Hydrolytic activity of amylase produced in solid - state fermentation by a local isolate of *Aspergillus niger*

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Abstract- The increase in demand for local enzymes for industrial activities has stimulated the exploration of the extracellular enzymatic activities of several microorganisms. Using validated values from a central composite designed experiment, amylase from a locally isolated strain of *Aspergillus niger* was produced in a larger quantity by solid-state fermentation for characterization and its subsequent application in the hydrolysis of raw native starches. The optimum activity of the enzyme was 30.96 U/ml-min. Partial purification using ammonium sulphate saturation was attained at 50% $(NH_4)_2SO_4$. The enzyme was stable over a wide range of pH (4 - 8) and temperature (30^oC – 60^oC). Other optimum values determined were; Ca^{2+} concentration – 5 mM, starch (substrate) concentration – 3%, enzyme - substrate reaction time – 6 min and enzyme dosage – 4 mg total protein / 3% starch solution. The enzyme was able to hydrolyse the raw starchy substrates studied, producing glucose concentrations of 31.90 mg/ml, 24.78 mg/ml, 19.31 mg/ml and 8.85 mg/ml from maize, sweet potato, yam, and cassava respectively. The optimal substrate concentration of these substrates investigated also showed that different starchy substrates behaved differently towards hydrolysis by amylase. The study has shown the possible production of a local enzyme that was capable of hydrolyzing raw native starches with good activity. This result indicates the possibility of less dependence on imported enzymes.

Index Terms- Amylase, *Aspergillus niger*, Hydrolysis, Native starch, Solid-state fermentation

I. INTRODUCTION

The application of biotechnology to produce industrially competent enzymes has stimulated the quest for the extracellular enzymatic activities of several microorganisms (Saranraj and Stella, 2013). Amylase is a very common enzyme found in most starch processing industries due to its hydrolytic action on starches to produce simple sugars (Reddy *et al.*, 2003). The potential technological importance and economic profits of amylases have made it receive a great deal of attention in developing countries (Suganthi *et al.*, 2011). Amylase sourced from molds, bacteria, and yeasts have been reported and their biochemical characteristics documented (Buzzini and Martini, 2002; Liaquat *et al.*, 2015; Suganthi *et al.*, 2011). Molds are identified to produce high amounts of amylase (Suganthi *et al.*, 2011) and amongst these, *Aspergillus niger* is seen as the most important source of industrial enzymes (Sakthi *et al.*, 2012). Commercially, fungal amylases have been reported to be more stable than bacterial amylases (Suganthi *et al.*, 2011); this has therefore called for several researchers to optimize the culture conditions of suitable fungal strains (Abu *et al.*, 2005).

Solid - state fermentation (SSF) holds unlimited potential for the production of enzymes (Pandey *et al.*, 1999), particularly when fungi are involved (Bhargav *et al.*, 2008). SSF present benefits such as; simplified operations, high volumetric production, and the requirement for low initial capital (Omemu *et al.*, 2005; Pandey *et al.*, 1999).

Starchy tubers (such as yam, cassava, sweet potato, and cocoyam) and cereals occur abundantly in most developing countries in the tropics, with the former being the most cultivated; since these remain the important staple foods in the diet of most people living in these developing countries (Okolo *et al.*, 1995). Annual losses of large proportions of these tubers to spoilage agents are encountered due to inadequate storage facilities and improper post-harvest practices (Okolo *et al.*, 1995; Omemu *et al.*, 2005; Owusu - Darko *et al.*, 2016). These tubers, rich sources of starch, could be put to an alternative use; enzymatic conversion into reducing sugars by saccharification (Omemu *et al.*, 2005). Enzymatic hydrolysis has been shown to be economically superior in terms of process simplicity and energy utilization as compared to the conventional method that uses pregelatinized starch as substrates (Okolo *et al.*, 1995; Omemu *et al.*, 2005). Resistance to amylase enzymatic action has been observed in starches from tubers as compared to the susceptibility in cereal starches (Omemu *et al.*, 2005).

This paper reports on some properties of amylase produced by a locally isolated strain of *Aspergillus niger* as well as its hydrolytic characteristics on some raw native starches.

II. MATERIALS AND METHODS

Preparation of starches from tubers

Cassava, sweet potato and yam starches were prepared according to the procedure outlined by (Rasper, 1969). Maize grains were steeped in water for 3 days with intermittent changes of the water. It was milled using a double disc attrition milling machine and the resulting dough was mixed with adequate water. The suspension was filtered through a 250 µm sized mesh gauze. The filtrate was allowed to stand for 3h and the water was decanted leaving the settled starch slurry at the bottom. The starch was carefully dried in circulating air temperatures below 45^{o}C. Corn-derived soluble starch (Sigma – Aldrich) was used as the standard. All other chemicals were of analytical grade and obtained from Sigma – Aldrich St. Louis, MO, USA.

