Extracellular Amylase Production of a Thermotolerant *Fusarium* sp. Isolated from Eastern Nigerian soil

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ABSTRACT

In this work, an α- amylase producing Fusarium sp. *was isolated from the soil at 50 ^0C. Growth and enzyme production occurred at 30, 45 and 55 ^oC. Soybean meal at 1% concentration, supplemented with 0.2% NH_4Cl and 2.5% corn starch elicited the highest amylase yield. Optimum pH for the enzyme was pH 6.5 which retained over 60% of its activity after 24 h incubation at the pH range of 4.5-7.0. The enzyme showed high activity from 40-70 ^0C with optimal activity at 50 ^oC and 78% activity was retained after incubation at 70 ^oC for 30min. Catalytic function of the crude amylase was stimulated by Mg (136%), Ca (118%) and Zn (118%) at 2mM concentrations. The enzyme hydrolyzed cassava, potato and yam starches effectively.*

Key words: hydrolysis, starch, fungal growth, amylase synthesis

INTRODUCTION

Starch hydrolyzing enzymes, such as amylases are amongst the most important industrial enzymes and account for over 25% of industrial enzymes (Reddy et al., 2003, Cordeiro et al., 2002). Amylases are used in numerous biotechnological processes, including biofuel, textile, medical, chemical and analytical fields, paper and detergent production (Fossi et al., 2005). Other applications include use in the sewage treatment to reduce disposable solid content of the sludge and for pretreatment of animal feed to improve the digestibility (Kokab et al., 2003; Regulapati et al., 2007, Saxena et al., 2007). Starch is composed of two high molecular weight components, including amylose (15-25%), a linear polymer made up of α-1,4-linked glucopyranose residues and amylopectin (75-85%), a branched polymer made up of α-1,4 and α-1,6 linked glucopyranose residues (Dock et al., 2008). Starch degrading enzymes include endo-amylases (α-amylase), exo-amylases (β-amylase), debranching enzymes and glycosyltransferases (Cereia et al., 2006, Khajeh et al., 2006). Depending on the type of amylase, starch is degraded to simple sugars such as glucose, maltose or to oligosaccharides, maltooligosaccharides or dextrins (Abou-Elela et al., 2009).

Microbial enzymes are preferred to plant enzymes due to their short growth period, higher productivity and thermostability (Mishra and Behera, 2008). Microbial growth and amylase production is dependent on growth conditions, such as type and concentration of carbon and nitrogen substrate, metal ion requirement, pH and temperature of growth (Cherry et al., 2004; Ghasemi et al., 2010). Though many microorganisms can grow on a wide range of

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carbon and nitrogen sources, it is economically more viable to utilize the cheap and easily available resources as substrates for amylase production (Pandey et al., 2000). Brans, straws and flours of different grains and tubers, such as barley, corn, cassava, potato, rice, sorghum and wheat, have been used as carbon sources in the fermentation medium, while protein sources used include soybean meal, yeast extract, peptone, and meat extract (Oliveira et al., 2007). Industrial enzymatic hydrolysis is influenced by a number of factors amongst which are environmental conditions of pH, temperature and presence of metal ion (Riaz et al., 2007).

Amylases have been reported to be produced by a number of fungi, including Aspergillus, Rhizopus, Fusarium, Candida, Penicillium, Thermomucor, basidiomycete Fomitopsis and Thermomyces (Balkan et al., 2005, Kumar et al., 2007, Kunamneni et al., 2004, Mohamed et al., 2007, Yoon et al., 2006). Majority of the studies on fungal amylases are based on mesophiles, rarely on facultative thermophiles (Maheswari et al., 2000). Current researches focus on thermotolerant enzymes from thermophilic microbial strains. Bhatti et al. (2007) and Figueira and Hirooka (2000) have reported amylases from mesophilic *Fusarium* species. This work reports the isolation of a facultative thermophilic *Fusarium* sp. for the production of alpha amylase.

