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PRODUCTION AND PURIFICATION OF β - AMYLASE FROM THE MUSHROOM, *Pleurotus ostreatus*.

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Abstract
Production of â-amylase from edible mushroom Pleurotus ostreatus was carried out using agricultural wastes namely, sawdust, rice bran, wheat bran and palm kernel pericarp as substrates. â-amylase isolated from Pleurotus ostreatus was purified by ion exchange chromatography on DEAE Sephadex A 50 and Gel filteration. The effect of variation in pH, temperature and different salts on the activity of the enzyme were investigated. The optimum pH and temperature was recorded to be 5.5 and 55^{0}C respectively. The Michaelis-Menten constant (Km), was 3.57umol/min/ml while the maximum velocity (Vmax), was 0.21 µmole/min/ml. The effect of salts showed Hg Cl $_2$ to be the most potent inhibitor and CaCl$_2$ the least. â-amylase from Pleurotus ostreatus could favorably be substituted for the ones obtained from other sources since they are becoming too expensive for industrial applications owing to the global economic melt-down.

Keywords: Agricultural wastes, *Pleurotus ostreatus*, β -amylase, temperature, pH.

Introduction

Mushrooms are filamentous fungi that produce tubular structure called hyphae at the tips or at specialized region where branches arise. Through branching or fusion of hyphae, a network of these filaments is formed which is called the mycelium. (Kuforiji, 2006). The fruiting body of the mushroom is also a potential source of lignin and phenol degrading enzymes (Fountoulakis *et al.*, 2002). Pleurotus species is third place in worldwide production of edible mushrooms, after *Agaricus bisporus* and *Lentinula edodes* (Chang, 1990). Mycelia growth of *Pleurotus ostreatus* is fast and various lignocellulosic waste products can be used as culture substrate (Yildiz *et al.*,2002).This fungus produces several extracellular enzymes involved in degradation and are also commercially important (Ait-Lahssen *et al.*, 2001). Different types of substrate have been employed for the cultivation of microorganisms to produce host of enzymes. These include sugar cane baggase, wheat bran, rice bran, wheat straw, soybeans and saw dust.

Although commercial production of amylase is carried out using both fungi and bacteria, amylase occurs widely in higher plants (Aunstrup, 1998). This enzyme has found applications in processed food industry, fermentation technology, textile and paper industry. The commercial applications of beta amylase particularly from mushroom deserve an urgent attention as it may be a favoured alternative to other microbial amylases. This work focused on the production and purification of beta amylase from the edible mushroom, *Pleurotus ostreatus*

Materials and Methods

Collection and identification of Pleurotus ostreatus .
Freshly fruiting bodies of mushroom *Pleurotus ostreatus* were harvested from the wild and identiified according to the methods of Zoberi (1972) and Staments (2000).

Production of Pleurotus ostreatus mycelium
The mycelium of *Pleurotus ostreatus* used was obtained by tissue culture from the pileus of a young sporophore by the method of Kuforiji (2006).

Production of crude enzyme.
Crude enzyme extraction was carried out according to the method of Arotupin, (2007). At different time interval the supernatant was taken to determined enzyme activities (Mussarat *et al.,* 2008).*Beta amylase production and assay*

The b-amylase activity was assayed by the methods of Bernfeld (1955). Enzyme solution (0.5 ml) was mixed with 0.5 ml of 1 % (w/v) soluble starch dissolved in 0.02M sodium acetate buffer solution (Batholomew et al., 2000). *Purification of beta-amylase by ion exchange chromatography*

Fifteen milliliter of acid treated crude extract was applied to a column eluted with buffer as described by Adewale and Afolayan, (2004). Fractions of 5ml tube were collected and the protein content determined with UV spectrophotometer at 280nm as for column calibration. The fractions containing â-amylase activity unbound to the gel were pooled and the bound proteins were eluted with a linear salt gradient (0-1M NaCl) in the same buffer. Protein determination was done by method of Lowry *et al.,* (1951).

Gel-filtration on Sephadex G-150
Ten gram of Sephadex G-150 was allowed to swell in 0.02m sodium acetate buffer 4.7 for two days before it was packed into 1.5x 90 cm column. Fractions which exhibited appreciable enzyme activity from ion exchange chromatography on diethylaminoethyl purification were pooled and concentrated. The pooled concentration fractions from a ion exchanger column (2.5x40) chromatography DEAE-Sephadex G-50 was applied to sephadex G-150 column.