Source of fungal culture

Five (5) fungal colonies were isolated from a serial dilution of a mouldy bread sample and they were identified as *Aspergillus niger* by employing the lactophenol cotton blue staining technique and studying morphology using standard identification manuals (Barnett and Hunter, 1972). These fungal colonies were screened for their ability to synthesize amylase on potato dextrose agar (PDA) amended with 2% (W/V) soluble starch medium (Uguru *et al.,* 1997). The ability of individual colonies to produce amylase was indicated by the zone of clearance shown on the medium. The isolate, *Aspergillus niger* KV5B showed the highest clearance zone (29 mm) and thus was selected for further studies. *Aspergillus niger* KV5B was maintained on PDA slants and stored in the refrigerator at 4^{o}C.

Culture conditions and solid state fermentation

A spore suspension of *Aspergillus niger* KV5B was obtained from a 5-day old culture grown on PDA plates at room temperature by adding 10 ml of sterile distilled water to the spores within a 1 cm cork borer and making the suspension to a 60 ml mark (Bentil *et al.,* 2015). Two (2 ml) of the spore suspension, containing about 3.96 x 10^{6} cells/ml, was used as the inoculum. Yam peels, ground into about 0.3 mm particle sizes, was used as the substrate for the amylase production. The enzyme was produced by SSF using the central composite designed optimized conditions (pH - 5.95, Temperature - 49.53^{o}C and incubation time – 104 h) as previously described (Kwatia *et al.,* 2017). The fungi were grown in a 100 ml Erlenmeyer flask containing 5 g of the ground yam peel and moistened with 5 ml of a mineral solution (comprising KH_2PO_4 – 0.35 g, NH_4NO_3 – 2.5 g, KCl – 1.25 g, $MgSO_4.7H_20$ – 0.025 g, $FeSO_4.7H_2O$ – 0.0025 g, soluble starch – 5 g in 250 ml of distilled water) (Sethi and Gupta, 2015).

Extraction of the enzyme from the fermentation medium

The enzyme was extracted using 50 ml of 0.1 M phosphate buffer (pH 6). The buffer on the substrate bed was shaken on an orbital shaker for 30 min at 250 rpm, the resulting mixture was filtered through cheesecloth and the filtrate centrifuged at 3600 g for 15 mins (Abu *et al.,* 2005). The supernatant was decanted and used as the crude enzyme.

Amylase assay

The activity of amylase was assayed as described by (Uguru *et al.,* 1997). The reaction mixture comprised of 0.5 ml of the crude enzyme, 0.5 ml of 1% (W/V) starch solution in 0.02 M phosphate buffer with 0.006 M NaCl (pH 6.9). The reaction was incubated for 3 min at 37^{o}C and was terminated using 1 ml of 3, 5 dinitrosalicylic acid (Miller, 1959), followed by boiling for 5 min, cooled and absorbance were taken at 540 nm. The amount of reducing sugars liberated (Miller, 1959) was estimated using glucose as standard. A unit of amylase activity was expressed as the amount of enzyme that released 1 µmol of reducing sugars (maltose/glucose) per minute under the assay conditions (Sakthi *et al.,* 2012).
The protein content (Lowry *et al.,* 1951) of the extract was also determined using bovine serum albumin as the standard at 540 nm.

Partial Purification of the crude amylase

Precipitation of the crude enzyme was carried out using ammonium sulphate ($(NH_4)_2SO_4$). The extracted crude amylase was saturated with ammonium sulphate up to 80%. Precipitates were obtained by gently stirring the mixture and leaving on ice for 30 min after which the mixture was centrifuged at 3600 g for 15 min. The resultant precipitates were solubilized in 0.1M phosphate buffer (pH 6) and their activities and protein concentrations determined.

Characterization studies

Effect of pH on enzyme activity and pH stability of the enzyme

The effect of pH on the enzyme activity was investigated by dissolving 1% (W/V) soluble starch in 0.1 M sodium citrate buffer (pH 4 – 6), 0.1 M phosphate buffer (pH 7 – 8) and glycine NaOH buffer (pH 9 – 10) (Shah *et al.*, 2014). A fraction of the enzyme was incubated at different pH values (4 – 10) without the substrate in order to determine the pH stability of the enzyme. The DNS method was used to estimate the enzyme activity using 0.5 ml of the crude enzyme.

Effect of temperature and temperature stability of the enzyme

An enzyme substrate reaction mixture was incubated at different temperatures of 30^{o}C – 90^{o}C for 3 min to determine the optimum temperature of the enzyme. Thermostability of the enzyme was also determined by incubating the enzyme fraction without a substrate at temperatures of 30^{o}C – 80^{o}C. The DNS method was used to estimate the enzyme activity using 0.5 ml of the crude enzyme.

Effect of calcium ion concentration on the stability of the enzyme

Amylase stability at 60^{o}C was investigated by incubating the enzyme-substrate reaction mixture with different concentrations of $CaCl_2$ (2 mM, 5 mM, 7 mM and 10 mM). An untreated sample was used as the control.

Effect of substrate (starch) concentration on amylase activity

Starch concentrations of 1%, 2%, 3%, 4%, and 5% suspended in 0.1 M phosphate buffer (pH 6. 9) with 0.05 M NaCl were used to investigate the effect of starch concentration on amylase activity. The DNS method was used for the amylase activity determination.