MATERIALS AND METHODS

Isolation of microorganism
Soil sample collected from a honey processing area in Eastern Nigeria was cultivated in potato dextrose agar supplemented with 0.5 % (w/v) starch and incubated at 50 ^{o}C. Amylolytic isolates were selected by flooding the agar plates with Gram's iodine solution. The isolates which had a higher ratio of clearing zone to colony size were grown in liquid culture and the level of amylase production was determined from cell free culture supernatant. The characterization and identification of the isolate was made following the methods of Raper and Fennel (1965).

Nitrogen sources
Soybean meal and cowpea meal were freshly prepared. They were soaked overnight and 1 h, respectively. This was followed by proper washing to remove their test coat. The seeds were promptly

dried in an air drier, ground to flour and stored in airtight containers.

Starch
Cassava (*Manihot utillisima*), cocoyam (*Xanthosoma saggitifolium*), yam (*Dioscorea rotundata*), corn (*Zea mays*), rice (*Oryzae sativa*) and sorghum (*Sorghum sativa*) starches were prepared according to standard procedures (Corbishley and Miller, 1984; Watson, 1984). These starches were completely transformed to non-reducing forms by reacting with $NaBH_4$. Other materials used were of analytical grade.

Production of crude enzyme
The inocula were prepared by growing the fungus on potato dextrose agar (PDA). Fermentation medium contained (gL^{-1}): $(NH_4)_2SO_4$, 1.4; $KHPO_4$, 2.0; $CaCl_2$, 0.3; $MgSO_4.7H_2O$, 0.3; Yeast extract, 10.0; starch, 20.0; Tween-80, 2.0; Mineral solution, 1mL in deionized water. The mineral solution contained (gL^{-1}): $FeSO_4.7H_2O$, 5.0; $MnSO_4$, 1.6; $ZnSO_4.7H_2O$, 1.4; $CoCl_2$, 2.0 (Takao et al., 1986).

Effect of the cultivation time and temperature on microbial growth and amylase production
To prepare the inoculum, agar plug of profuse growth of isolate was inoculated into 100 mL Erlenmeyer flask containing 20 mL of the fermentation medium and incubated for 24 h. A suspension containing 10^5 of fungus spores in 1 mL solution served as inoculum. The cultures were incubated at 30, 45 and 55 ^{o}C in a Gallenkamp orbital incubator at 120 rpm for five days. The crude enzyme was extracted from the growth media by filtration through Whatman No 1 filter paper. The extract was centrifuged at 10,000 rpm (25,900×g) for 15 min at 4 ^{o}C to remove the suspended particles. Samples were collected daily for the determination of growth rate, extracellular protein accumulation, reducing sugars and enzyme activity. Absorbance of reaction solutions was read using a Spectronic 20 UV Spectrophotometer. The mycelium was separated from the culture medium by filtration and the filtrate was used to determine the enzymatic activity. Dry weight was determined by drying the mycelium at 105 °C for 24h.

Enzyme assay
The amylase activity was assayed using a reaction mixture containing 0.5 mL of 1% (w/v) soluble starch as the substrate in 0.2 M citrate-phosphate

buffer at pH 6 and incubated for 10 min at 40 ^{o}C. Reducing sugar liberated was estimated using the method described by Sandhu et al. (1987). One unit of amylase (U) was defined as the amount of enzymatic extract that liberated 1μmole of reducing sugar per minute under the assay conditions.

Effect of organic nitrogen source on amylase production

The effect of 1% yeast extract, tryptone, cow blood meal, lablemco powder, casamino acid, soybean meal and cowpea meal on amylase production by the *Fusarium* sp. was studied.

Effect supplementing organic nitrogen source with inorganic nitrogen

The effect of the addition of 0.2% KNO_3, NH_4Cl, $(NH_4)_2SO_4$ and $NaNO_3$ each to the growth medium containing soybean meal (1%) was evaluated for amylase production.

Effect of carbon sources on amylase production

Cassava, sorghum and potato starch each at 1% individually was added to the production medium to determine the effect on amylase production. Furthermore, to obtain detailed information about the synthesis of amylolytic enzyme by this organism, the effects of other carbon sources, xylose, pectin, mannose, cellulose, glucose, maltose and lactose were also evaluated.

