C after which there was a decline. When the enzyme was heated at 80^{o}C for a period of 0-30 minutes, amylase activity gradually declined as the period of heating increased. Activity was completely lost at 30 minutes as shown in Table 4. Table 5 shows the effect of pH. Within the pH range of 3.0 to 8.0, amylase activity gradually increased. Optimum activity was observed at pH 6.0 after which there was a decline. Table 6 shows the effect of cations. Within a concentration range of 0-30mM of Al^{3++} and Fe^{3+} respectively, amylase activity gradually increased. Optimum activities were observed at 30mM for the two cations. Result of the effect of chemicals is presented in Table 7. Within a concentration range of 0mM – 10mM of p-chloromercuric benzoate and iodoacetamide, amylase activity gradually decreased. Activity was completely lost at 8mM concentration of iodoacetamide. There was a gradual decrease in enzyme activity as concentration of p-chloromercuric benzoate increased.There was 96.5% percent decrease in activity at 10mM concentration.

Table 1 Effect of Incubation period on the activity of amylase produced by *Aspergillus fumigatus* in infected rice

Incubation period	Enzyme activity (units)
Day 1	20
Day 2	98
Day 3	350
Day 4	560
Day 5	790
Day 6	770
Day 7	720
Day 8	700
Day 9	610
Day 10	580

Table 2 Effect of substrate concentration on the activity of amylase produced by *Aspergillus fumigatus* in infected rice

Substrate concentration (%)	Enzyme activity (units)
0.02	130
0.04	340
0.06	530
0.08	610
0.10	790
0.12	730

Table 3 Effect of temperature on the activity of amylase produced by *Aspergillus fumigatus* in infected rice

Temperature	Enzyme activity (units)
20^{o}C	200
30^{o}C	560
35^{o}C	780
40^{o}C	620
45^{o}C	430

Table 4 Effect of heat on the activity of amylase produced by *Aspergillus fumigatus* in infected rice

Heat (minutes)	Enzyme activity (units)
0	820
5	470
10	30
15	25
20	14
25	3
30	0

Table 5 Effect of pH on the activity of amylase produced by *Aspergillus fumigatus* in infected rice

pH	Enzyme activity (units)
3.0	180
3.5	290
4.0	320
4.5	400
5.0	610
5.5	730
6.0	800
6.5	720
7.0	520
7.5	470
8.0	330

Table 6 Effect of cations on the activity of amylase produced by *Aspergillus fumigatus* in infected rice

Al^{3++} (Concentration)	Enzyme activity (units)
0 mM	790
5 mM	820
10 mM	840
15 mM	880
20 mM	910
30 mM	980

Fe^{3+} (Concentration)	Enzyme activity (units)
0 mM	770
5 mM	790
10 mM	830
15 mM	860
20 mM	880
30 mM	900

Table 7 Effect of chemicals on the activity of amylase produced by *Aspergillus fumigatus* in infected rice

Iodoacetamide (Concentration)	Enzyme activity (units)
0 mM	790
2 mM	61
4 mM	52
6 mM	10
8 mM	0
10 mM	0

P-chloromercuric (Concentration)	Enzyme activity (units)
0 mM	730
2 mM	42
4 mM	38
6 mM	30
8 mM	28
10 mM	25

DISCUSSION

The amylase produced by *Aspergillus fumigatus* showed optimum activity at pH 6.0. A similar result was reported by Abe et al., (1998) for amylase produced by some microorganisms. Optimum pH for growth and amylase production by many fungi has been reported to range from pH 5 to 6 (Khoo *et al*., 1994). Hydrogen ions concentration has been found to affect the optimum activity of the enzyme and that the non stability of the enzyme outside its maximum pH makes it loose its tertiary structure (Dixon and Webb, 1971).

Optimum activity of the amylase produced by *Aspergillus fumigatus* was observed at 35^{o}C. Bilal and Figen (2005) observed that *Penicillum chrysogenum* produced an amylase with optimum activity at a temperature range of 30-40^{o}C. The rate of enzyme catalysed reactions generally increases with the temperature range at which the enzyme is stable and retains full activity (Prescott *et al*., 2008). The effect of temperature on enzyme activity may be due to several causes which include an influence on the activity of the enzyme substrate affinity or the effect of increasing rate of thermal denaturation of the enzyme above the critical temperature (Prescott *et al*., 2008).

The amylase obtained in this study declined gradually in activity after being heated at 80^{o}C within 0 – 30 minutes. According to Bernhardsdotter *et al*., (2005), an alkali *Bacillus spp* isolate produced an alpha-amylase which was completely inactivated at 55^{o}C. When an enzyme is heated or is at a temperature above optimum, the enzyme has a tendency to be denatured (Stryer, 2002). Enzymes are heat labile and denaturation of the enzyme protein by heat results in gradual loss of its catalytic properties (Reed, 1995).