Enzyme substrate reaction time

The effect of reaction time on amylase activity was investigated by incubating the enzyme-substrate reaction mixture at different time intervals of 0, 3, 6, 9, 12 and 15 min and the enzyme activity assayed using the DNS method.

Effect of enzyme concentration

The enzyme saturated at 50% of $(NH_4)_2SO_4$ with a protein concentration of 1.90 mg/ml (approximated to 2 mg/ml). Thus, multiples of 2 mg/ml i.e. 2 ml (4 mg of protein), 3 ml (6 mg of protein), 4 ml (8 mg of protein) and 5 ml (10 mg of protein) were used to determine the effect of enzyme concentration on amylase activity. The enzyme assay was by the DNS method.

Hydrolysis of starchy substrates

The ability of the enzyme to hydrolyze starch was investigated by using starches from cassava, sweet potato, yam, and maize. Commercial corn – derived soluble starch (Sigma – Aldrich, St. Louis MO, USA) was used as the standard. The starch hydrolysis was investigated using the optimized conditions that were obtained after the characterization. The rate of hydrolysis of each starch was evaluated based on the quantity of reducing sugars (mg /ml) produced.
In order to extend the substrate range of the substrates industrially, the optimal substrate concentrations of the native starches were also investigated with starch concentrations of 1 %, 2 %, 3 %, 4% and 5 %. The amylase activity was determined using the DNS method after incubating the enzyme – substrate mixture for 30 min.

Statistical analysis and experimental design

All determinations were of 3 independent experiments. Mean values of each experiment were reported and the least significant difference (LSD) test was used to identify means that differed significantly. Sigma plot (Ver. 10.1) software was used for the analysis and graphs.

III. RESULTS AND DISCUSSION

Partial purification of amylase

The stages of purifying amylase produced by *Aspergillus niger* KV5B using ammonium sulphate $(NH_4)_2SO_4$ is shown in table 1. Table 1 indicates that the specific activity of amylase increased from one purification step to another until the highest activity (18.04 U/mg) was reached at 50% $(NH_4)_2SO_4$ saturation. The increase of specific activity from 0% to 50% $(NH_4)_2SO_4$ saturation was an indication that the purification steps used were effective in eliminating contaminant proteins. The ability of the $(NH_4)_2SO_4$ to concentrate enzymes makes it employable even in later stages of purification to concentrate enzymes from dilute solutions.

Table 1: Purification table for the crude extracted amylase using ammonium sulphate

Purification step	Total activity (U/ml-min)	Total protein (mg/ml)	Specific activity (U/mg)	Purification fold (X)
Crude extract	16.06±2.02	8.71±2.00	1.93±0.66	1.00
20% $(NH_4)_2SO_4$	30.83±0.49	6.99±1.29	4.52±0.87	2.34
50% $(NH_4)_2SO_4$	35.16±1.44	1.96±2.20	18.04±1.46	9.33
80% $(NH_4)_2SO_4$	39.05±2.22	4.89±1.60	8.67±3.15	4.48

Effect of pH and pH stability of the enzyme

Figure 1 shows the effect of pH as well as pH stability on the activity of the enzyme. The highest activity (31.88 U/ml-min) was observed at pH 5. A pH of 28 also recorded a high activity of 29.91 U/ml-min. This pH range shows the versatility of the enzyme for several process conditions. These optima observed indicated the enzyme was at its satisfactory conformation (Sachdev *et al.*, 2016). Kumar and Duhan (2011) and Alva *et al.* (2007) reported similar pH ranges of (4.2 and 8.4) and (5.8 and 9) for *Aspergillus niger* MTCC – 104 and *Aspergillus sp* JG112 amylases respectively. They attributed such observations to the existence of at least two amylolytic activities, i.e. glucoamylase and α-amylase since both enzymes synergistically degrade starch molecules.
The enzyme retained more than 80% of its activity across a pH range of 4 – 8. This implied the enzyme was stable over the pH ranges 4 – 8. The drop in activity at pH 9 and 10 (Figure 1) could be attributed to severe denaturation of the structure of the enzyme due to the changes in pH thus leading to the reduction of activity at such pH levels (Kumar and Duhan, 2011).