Effect of metal salt in enzyme production

The effects of the addition of 0.005M of each of the following salts were studied: $FeSO_4.7H_2O$, $CoSO_4$, $ZnSO_4.7H_2O$ and $MnSO_4.H_2O$. Results were compared for amylase production when 1 mL of mineral solution containing (gL^{-1}): $FeSO_4.7H_2O$, 5.0; $MnSO_4$, 1.6; $ZnSO_4.7H_2O$, 1.4; $CoCl_2$, 2.0 (Takao et al., 1986) was used. A basal medium containing (gL^{-1}): NH_4Cl, 0.4; KH_2PO_4, 2.0; $CaCl_2$, 0.3; $MgSO_4.7H_2O$, 0.3, soybean meal, 10.0; Tween 80, 2.0 and corn starch 20.0 was used.

Characterization of crude enzyme

The effect of temperature on the crude amylase activity and stability was assayed at 30 - 90 ^{o}C. The reaction mixture containing 0.5 mL of crude enzyme, 0.5 mL of 1% (w/v) soluble starch solution in 5 mL of 0.2 M phosphate buffer (pH 6) was incubated for 10 min at the test temperatures. Thereafter, the reaction mixture was promptly

cooled on ice before the enzyme activity was assayed as described earlier. To determine the enzyme thermostability, the crude enzyme was pre-incubated at various temperatures from 30 to 70 ^{o}C for 30 min and then promptly chilled on ice, after which the residual activity was determined under normal condition as described earlier. The effect of pH on the crude amylase over the range pH 2.5 to 9.0 was also studied. The buffers were prepared as described by McIlvaine for citric acid-Na_2HPO_4 buffer solution, ranging from pH 2.5-7.5 and phosphate buffer, ranging from pH 8.0-9.0. The pH activity profile was determined by incubating 0.5mL of the enzyme solution with 0.5mL of 1% (w/v) corn starch in buffer at 40 ^{o}C for 10 min. Reducing sugar production was assayed as earlier described. The pH stability profile was determined by suspending the diluted crude enzyme in appropriate buffer of pH 2.5-9.0 and pre-incubated for 24 h at room temperature. After incubation, the residual enzyme activity was assayed as described earlier.

Effect of metal ions on enzyme activity

The effect of various divalent cations (Ca^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, Hg^{2+}, Bi^{2+} and Cu^{2+}) on the crude amylase was evaluated. The amylase enzyme was pre-incubated with 2mM of each metal ion in citrate-phosphate buffer (pH 6) at 40 ^{o}C for 10 min and then assayed for the residual amylase activity.

Enzyme hydrolysis of various starches

The ability of the crude amylase to hydrolyze different native starches was studied using cassava, corn, sorghum, yam and potato starches. Commercially available soluble starch was used as standard. The assay mixture consisted of 10 gL^{-1} of various starch in 0.2 M citrate-phosphate buffer (pH 6) and 0.5 mL of the enzyme. Enzyme assay was carried out after incubation at 40 ^{o}C for 10 min.

Tests were conducted in triplicates, and the standard deviation was calculated using Microsoft excel and mean values used.

RESULTS

### Time course of growth and enzyme production by *Fusarium* sp. at different temperatures

The optimal growth for the *Fusarium* sp. was recorded at 30 ^{o}C as shown in Figure 1. Microbial

growth was slightly less at 55 °C compared to growth at 45 °C. Growth peaked after 72 h cultivation, followed by a gradual decline as shown in Figure 1.

Figure 2 showed that amylase production was highest at 30 °C, followed by 45 °C and 55 °C. While highest amylase production at 30 °C

occurred after cultivation for 120 h, highest production at 45 °C and 55 °C was observed after 72 h.