Determination of apparent molecular weight of enzyme
The apparent molecular weight of the beta amylase was estimated under non denaturing condition by gel filtration on a column of Sephadex-150(1.5´90cm) using the different protein (Ray, 2000).

Determination of kinetic parameters
Michaelis – Menten Constant (Km), and the maximum velocity (Vmax) was determined for the enzymes (purified) by varying the concentration of soluble starch (0.5mg/ml). Aliquot of 0.5ml of the desired concentration of starch was incubated with 0.5ml of the enzyme (Lineweaver and Jansen, (1951).The values of Km and Vmax are estimated from the double reciprocal plot of 1/v against 1/

Effect of variation in temperature, pH and inhibitory salts on beta -amylase activity.
These were determined according to the method of Mussarat *et al.* (2008).

Results
Figure 1 shows the beta-amylase activity of the culture filtrates of *Pleurotus osreatus* mushroom grown on different agro-industrial substrates. Rice bran had the highest beta-amylase activity of 220 µmole/min/mg at 72hrs, wheat bran 90µmole/min/mg, saw dust had the least 30 µmole/min/mg and the lowest, 10 µmole/min/mg was recorded in palm kernel pericap(Figure 1).

Enzyme purification by ion exchange chromatography
Ion exchange Chromatography on DEAE sephadex A-50 produced multiple protein peaks designated as W X Y and Z with the major protein peak designated as X. The major peak X component of *Pleurotus ostreatus* mushroom elution profile exhibited a single beta-amylase activity peak, while other peaks W Y and Z lack such enzymatic activity (Figure2). Further fractionation of the components of peak X of *Pleurotus ostreatus* mushroom from the DEAE sephadex A-50 on gel filtration yielded four distinct protein peaks of absorption shown as Xa, Xb, Xc and Xd, respectively (Figure 3). Only the components of peak Xc exhibited beta-amylase activity, while those of Xa, Xb and Xd lack such enzymatic activity. The molecular weight of this component from the elution volume was approximately 70,794.58 Daltons.

Time interval (hrs)

Fig. 1. Beta-amylase activity of different substrate by *Pleurotus ostreatus*.

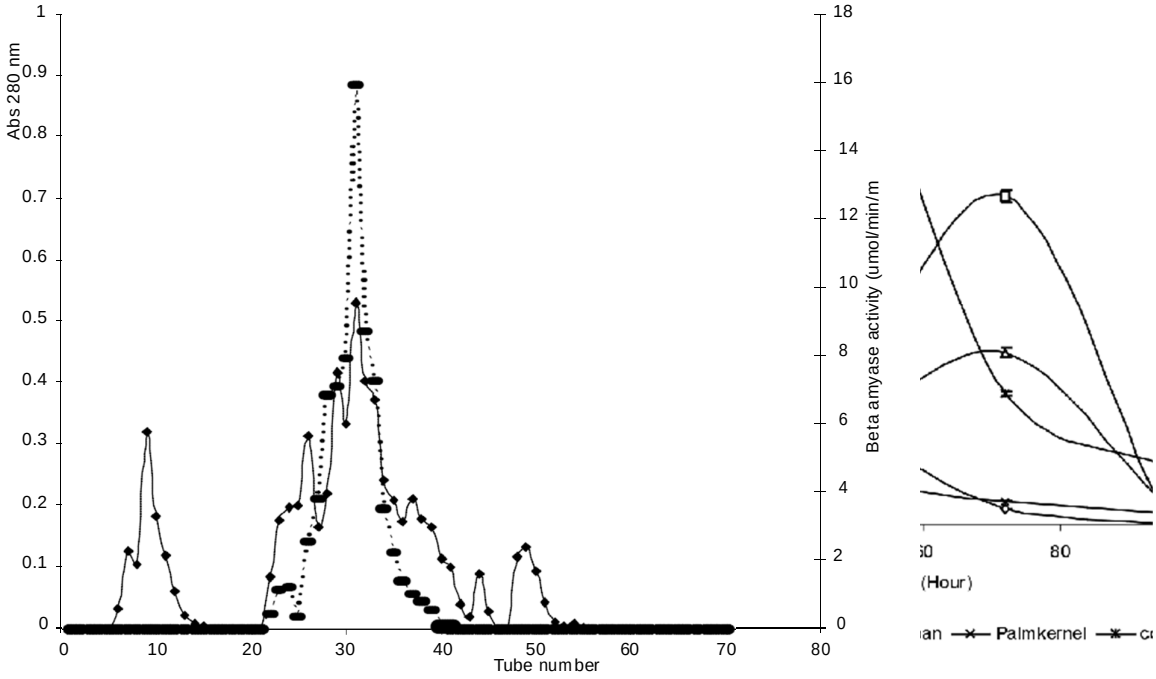

Fig. 2. Elution profile of crude extract of â-amylase on ion exchange DEAE sephadex A-50.