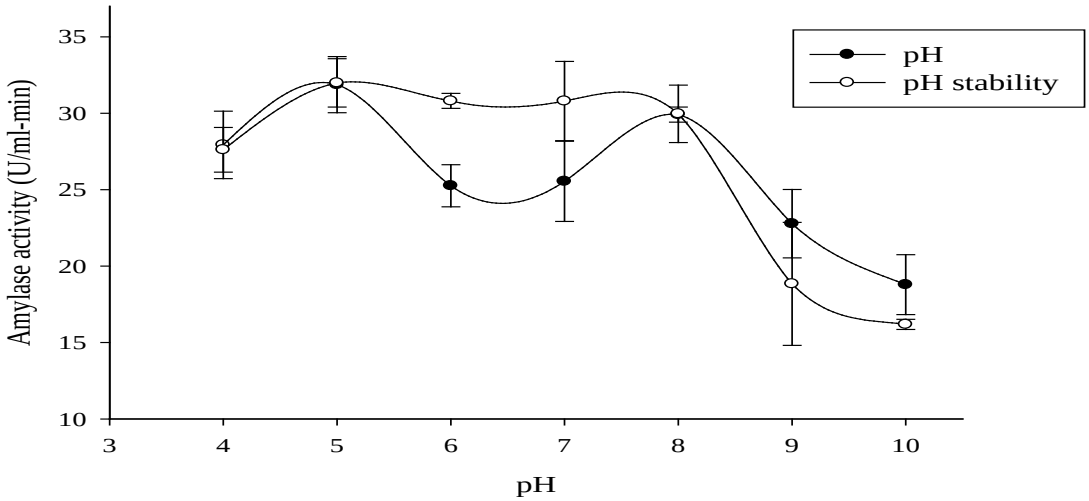

Figure 1: Effect of pH and pH stability of *A. niger* KV5B enzyme activity

Effect of temperature and temperature activity of the enzyme

The effect of temperature on the activity of amylase showed that the enzyme activity increased progressively with an increase in temperature from 30 ^{o}C (16.73 U/ml-min) to a maximum of 28.41 U/ml-min at 60 ^{o}C (Figure 2). Reduction in amylase activities was observed at temperatures below 60^{o}C. The decrease in amylase activity may be attributed to the disruption of the secondary, tertiary and quaternary bonds of the amylase enzyme (Sachdev *et al.,* 2016; Schokker *et al.,* 1998). An optimum temperature of 70 ^{o}C was recorded for an amylase produced by *Aspergillus niger* on yam peels (Uguru *et al.,* 1997). The enzyme showed thermal stability across a temperature range of 30 ^{o}C – 60 ^{o}C, beyond which the activity decreased (Figure 2), this may probably be the result of amino acids destruction, peptide chain hydrolysis and aggregation thus causing incorrect conformation of the enzyme (Alva *et al.,* 2007).

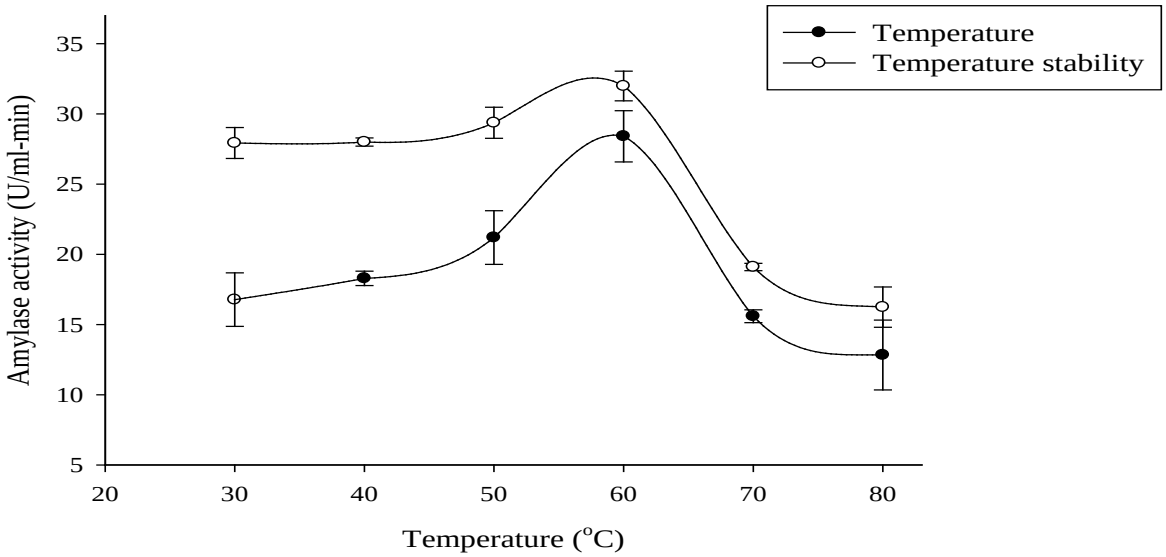

Figure 2: Effect of temperature and temperature stability of *A. niger* KV5B amylase activity

Effect of calcium concentration on the stability of the amylase

The influence of Ca^{2+} varied with concentrations of calcium that the enzyme was exposed to at 60^{o}C (Figure 3). A concentration of 5 mM of calcium ions recorded the highest activity (39.51 U/ml-min) and the least was found with 2 mM calcium concentration. Amylase is a metalloenzyme and is stabilized in the presence of calcium ions (Konsoula and Liakopoulou-Kyriakides, 2007; Prajapati *et al.,* 2015). The results of this study indicated that amylase from *Aspergillus niger* KV5B was stable in the presence of Ca^{2+}.
The stability of an enzyme from *Aspergillus sp* cmst 04 at 5 mM concentration has been reported (Ajikumar *et al.,* 2014). An inhibitory action of Ca^{2+} at 10 mM was also reported by (Babu and Satyanarayana, 1993). Other reports also suggest that Ca^{2+} does not affect the enzyme activity (Asgher *et al.,* 2007).

