GLOBAL JOURNAL OF PURE AND APPLIED SCIENCES VOL. 9, NO. 1, JANUARY 2003, 53-58
 ISSN 1118-0579

AMYLOLYTIC STUDIES OF PLEUROTUS TUBER-REGIUM

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(Received 18 October 2001; Revision accepted 12 November 2001).

ABSTRACT

The alpha amylase of the sclerotium of *Pleurotus tuber-regium* was studied. The enzyme was purified from the fresh sclerotium through dialysis, ammonium sulphate fractionation and column chromatography of CM sephadex. The enzyme showed 70% of it's optimal activity between p.H 4.0 to 8.0. Acid and thermal stability was found at p.H 2.0 and temperature of 60°c respectively. Michealis Menton constant (km) and maximum velocity (Vmax) of the enzyme with starch as substrate was 1.25mg/ml and 5.0ug/ml/min, respectively. The paper chromatography of the hydrolytic products in starch showed hydrolytic products like glucose, maltose and dextrin, suggesting an alpha-amylase type of activity. Maltose is not hydrolysed by this enzyme.

Keywords: *Pleurotus tuber-regium*, Amylase, Sclerotium, fungus.

INTRODUCTION

Pleurotus tuber-regium is a tropical basidiomycete which grows widely in Nigeria. It is a macro fungus characterized by a large globoid sclerotium, which is whitish in colour inside and darkish-brown outside (Zoberi, 1973; Oso, 1977). P. tuber-regium grows cheaply on breadfruit tree (*Treculia africana*), palm tree (*Elaeis guineensis*) and on farm wastes (Okhuoya and Okogbo, 1991; Peterson, et al 1997).

The *sclelotium* of *P. tuber-regium* under moist condition, produces fruit bodies (Okwujiako, 1999). Total world production of edible mushrooms in 1994 was 4.9million tones (Chang, 1996), with Pleurotus specie, mostly *Pleurotus astreatus* accounting for 25% (Zervakis and Balis, 1996).

There is a growing need for vegetative production of the sclerotal and fruit bodies. Currently many studies are going on to enhance the vegetative production, spore germination and disease control of this fungus (Oso, 1977, Nwokolo, 1987; Okhoya and Okogbo, 1990; Fasidi and Ekuere, 1993; Okwujiako, 1999; Okwujiako, 1992).

Oso, (1977), reported that *Pleurotus tuber-regium* could utilize carbohydrates like glucose, fructose, xylose, detrin and starch as common sources for growth, indicating the presence of carbohydrate degrading enzymes. Recently, the fruiting body was used to produce total amylase, alpha-amylase, proteinase, lipase, peroxidase, catalase and polyphenol oxadse (Kadiri and Fasidi, 1990).

The need, for production of enzymes from local and cheap sources is necessary and will help reduce dependence on foreign enzyme kits. Amylases are enzymes involved in the degradation of starch and starch like polysaccharides.

Pleurotus tuber-regium growing naturally on decaying wood is expected to produce extracellular hydrolytic enzymes necessary to mobilize carbon and nitrogen for growth. Attempt is therefore made to study the properties and hydrolytic patterns of the amylase of *Pleurotus tuber-regium* not in the fruiting body but in sclerotium. The aim is to see whether such amylase, if present, can be introduced into starch producing industries and thus reduce cost.

MATERIALS AND METHODS

The soluble starch and carboxymethyl sephadex-G50 used were products of sigma chemical company, U.S.A. All other reagents used were of analytical grade. The sclerotia of *Pleurotus tuber-regium* were purchased from Ose market, Anambra State, Nigeria.

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Amylase Assay
Amylase activity was determined by Nelson colorimetric method (Nelson, 1944). Assay mixture contained 1ml of 1% soluble starch in 0.1m phosphate buffer p.H 6.9 and 1ml of enzyme extract. After incubation for 3o minutes, at 30°c, 1ml of assay mixture was used to reduce Nelson's reagent. Absorbance was monitored at 600nm and reducing equivalent extrapolated from a standard curve prepared with glucose. Each assay was performed in duplicate. One unit of amylase activity was defined as the activity that liberates 1 microgram glucose equivalent per minute.

Protein Determination
Protein was estimated by the method of Lowry, et. al. (1951).

Determination of pH Optimum
The pH optimum for the reaction was determined as described for amylase assay. Buffers used were acetic acid-sodium acetate from pH 4.0 to 6.0; phosphate buffer from 6.0 to 7.0;. Tris-Hcl buffer from pH 7.0 to 9.0; and sodium bicarbonate – sodium carbonate for pH 10.0. All buffers had molarity of 0.1m, no specific buffer effect was observed.