It was also evident from Figure 2 that amylase production by *Fusarium* sp. at 30, 45 and 55 °C was optimal during the stationary phase of growth.
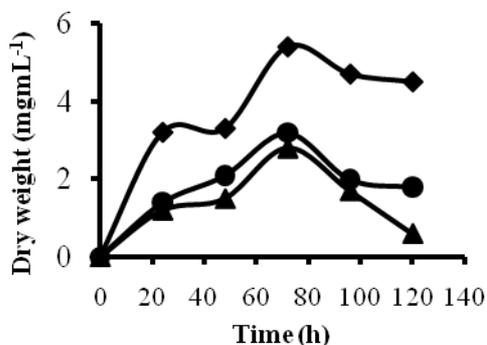
Figure 1 - Effect of temperature on the growth of *Fusarium sp*. Symbols: diamond (30°C), circle (45°C), triangle (30°C).
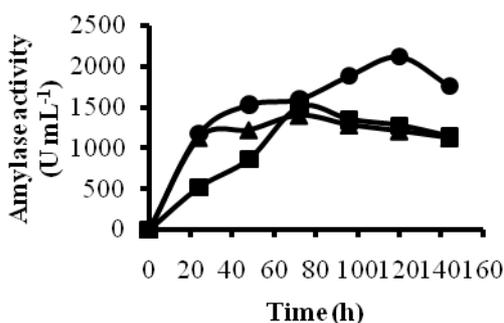
Figure 2 - Effect of time of cultivation on amylase production of *Fusarium* sp at different temperatures. Symbols: circle (30°C), square (45°C), triangle (30°C).

Effect of organic nitrogen source, carbon source and mineral salts on amylase production of *Fusarium* sp.

Different nitrogen sources, including soybean meal, casamino acid, yeast extract, tryptone, cowblood meal, lablemco powder and cowpea (1%

each) were used as organic nitrogen source for amylase production. Soybean meal elicited the highest production of amylase (1808 UmL^{-1}) and yeast extract (1554 UmL^{-1}) as shown in Table 1. Cowpea meal did not favour amylase production and gave the lowest yield.

Table 1 - Effect of different sources of organic nitrogen on amylase production

Nitrogen source (1%)	Amylase activity (UmL^{-1}) (mean±SD)
Soybean meal	1808 ± 3
Casamino Acid	1752 ± 4
Yeast extract	1554 ± 1
Tryptone	1588 ± 2
Cowblood meal	1236 ± 1
Lablemco powder	1232 ± 7
Cowpea meal	321 ± 5

*SD= standard deviation

Supplementing soybean meal with 0.2% concentration of various inorganic nitrogen salt had varying effects on amylase production. Supplementation of soybean meal with KNO_3 (1880 UmL^{-1}) and NH_4Cl (2097 UmL^{-1}) stimulated amylase production (Table 2). However, $NaNO_3$ had an inhibitory effect observed by the reduction in extracellular amylase production in the culture fluid from a concentration of 1808 UmL^{-1} to 1701 UmL^{-1}.

Table 2 - Supplementation of soybean meal with various sources of inorganic nitrogen.

Nitrogen source (UmL^{-1})	Amylase activity (0.2%) (mean $\pm$ SD)
KNO_3	1880 $\pm$ 6
NH_4Cl	2097 $\pm$ 3
$(NH_4)_2SO_4$	2001 $\pm$ 2
$NaNO_3$	1701 $\pm$ 1

*Soybean meal (1%) was used as the organic nitrogen source in all cases.
*SD= standard deviation

Corn starch gave an amylase yield of 2227 UmL^{-1}, followed by cassava starch, 2195 UmL^{-1} and sorghum starch, 2013 UmL^{-1} (Table 3). Though amylase was produced using mannose (856 UmL^{-1}), pectin (1298 UmL^{-1}), xylose (1343 UmL^{-1}) and lactose (634 UmL^{-1}) as carbon sources, these figures were low compared to amount produced using native undefined starches as carbon source. Amylase production by the *Fusarium* sp. increased with increase in corn starch to a concentration of 2.5% after which extracellular production gradually declined (Figure 3). There was an increase in amylase synthesis in the culture medium of the fungus following an increase in the concentration of soybean meal from 0.5% - 1.5%, after which there was a rapid decline in enzyme synthesis (Figure 3).

Table 3 - Effect of different carbon sources on amylase production of the *Fusarium* sp.