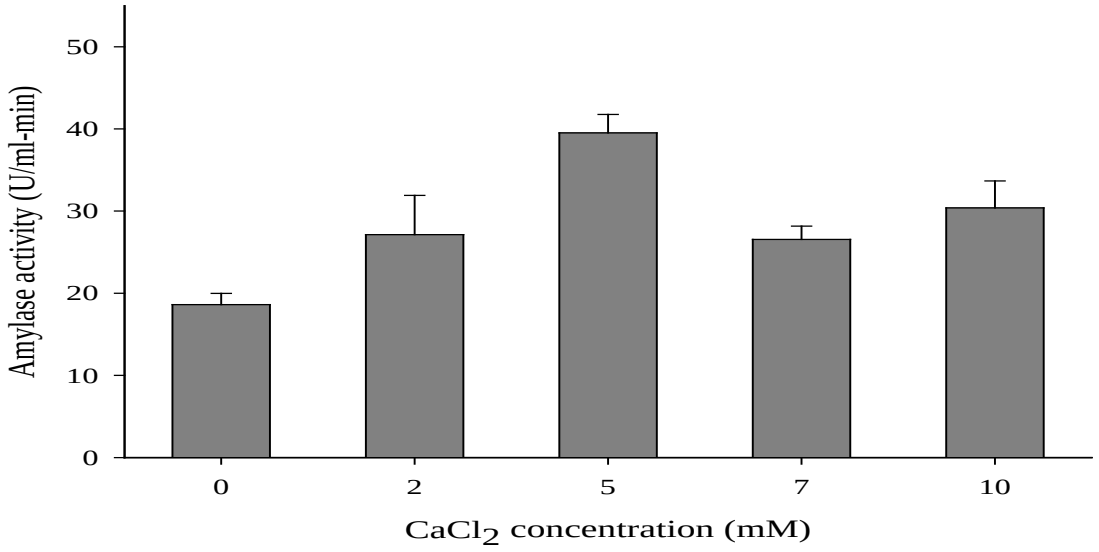

Figure 3: Effect of calcium concentration on *A. niger* KV5B amylase activity

Effect of substrate (starch) concentration on amylase activity

Amylase activity increased with increase in starch concentrations from 1 % (21.88 U/ml-min) to 3% (37.92 U/ml-min), but the activity decreased beyond 3% (Figure 4). The active sites of the enzyme were not saturated at low substrate concentrations; therefore, increase in activity was expected when the substrate concentration increases (Kumar and Duhan, 2011). Low activities recorded beyond 3% might have been a result of blockage of the active site of the enzyme due to competition for these sites by the substrates, thus preventing other molecules from binding (Nyamful, 2013).

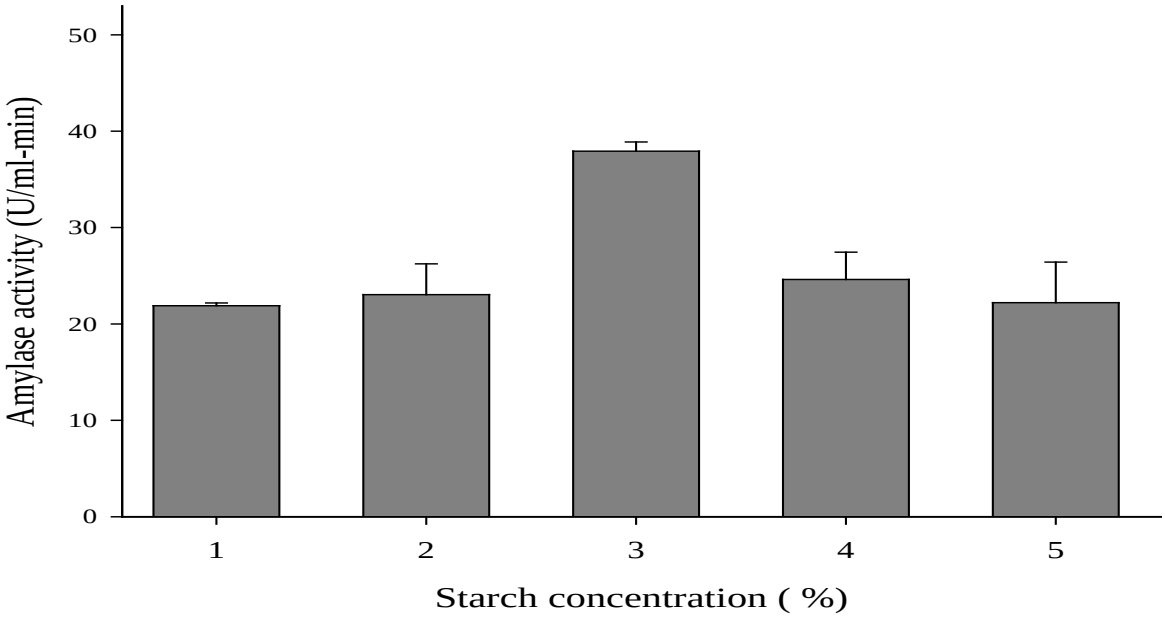

Figure 4: Effect of starch concentration on *A. niger* KV5B amylase activity

Effect of enzyme-substrate reaction time

The time allowed for starch hydrolysis increased the enzyme activity from 3 min (25.35 U/ml-min) to a maximum of 6 min (32.41 U/ml-min) (Figure 5). Incubation beyond 6 min, however, decreased the activity (Figure 5). The enzyme showed efficiency within a relatively shorter time, thus saving time and energy and also a possible prevention of the formation of undesirable products (Dzogbefia *et al.*, 2008).