Acid Stability (Minoda, et al. 1968)
Partially purified amylase extract was incubated in HCl – acetate buffer of p.H. range 1- 4 for 80 minutes, 5ml of enzymes was titrated to pH 4.0 with sodium acetate solution. Amylase activity was determined as described above.

Thermal Stability (Glymph and Stutzenberger, 1977)
Amylase extract was dissolved in 0.1m phosphate buffer pH 6.9 and incubated at various temperatures for 60 minutes. At intervals of 0,10,30 and 60 minutes, amylase activity was determined as described above.

Paper chromatography of enzymatic products
Paper chromatography was carried out on Whatmann No 1 paper by ascending method. The developing solvent was n-propanol : ethylacetate : water, 14:2:7. The silver dip method was used to identify the sugar spots.

Extract and Partial Purification of Amylase (Depinto and Campbell, 1980).

Extraction: 60g of powdered sclerotium was blended with 220ml of ice-cold 0.01m phosphate buffer pH 6.9 for 3 minutes at high speed. The homogenate was passed through muslin cloth and the filtrate was centrifuged at 5000rpm for 10 minutes to remove fine particles.

Ammonium Sulphate Fractionation
To the clear filtrate, solid ammonium sulphate was dissolved to 25% saturation and left for 2 hours at 10°C. The precipitate was removed by centrifugation, at 5000g and the supernatant was made 50% saturated with solid ammonium sulphate and left at 10°C for 8 hours. It was centrifuged at 5000g for 15 minutes and the precipitate dissolved in minimum amount of buffer. It was dialysed in four litres of 0.01m phosphate buffer (pH 6.9) for 18hours, with two changes of buffer.

Carboxymethyl Sephadex – G50 Chromatography
The slurry of swollen CM – Sephadex was poured into a column of 10cm x 1cm and washed with 100ml of 0.01m potassium phosphate buffer pH 8.0. The dialysed enzyme extract containing about 2mg protein was eluted through the column with a 0 to 0.4m NaCl gradient in 0.01m phosphate buffer pooled together and concentrated with polythlene glycol. The eluate was then dialysed in 4 litres of 0.01m phosphate buffer pH 6.9 for 8 hours, at 10°C with two changes of buffer.

RESULTS

The three-step purification procedure, used to extract amylase from the sclerotium, increased the specific activity 20 times. It was difficult to obtain high yield of the enzyme because extracts of the sclerotium have low protein content.

PH Optimum
The amylase exhibited a broad pH optimum in the range of 0.6 to 8.0 (Fig. 1). The activity of the amylase fell rapidly from pH 8.0 to pH 10.0 and at pH 10.0 only 18% of its optimal activity was left.

Acid Stability
Result on acid stability of *Pleurotus tuber-regium* amylase indicated that it is stable at pH 2.0, retaining 70% of its original activity at

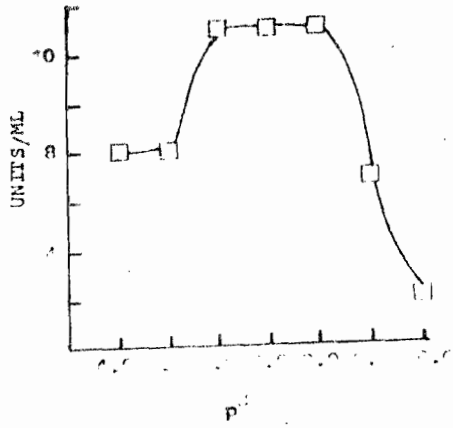

Fig.1. Effect of p^H on the activity of amylase from Pleorutus tuber-regium

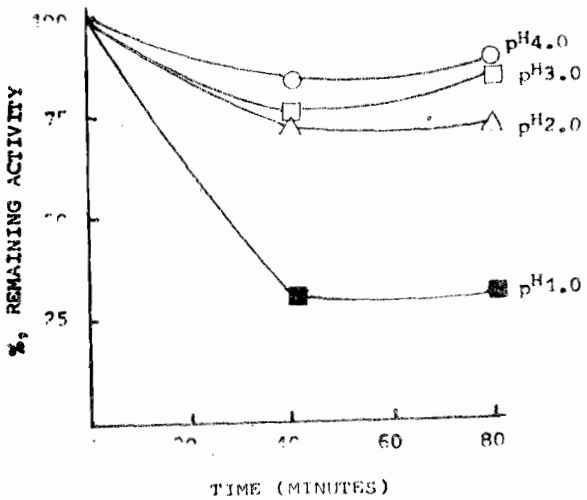

Fig. 2. Acid stability of amylase from Pleorutus tuber-regium. Amylase were incubated at various acid buffer solutions at 30°C and the activities remaining were assayed at p^H 4.0.