Fig. 3 Elution profile of pooled fraction from ion exchange chromatography on gel filtration sephadex G-150.

Effect of temperature on the activity of β -amylase

The influence of temperature on the enzyme activity is shown in Figure 4. Beta amylase activity increased with increase in temperature up to 55^{0}C after which there was a decline in activity. Exposure to temperature of 60^{0}C resulted in a loss of the maximum activity of the enzyme. Relative activity was expressed in comparison with activity at 50^{0}C which was taken as 100%.

Effect of pH on the activity of β -amylase

The effect of pH on the activity of â-amylase is shown in figure 5. The enzyme was found to be active in pH range of 3-9. Beta-amylase activity increase in pH after which there was a decrease with increase in pH value and the optimum pH recorded at 5.5 which was the pH at which activity was maximal. Relative activity was expressed in comparison with the activity at pH 5.5, which implies activity at pH 5.5 was taken as 100%.

Effect of inhibitory salts on the activity of β -amylase

The effects of inhibitory salts on the activity of beta-amylase are shown in Table1. Inhibitory salts namely $CaCl_2$, $CuSO_4$, NaCl, EDTA, $MgCl_2$ and HgCl had inhibitory effect on activity of the enzyme. HgCl was the most potent at concentration of 40mM while $CaCl_2$ had least inhibitory effect on the enzyme.

Kinetic parameters

The Michealis- menten constant, Km was estimated from the (Line weaver-Bulk) (Figure 6) double reciprocal plot to be 3.57mg/ml and a maximum velocity, Vmax, was found to be 0.21μmole/min/ml.

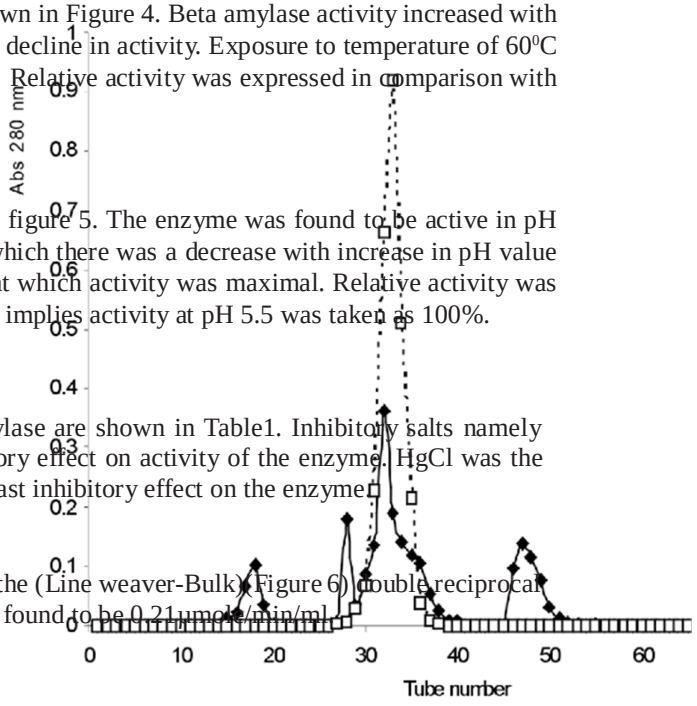

Fig. 4: Effect of temperature on beta-amylase activity from *Pleurotus ostreatus*