Carbon sources (1%)	Amylase Activity (UmL^{-1}) (mean $\pm$ SD)
Cassava starch	2195 $\pm$ 1
Corn starch	2227 $\pm$ 2
Sorghum starch	2013 $\pm$ 6
Potato starch	1976 $\pm$ 4
Soluble starch	1806 $\pm$ 3
Cellulose	1405 $\pm$ 6
Xylose	1343 $\pm$ 4
Pectin	1298 $\pm$ 8
Glucose	910 $\pm$ 7
Maltose	1555 $\pm$ 2
Mannose	856 $\pm$ 3
Lactose	634 $\pm$ 5

*SD = standard deviation
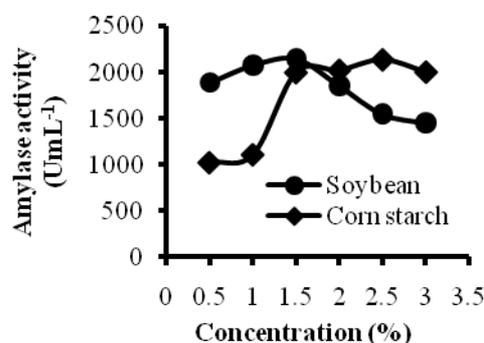
Figure 3 - Effect of concentration of sorghum meal and corn starch on the amylase production by *Fusarium* sp. when grown at 30 °C for 72 h.

A remarkable increase occurred in amylase secretion by the culture as a result of presence of metal ion; amylase yield increased from 1075 UmL^{-1} in absence of metal salt to 2143 UmL^{-1} following the addition of the mineral salt solution (Figure 4).

**Influence of pH, temperature and metal salt on enzyme activity and stability**

Optimum pH for amylase produced by *Fusarium* sp. was 6.5, with 1% starch as substrate (Figure 5)

though there was marginal difference in amylase activity at pH 6.5- 8.5. The amylase was relatively stable and retained over 60% of its activity after 24 h incubation at pH 4.5 to 7.0.

Figure 6 indicated that the *Fusarium* sp. amylase showed a high activity level with very little difference in exact values at a broad temperature range (40 °C – 70 °C) with optimal activity at 50 °C. The crude amylase extract was temperature stable and retained over 78% of its activity at 70 °C after 30 min incubation.
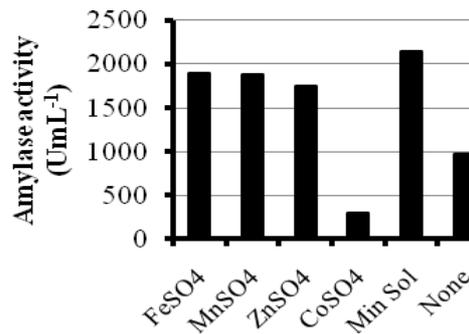
Figure 4 - Effect of mineral salts on amylase synthesis by the *Fusarium* sp. when grown at 30 °C. for 72 h. Concentration of metal salt (0.005M) each, mineral solution contained all four in 1mL solution.*Min Soln= Mineral solution
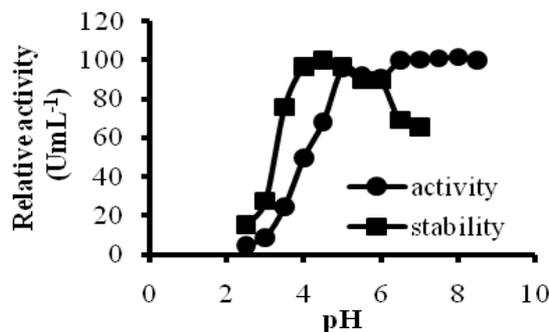
Figure 5 - Effect of different pH on the activity and stability of the amylase
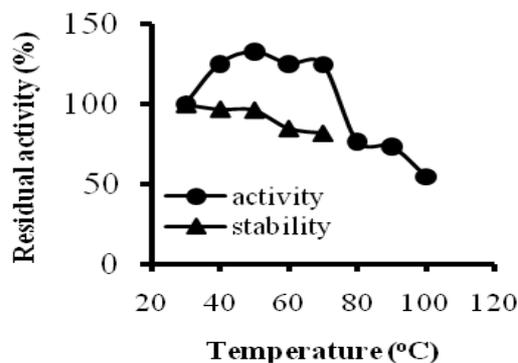
Figure 6 - Effect of different temperatures on the activity and stability of the amylase