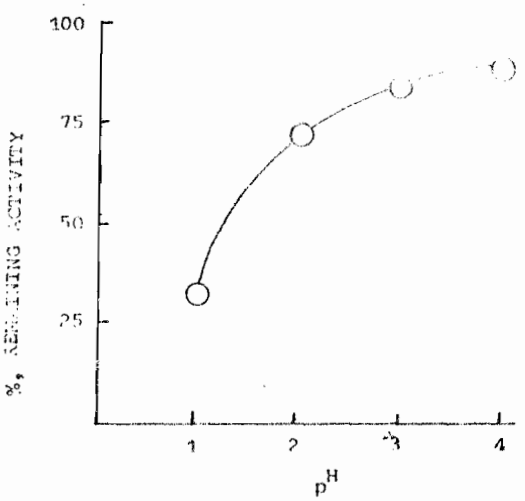

Fig. 3. Activity remaining after incubation of amylase at various buffer solutions of p^H 1 - 4 for 80 minutes at 30°C.

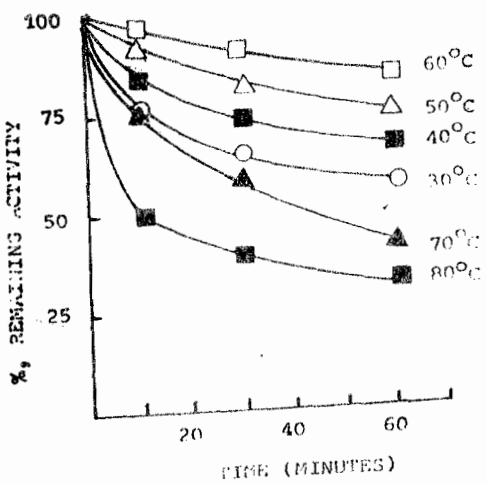

Fig. 4. Thermal stability of amylase from Pleorutus tuber-regium. Amylase were incubated at various temperatures and p^H6.9, and the activities remaining were assayed.

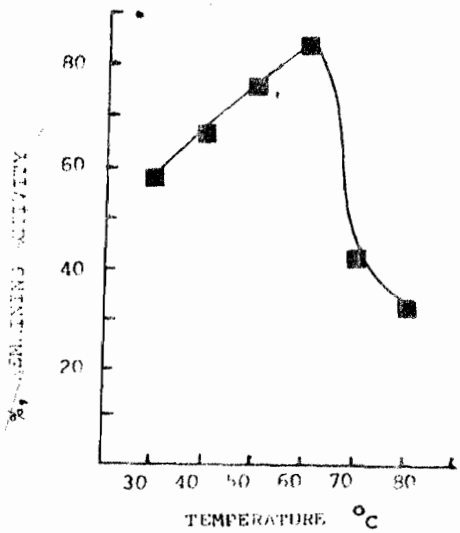

Fig. 5. Activity remaining after incubation of amylase at various temperatures for 60 minutes at p^H 6.9.

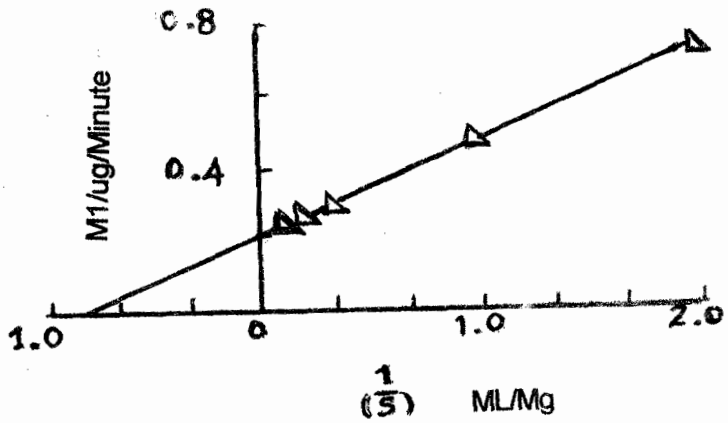

Fig. 6: Lineweaver-Burk plot for the determination of the Km value for starch with partially purified, P. *tuber-regium* amylase. The line was fitted to the points by the least-squares method.