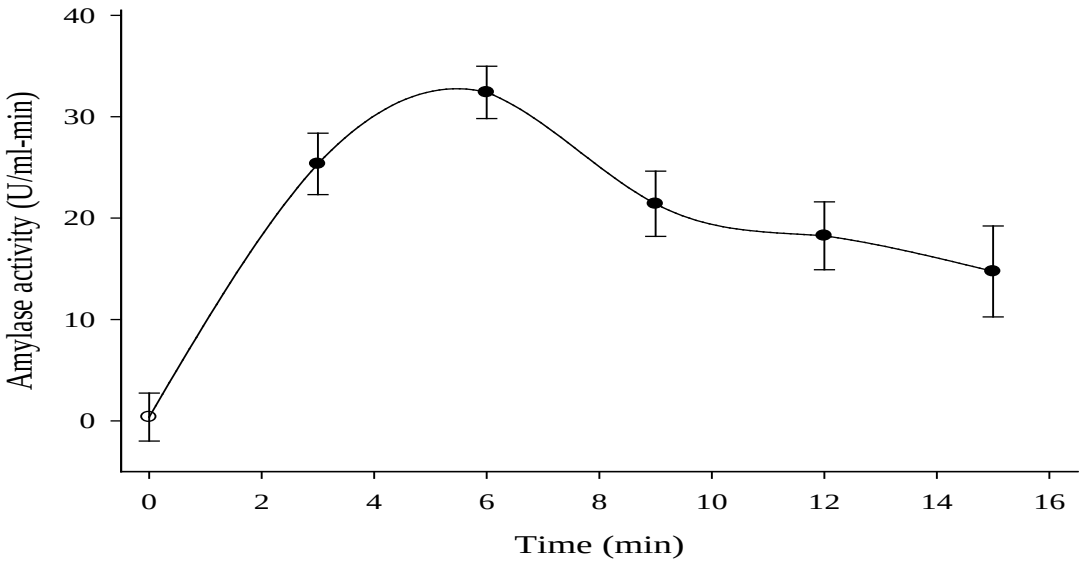

Figure 5: Effect of reaction time on *A. niger* KV5B amylase activity

Enzyme dosage

Figure 6 depicts the effect of enzyme dosage on the hydrolysis of starch. Two (2) ml of the enzyme extract (i.e. 4 mg of total protein) per 3% starch solution gave the highest activity of 58.10 U/ml-min and the least activity was observed with 1 ml of the enzyme extract (i.e. 2 mg of total protein). A probable inhibitory action of the enzyme beyond 4 mg total protein / 3% starch solution as a result of increased enzyme dosage thus introducing competition for binding could have resulted in the decrease in amylase activity. The enzyme application could be cost-effective since lower concentrations will be required if it should be used by industries for starch hydrolysis.