Catalytic function of the crude amylase was stimulated by Mg^{2+} (136%), Ca^{2+} (118%), Zn^{2+} (118%) and to a lesser extent Cu^{2+} (109%) and Co^{2+} (107%) at 2 mM concentrations (Table 4). Mn^{2+} (80%) and Fe^{2+} (89%) slightly inhibited the amylase activity.

Relative rate of hydrolysis of different starches by the amylase

The rate of the amylase hydrolysis of different native starches was evaluated. Figure 7 showed that the amylase from the *Fusarium* sp. was able to hydrolyze different starches to varying degrees. Highest rate of hydrolysis was observed for cassava (76%), followed by potato starch (65%). Lower values of 53% and 44% were observed for yam starch and corn starch, respectively.

Table 4 - Effect of different metal ions on amylase activity.

Metal salt concentration (2 mM)	% Relative activity (mean $\pm$ SD)
$BiSO_4$	104 ± 4
$MgSO_4 \cdot 6H_2O$	136 ± 6
$ZnSO_4 \cdot 7H_2O$	118 ± 1
$MnSO_4 \cdot H_2O$	80 ± 3
$HgCl_2$	100 ± 2
$CuSO_4$	109 ± 2
$CaCl_2 \cdot 2H_2O$	118 ± 4
$CoCl_2 \cdot 6H_2O$	107 ± 1
$FeSO_4 \cdot 7H_2O$	89 ± 3
None	100 ± 5

*SD = standard deviation
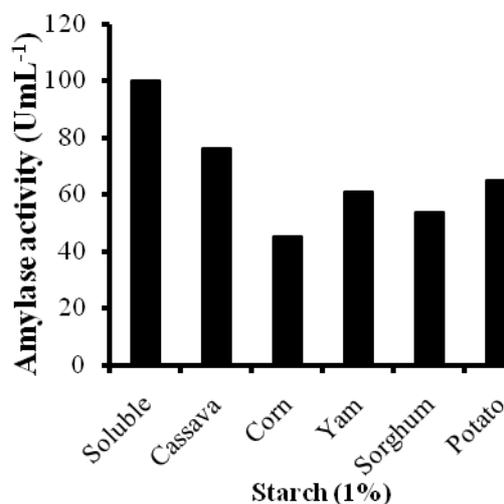
Figure 7 - Substrate specificity of crude amylase extract from *Fusarium* sp. Value for soluble starch was taken as 100% hydrolysis.

DISCUSSION

Detailed production studies for media optimization though not often reported are necessary steps in the development of enzyme biotechnology (Quang et al., 2000, Kathiresan and Manivannan, 2006).

Factors such as composition of culture medium, cultivation conditions and properties of the microbial strain are known to exert control on enzyme regulation during microbial fermentation. Existing reports indicate that changes in carbon source and carbon nitrogen ratio used during

enzyme production effect variations in multiple forms and molecular species of amylases (Abu et al., 2005).