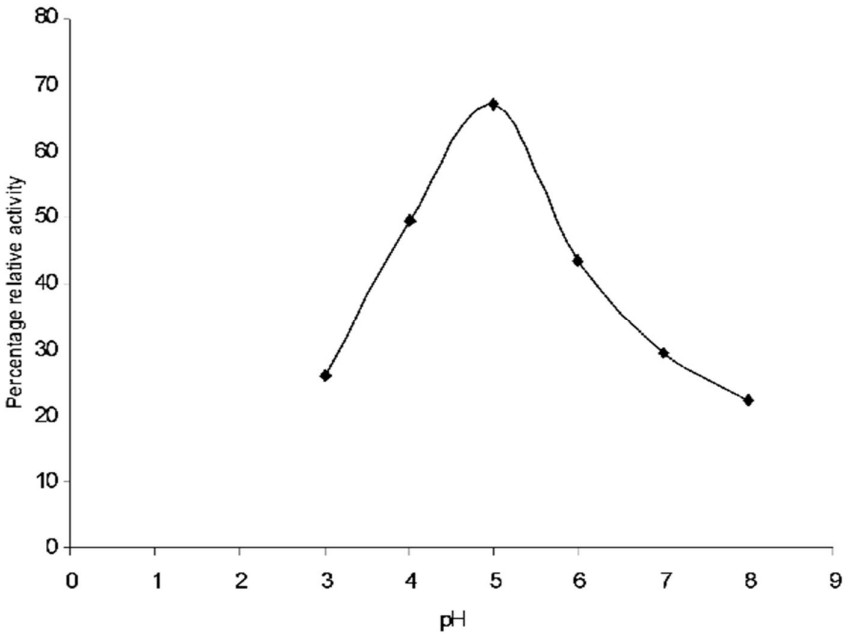

Fig. 5. Effect of pH on beta-amylase activity from *Pleurotus ostreatus*

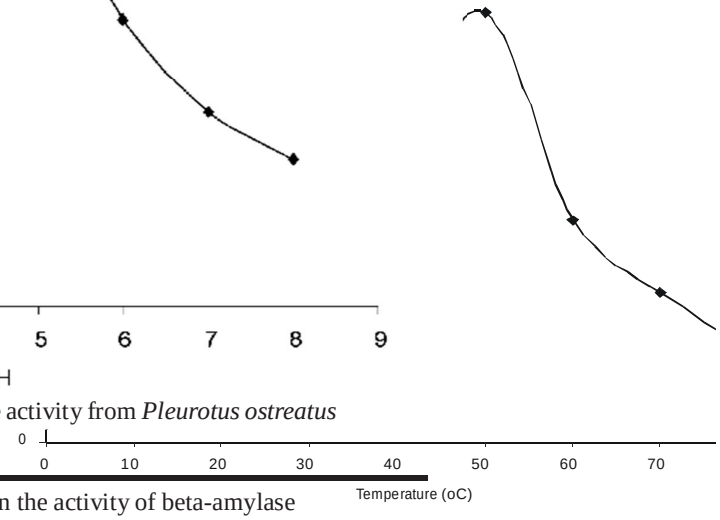

Table 1. Effect of inhibitory salts on the activity of beta-amylase

Salts	% Residual activity
Control	100
$CaCl_2$	93.90
$CuSO_4$	43.90
NaCl	69.51
EDTA	40.24
$MgCl_2$	54.27
HgCl	10.98

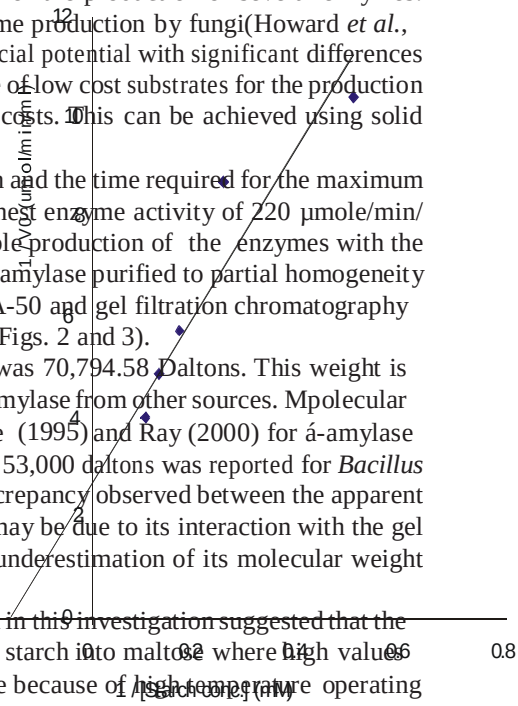

Fig. 6. Lineweaver-burk plot for the purified beta-amylase of *Pleurotus ostreatus*

Discussion

Agro-industrial wastes are available in large amounts have been used for the production of several enzymes. These wastes have been evaluated as substrates for extra cellular enzyme production by fungi(Howard *et al.*, 2003). Filamentous fungi grew well on various raw materials of commercial potential with significant differences in the rate of enzyme production (Mabrouk and Ahwanyi, 2008). The use of low cost substrates for the production of industrial enzymes is one of the ways to greatly reduce production costs. This can be achieved using solid agricultural waste materials as substrates (Wizani *et al*., 1999).