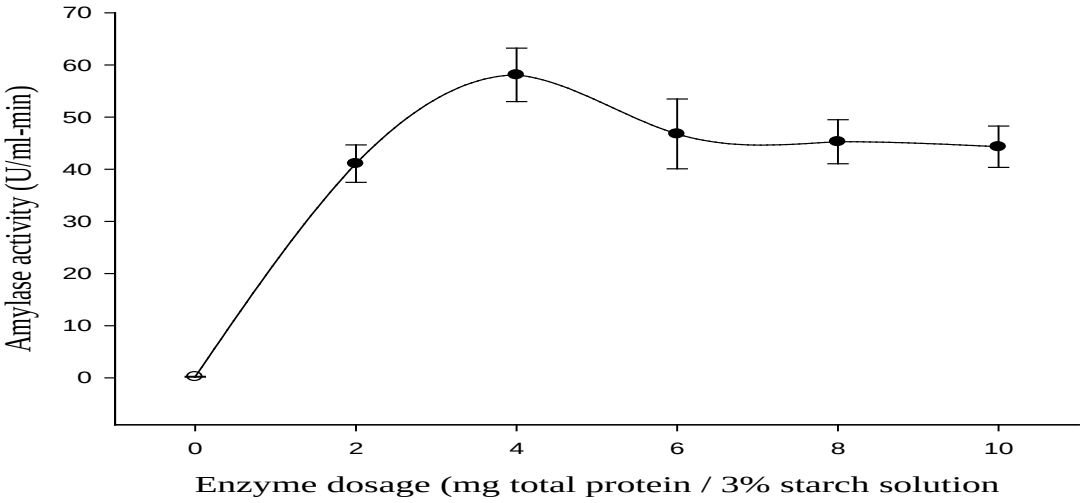

Figure 6: Effect of enzyme dosage on *A. niger* KV5B amylase activity

Hydrolysis of native starchy substrates

The ability of the amylase to hydrolyse starches from tubers (cassava, yam, and sweet potato) and cereal (maize) was studied using corn-derived soluble starch as a standard. The results that portrayed the amount of reducing sugars (mg/ml) produced are shown in Figure 7. The corn-derived soluble starch was also used as a standard (100%) to evaluate the susceptibility of each starchy source to amylase hydrolysis. By keeping all the previously determined parameters at their optimized levels, rapid hydrolysis was observed on the corn-derived soluble starch and maize to give reducing sugar concentrations of 30.68 mg/ml and 31.90 mg/ml respectively. In general, the order of starch hydrolysis was maize > soluble starch > sweet potato > yam > cassava (Figure 7). Statistically, reducing sugars produced by maize, sweet potato and yam were significantly higher ($p<0.05$) than that produced by cassava starch within the six minutes of hydrolysis. However, no statistical difference ($p>0.05$) was observed between hydrolysis of sweet potato and yam starches. The conversion efficiency of the substrates as shown in Table 2 indicated that starch from maize was rapidly hydrolyzed (103. 98%) followed by sweet potato, yam, and cassava with conversion efficiencies of 80.76%, 62.56%, and 28.83% respectively.
The optimal substrate concentration of each substrate after 30 min incubation is summarized in figure 8. Maize starch recorded the fastest optimal level and thus highest hydrolysis and activity at 2% concentration (65.29 U/ml-min) followed by soluble starch (60.55 U/ml-min), cassava (56.54 U/ml-min) and sweet potato (48.45 U/ml-min) starches, all at 3% concentration. Yam recorded the least hydrolysis and thus the least activity (34.24 U/ml-min) at 4% concentration (Figure 8). Results in figure 8 also showed that the ability of amylase of *Aspergillus niger* KV5B to digest raw starches is directly proportional to incubation time

Table 2: Conversion efficiencies of the native starchy substrates at 6 min of incubation

Starches	Amount of reducing sugars (mg/ml)	Relative conversion efficiency (%)
Soluble starch	30.69 ± 0.45	100.00
Maize	31.91 ± 0.48	103.98
Yam	19.31 ± 0.43	62.56
Sweet potato	24.78 ± 0.11	80.76
Cassava	8.85 ± 0.18	28.83