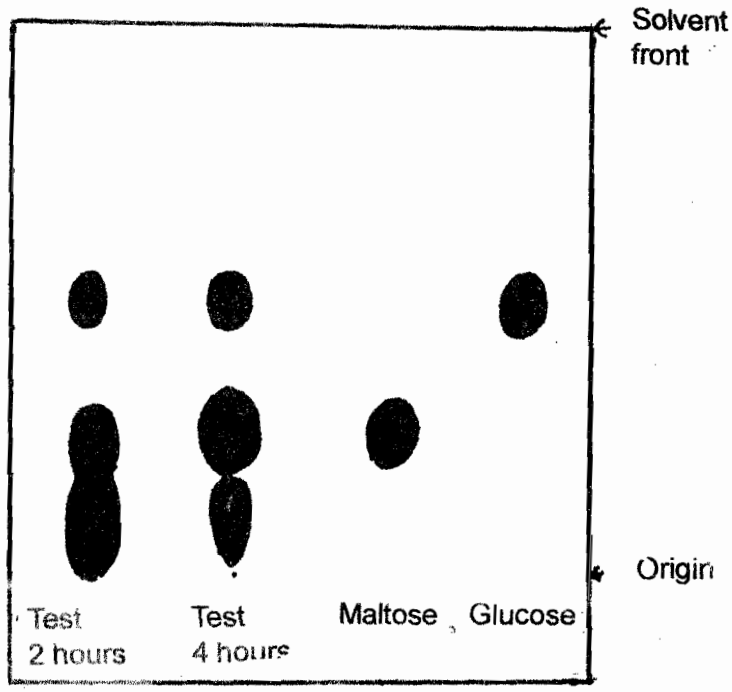

Fig. 7: Paper chromatogram of starch degradation products with P. *tuber-regium* amylase after 2 and 4 hours incubation.

stability increased from pH 2.0 to pH 4.0. The amylase was inactivated at pH 1.0.

Thermal Stability

The amylase was stable at 60°C after 60 minutes of incubation (Figs 4 & 5). Thermal stability of the amylase gradually decreased from 50°C to 30°C. The enzyme was unstable at 70°C and 80°C.

Kinetic Parameter

The km and Vmax values were deduced from the Lineweaver-Burke plot (Fig. 6). The km for the amylase with starch as substrate was 1.25mg/ml and Vmax was 5.0ug/ml/min.

Hydrolytic pattern of the amylase

Chromatography of products of the amylase action on starch, under routine assay conditions showed that glucose, maltose and dextrins were major products (Fig. 7). The enzyme does not hydrolyse maltose.

DISCUSSION

The result of this study showed that the amylase of *P. tuber-regium* was found to be acid-stable at pH 2.0 and thermostable at 60°c. The amylase also maintained a high activity of above 70% of the optical activity between a pH range of 4.0 to 8.0. This compares well with other acid-stable fungal amylases like *Aspergillus niger* (Minoda et. al. 1968); *Thermostable* amylase like *Bacillus macerans* (De Pinto and Campbell, 1980); *Thermonospora curvata* (Glymph and Stutzenberger, 1977), thermostable actinomycete (Obi and Odibo, 1984).

Castro, et. al. (1993) showed that physiochemical factors such as p.H. and/or temperature affect enzyme yield. He demonstrated this in a continuous culture (on soluble starch medium) and showed that maximum production of alpha amylase and alpha-glucosidase occurred at 36% and pH 6.8. Hence such combinations of broad optimum pH of 6.0 to 8.0, stable temperature of 60°c and acid stability of 2.0, encourages high industrial application.

The production of maltose, glucose and dextrin from starch suggests that the amylase has an alpha-amylase type of activity. Also it shows that *P. tuber-regium* amylase is capable of freeing glucose from oligosacharides and polysaccharides, without any action on maltose. This property is highly needed in our starch degrading industries. This can be applied in production of glucose and maltose syrup (Kent, 1981). In flour industries, amylase is extensively used for testing the quality of the flour. This depends on the ability of amylase to liberate maltose from the damaged flour (Barfoed, 1976). Also, during baking, gas production is needed. This depends on the quantity of maltose present. Inadequate gasing (maltose production less than 1.5mg/dl) may be due to lack of alpha-amylase (Kelly and Forgarty, 1980).

The observed effect after 4 hours of incubation is similar to that found in degradation of starch by alpha amylase in feeds (Cone and Viot, 1990) but found to be quite different from that of *Aspergillus niger* which is 48 hours at 30°c (Aurang-Zeb, et. al. 1991).

Amylase of *P. tuber-regium* obeys Michealis equation. Thus velocity increases hyperbolically as substrate concentration increases (Fig. 6). The Michealis Menten's constant (km) for starch (1.25mg/ml) was similar to that reported for *Baccilus-steatothemophilus*, which is 1.0mg/ml (Pfuffer and Elliot, 1969) and *Thermospora vulgaris* is 1.4mg/dl (Kuo and Hartman, 1966).

The results obtained so far have stimulated further investigations on production, purification and characterization of the amylase as possible enzymes for industrial applications.

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