The amount of beta amylase enzyme produced by the mushroom and the time required for the maximum production differs with each substrate (Fig. 1). Rice bran had the highest enzyme activity of 220 µmole/min/mg. All the tested agro-industrial wastes employed enhanced appreciable production of the enzymes with the least of 10 µmole/min/mg recorded in palm kernel pericarp. The beta-amylase purified to partial homogeneity by combination of ion-exchange chromatography on DEAE sephadex A-50 and gel filtration chromatography on sephadex G-150 showed a specific activity of 37.26 µmole/min/ml (Figs. 2 and 3).

The apparent molecular weight obtained for purified enzyme was 70,794.58 Daltons. This weight is within the various molecular weights that have been reported for beta-amylase from other sources. Mpolecular weight of 77,000 and 76,000 daltons were reported by Shili and Labbe (1995) and Ray (2000) for á-amylase from *Clostridium perfringes* and *Bacillus megaterium* respectively, while 53,000 daltons was reported for *Bacillus polymyxa* 26-1 by Sohn *et al.* (1996). It has been suggested that the discrepancy observed between the apparent molecular weight determined by gel filtration for the purified enzyme may be due to its interaction with the gel which usually result in the retardation of its mobility and subsequent underestimation of its molecular weight (Buonocore *et al.*, 1976)

The optimum temperature for â-amylase activity of 55°C reported in this investigation suggested that the enzyme can be employed in the food and beverage industries to convert starch into maltose where high values has been placed on the thermo stable and thermo activity of the enzyme because of high temperature operating condition, Bartholomew *et al.* (2000). An optimum temperature of 50°C was reported by Martinez *et al.*, (2002) whereas, the pea epicocyl beta-amylase was unstable above 40°C (Lizotte *et al.*,1990). Hyun and Zeikus (1985) reported that most of the beta-amylase earlier show optimum activity at low temperature are not thermo stable. Microbial beta- such as *Bacillus pollymixa* 26-1â-amylase has an optimum of 45°C while â-amylase isolated from *Bacillus spp* was also reported to have an optimum temperature of 60°C (Ray, 2000). Inactivation

of the enzyme due to high temperature has been reportedly associated with a two-step process.(Martinez *et al.*, 2002). The reversible thermal unfolding of an enzyme as a result of increase in vibration and rotational motion of reacting molecules, which may also lead to dissociation in case of multi-subunit enzyme (Khanderparkar and Bhosle, 2008).

A pH range of 3- 8 was recorded for beta-amylase from *Pleurotus ostreatus* and the optimum was 5.5. This was similar to pH of beta –amylase obtained from other sources . Obineme *et al*. (2003) obtained a pH range of 5.5-6.5 for the rice â-amylase from *Aspergillus oryzae,* while Ajele (1997) obtained an optimum pH of 4.5 for soybeans â-amylase. A pH 5.5 was reported by Martinez *et al.* (2000) and pH range of 6-9 by Bartholomew *et al.* (2000) for raw potatoes. The fact that the â-amylase is active over a wide pH range may imply that the enzyme will be useful in industrial processes that are subjected to wide pH changes which may be from the acidic to alkaline and vice-versa. Enzyme activity increased with temperature but decreased after the optimum while that of pH decreased with increase or decrease in value. This decline may be due to thermal inactivation resulting from denaturation of the enzyme protein or dissociation of essential cofactors, Kim (1996) and Sohn *et al.*(1996).

The addition of salts at 0.04M was inhibitory to the activity of beta-amylase produced by *Pleurotus ostreatus.* Kim (1996) and Martinez *et al.* (2002) have reported the inhibition of microbial enzymes by mercury and copper ions. Obineme *et al.* (2003) suggested that EDTA acted as inhibitor by chelating calcium ions with a resultant loss of catalytic activity of the enzyme. Also, the inactivation of the enzyme activity by these salts could be as a result of alteration in the pH of the reaction mixture and possible disruption of the enzyme structure. Akinyosoye *et al.* (1995) reported that, the inhibition of enzyme activity may due to changes in the pH either to higher or lower values.

The Michealis-Menten constant, Km, value obtained in this report was 3.57mg/ml and fall within the range reported by Khandeparkar and Bhosle (2008). The low Km value obtained compared with other organisms suggests that this purified enzyme has high affinity towards agro-industrial substrates utilized and could possibly be used for industrial saccharification. Line weaver and Jansen (1951) reported that a high Km value indicates weak binding and vice-versa. From these findings, P*leurotus ostreatus* contains â-amylase found to be thermo stable and thermo active. The enzyme could therefore, withstand some of the harsh conditions encountered during industrial operations. The production process of the enzyme from the mushroom is faster and less expensive than those obtained from chemical sources, making it a more favourable method in large scale processes.

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