The activity of amylase in this study was influenced by the concentration of the substrate with activity increasing with increase in starch concentration. In general, at low substrate concentration, the activity of the active sites of the enzymes is not saturated by the substrate and hence, the activity of the enzyme increases with increase in substrate concentration (Dixon and Webb, 1971). As the number of substrate molecules increases, the active site of the enzymes are covered to a higher degree until a saturation is observed where no active sites are available and hence no further increase in enzyme activity (Prescott *et al.,* 2008). Since the substrate molecules are too many around the enzyme, they may bind to the regions on the enzyme that are not active sites or may crowd on the active sites (Dixon and Kelly, 1979).

The results further showed that activity of the amylase produced by *Aspergillus fumigatus* in infected rice was stimulated by the cations Al^{3+} and Fe^{3+} but inhibited by p-chloromercuric benzoate and iodoacetamide.

CONCLUSION

The results of this study showed that *Aspergillus fumigatus* grew on a medium solely as rice. Amylases are important enzymes employed in starch processing industries and these enzymes are classified on how they break down starch molecules.

Based on the results obtained, it was considered possible to produce amylase from mouldy rice infected with Aspergillus *fumigatus*. Many researchers have implicated amylase in the degradation and denaturation of a number of starchy foods and food products (Poonam and Dalel, 1995).

REFERENCES

Abe, J.I., Bergmann, F.W., Obata, K. & Hizuhuri, S. (1998). Production of raw starch digesting amylase of *Aspergillus sp*. K-27. *Applied Microbiology and Biotechnology* 27: 447-450.

Adebiyi, C.A.B. & Akinyanju, J.A. (1998). Thermophilic amylase producers from the soil. *Nigerian Journal of Science and Technology* 11(1): 30- 38.

Akpan, I., Bankole, M.O., Adesemowo, A.M., Latunde-Dada, G.O. (1999). Production of amylase by *A. niger* in a cheapsolid medium using rice bran and Agricultural materials. *Tropical Science* 39: 77-79.

Bernhardsdotter, E.C. M.J., Ng, J.D., Garriott, D.K. and Pusey, M.L. (2005). Enzymatic properties of an alkaline, chelator-resistant alpha-amylase from an alkaliphilic *Bacillus sp* isolate L1711. *Process Biochemistry* 40: 2401-2408.

Bilal, & Figen, E. (2005). Production and properties of α-amylase from *Penicillium chrysogenum* and its application in starch hydrolysis. *Preparatory Biochemistry & Biotechnology* 35: 167-178.

Buzzini, P. & Martini, A. (2002). Extracellular enzymatic activity profile in yeast and yeast-like strains isolated from tropical environments. *Journal of Applied Microbiology* 93: 1020-1025.

Dixon, M. and Webb, E.C. (1971). *Enzymes*. Longmans, London. 950pp.

Dutta, A.C. (2007). *Botany for Degree Students*. Oxford University Press, New Delhi. 708pp.

Higuchi, Y., Ohashi, A., Imachi, H. and Harada, H. (2005). Hydrolytic activity of alpha-amylase in anaerobic digested sludge. *Water Science and Technology* 52(1-2): 259-266.

Hill, R. & Needham, J. (1970). *The Chemistry of Life*: *Eight Lectures on the History of Biochemistry*. Cambridge University Press, London, England. 17pp.

Khoo, S.L., Amirul, A.A., Kamaru, Z.M., Nazalani,N., Azizan, M.N (1994). Purification and characteisation of alpha-amylase from *Aspergillus flavus. Folia Microbiology* 39: 392-398.

Okolo, B.N., Ezeogu, L.I. & Mba, C.N. (1995). Production of raw starch digesting amylase by *Aspergillus niger* and *Bacillus alvei* grown on native starch sources. *Journal of Sci. Food Agric.* 69: 109-115.

Pandey, A., Nigman, P., Soccol, C.R, Soccol, V.T., Singh, D. and Mohan, R. (2000). Advances in microbial amylase. *Biotechnology and Applied Biochemistry* 31(2): 135-152.

Pfueller, S.L. and Elliott, W.H. (1969). The extracellular α-amylase of *Bacillus stearothemophilus. Journal of Biological Chemistry* 244: 48-54.

Poonam, N. and Dalel, S. (1995). Enzyme and microbial systems involved in starch processing. *Enzyme & Microbial Technology* 17: 770-778.

Prescott, L.M., Harley, J.P. & Klein, D.A. (2008). *Microbiology*. 8th Edition. McGraw Hill Inc., USA. Pp 789-810.

Reed, G. (1995). *Enzymes in Food Processing*. Academic Press, New York, London. Harcourt Brace Jovanovich Publishers. Pp 233-239.

Smith, B.D. (1998). *The Emergence of Agriculture*. Scientific American Library. A division of HPHLP, New York 54 (382): 577-583.

Sizer, F.S. & Whitney, E.N. (2000). *Nutrition*: *Concepts and Controversies*. Wadsworth, Thomson Learning, London. 567pp.

Stern, KR., Jansky, S. & Bidlack, J.E. (2003). *Introductory Plant Biology*. McGraw Hill Higher Education. New York. 624pp.

Stryer, L. (2000). *Biochemistry*. Freeman, W.H. and Company, New York. 1064pp.