Optimal temperature is known to influence the microbial growth, stability and enzyme production. Though the organism grew well and produced α-amylase at 30, 45 and 55 ^{o}C, optimal growth and enzyme production was at 30 ^{o}C. According to Maheswari et al. (2000), most fungi are unable to survive prolonged exposure to temperatures above 45 ^{o}C. The organism is probably a facultative thermophile, able to grow at mesophilic and thermophilic temperature ranges. Combining inorganic (soybean meal) and organic nitrogen (NH_4Cl) sources led to increased α-amylase production sp. The presence of readily available substrates has been noted to influence the biosynthesis of many extracellular enzymes via catabolite repression mechanism (Teodoro and Martins, 2000). The amylase production by *Fusarium* sp. was repressed by use of glucose as carbon source which was in agreement with Ramachandran et al. (2004). Though amylase production was slightly stimulated by maltose, it was remarkably induced by the native starches. Some reports indicate that maltose is an amylase inducer, though it does not elicit a high amylase yield (Moreira et al., 2001). This is probably the result of the easy breakdown of maltose to glucose which in turn represses amylase production (Ray et al., 1996). According to Nguyen et al. (2000), starch and its hydrolytic products induce amylase production. This correlated with the present findings as native undefined starch sources significantly stimulated enzyme production by the organism. Undefined starches are easily available and cheaper sources of starches in comparison to the more expensive industrially prepared starches. The present results showed that enzyme production was marginally pronounced when a combination of different metal salts was used which corresponded with the work of Morita et al. (1999). The presence of organic nitrogen sources elicited a higher extracellular α-amylase by the *Fusarium* sp, which was also reported by Hernandez et al. (2006). Aiyer (2004) reported that a *Rhizopus* sp could not grow or produce amylase in submerged culture without the presence of metal ions. Though growth was optimal after 72 h of incubation, amylase production reached its peak after 120 h signifying that α-amylase production was predominant during the stationary phase. This was contrary to the report of *Rhizopus*

microsporus var. rhizopodiformis amylase produced optimally during cell autolysis (Peixoto et al., 2003) α-amylase production increased with increase in starch concentration from 0.5-2.5% which was in agreement with the work of Ramanchandran (2004). Optimal amylase activity was recorded at pH 6.5-8.5, though enzyme was active at lower pH. This was a rare occurrence, most fungal amylase have been reported to be optimally active at the pH range of 4.5 - 6.0 (Okolo et al., 2001). The result does not correspond with the optimal pH of 3.0 - 5.0 reported for the glucoamylase from *Fusarium solani* (Bhatti et al., 2007). The amylase was relatively stable at the pH range of pH 4.5 to pH 7.0 maintaining over 66 % of its activity. Activity studies showed that the amylase was optimally active at 50 ^{o}C which was in variation with the glucoamylase from *F. solani* optimally active at 40°C (Bhatti et al, 2007). Chary and Reddy (1985) earlier reported the production of amylase from *F. oxysporum* and *F. scirpi* with optimal pH at 6.9; their optimal temperature of 30 and 40 ^{o}C, respectively did not correspond with that of the amylase produced by *Fusarium* sp in the present study. The *Fusarium* sp. was highly thermostable; 80% of initial enzyme activity retained at 70 ^{o}C after 30 min. incubation in the absence of substrate. This indicated that the *Fusarium* sp. amylase was relatively thermostable and was able to withstand the thermal inactivation. Obviously, the amylase produced by the *Fusarium* sp. was unique and did not correspond with the properties of the species earlier reported. Since the industrial application of amylases require high reaction temperatures for optimal efficiency with minimal contamination, thermostable amylases are now of utmost importance in biotechnological processes. The hydrolytic activity of the crude enzyme was highly stimulated by Mg^{2+}, Ca^{2+} and Zn^{2+} ions. Amylases are metalloenzymes which possess Ca^{2+} ions binding site for their activity and stability. However, enzymes are generally inactivated by heavy metals, with metal ions such as Hg^{2+} noted to cause irreversible inhibition of enzyme by binding strongly to their amino acid backbone (Chaplin and Bucke, 1990). The present results showed that amylase was stable in the presence of trace amounts of heavy metals. This could be an added advantage, as the sensitivity of amylase to heavy metal ions poses problems in some industrial processes due to the metal composition of reactors or presence of metal.

CONCLUSION

From the results it could be concluded that the amylase production by the *Fusarium* sp. was greatly influenced by the carbon, nitrogen and mineral sources used. Large amounts of amylase could be produced by the *Fusarium* sp. on cheap and easily accessible substrates. Moreover, the amylase was temperature stable while retaining the activity in the presence of low concentrations of heavy metal ions. Further studies and optimization could prove the amylase to be very useful in industrial processes requiring amylase or its hydrolytic products.

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