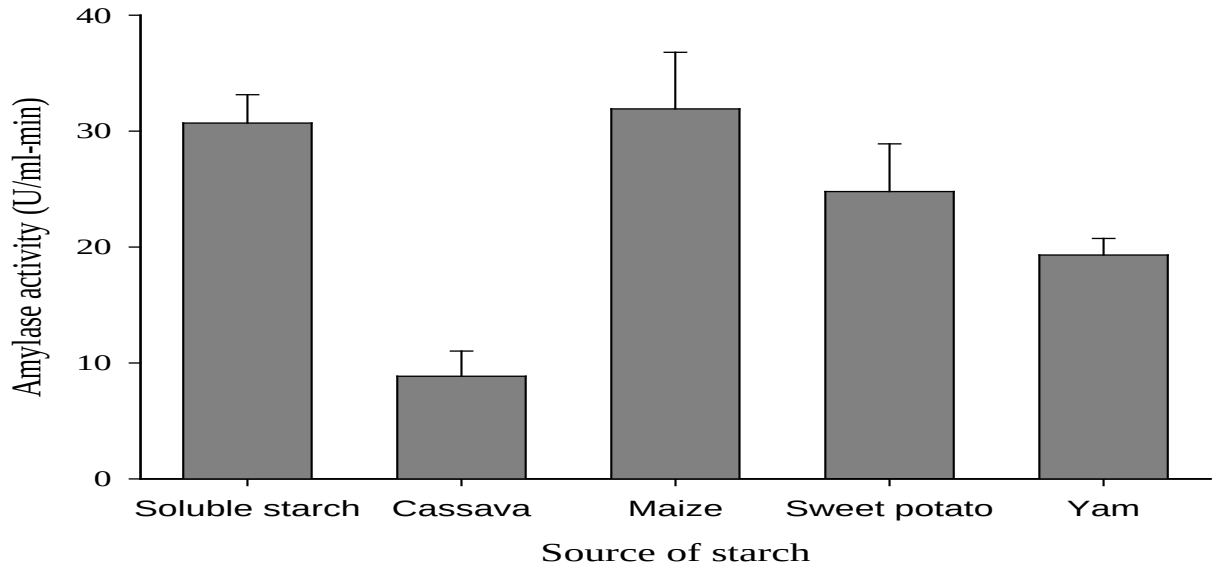

Figure 7: Hydrolysis of raw native starches by *A. niger* KV5B amylase

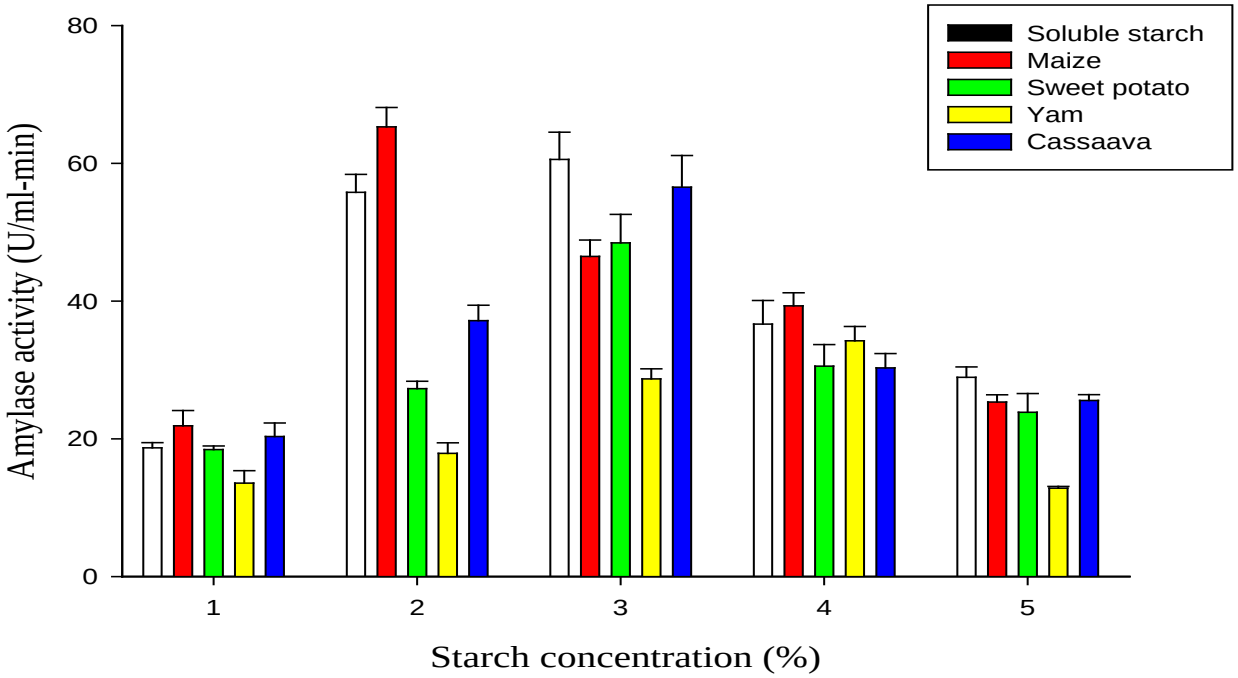

Figure 8: Optimum substrate concentration of the raw native starches upon hydrolysis by amylase *of A. niger* KV5B

Many factors have been attributed to the differences in enzymatic susceptibilities of starches; viz, starch source, extension of association between starch components, granule size, crystallinity, rate of amylose and amylopectin, polymeric type (A,B,C), amylose-lipid complex, type of enzyme and hydrolysis condition (pH, temperature, concentration)(Oates, 1997; Rocha *et al.,* 2010).
Maize starch granules have been identified to exhibit A –type crystal polymorphism with characteristics such as having a porous surface, faster chemical penetration and derivatization reactions, weak points and more susceptible to the enzyme-catalyzed reactions (Jane, 2009). Starches with such A polymorphism are more susceptible to hydrolysis by enzymes because the crystalline structure of such starches contains the A and B1 chains which are unstable and more vulnerable to rearrangement; therefore making them susceptible to hydrolysis (Rocha *et al.,* 2010).
Cassava and sweet potato starches also exhibited more susceptibility to the enzyme hydrolysis as compared to yam starches (Figure 8). The high susceptibility of cassava and sweet potato to the enzymatic hydrolysis may also be attributed to its A – type polymorphism as reported by (Rocha *et al.,* 2010). Yam starches, however, displays the B – type polymorphism which normally displays a nonporous internal granule structure thus, making them less susceptible to amylase hydrolysis (Jane, 2009). B – type starches present higher proportions of long B – chains, which extend for two or more clusters and stabilize the internal structure of granules thus becoming more resistant to enzymatic action (Jane, 2009; Rocha *et al.,* 2010).
Amylose content of maize, sweet potato, cassava and yam starches have been stated as 70 %w/w (Lim *et al.,* 1994; Morrison *et al.,* 1984), 28.9 ±0.35 %w/w (Tan *et al.,* 2006), 23.7±0.1 %w/w 8 -25 %w/w (Paes *et al.,* 2008; Yuan *et al.,* 2007) and 26.3±0.2 %w/w (Yuan *et al.,* 2007) respectively. A report by Tester *et al.* (2006) suggested that the amount of native starch hydrolysis by amylases is inversely proportional to the amylose content, indicating that high amylose starches are more resistant to amylase hydrolysis. This is supported by (Jane, 2009) who also reported that such amyloses are strongly associated with the periphery of the granules thus making them less susceptible to enzyme attack. These might be some contributing factors to the results recorded in this investigation.
Results from the study agree with reports made by Omemu *et al.,* (2005) and Okolo *et al.,* (1995) that starch susceptibility to amylase hydrolysis is dependent on the botanical source and the duration of the amylase treatment. The ability of the partially purified amylase of *Aspergillus niger* KV5B to hydrolyse roots starches especially cassava starch presents an incredible property since these roots are the most abundant in the tropics.

In conclusion, the amylase of *Aspergillus niger* KV5B selected for this study could be effectively used in hydrolysis of both native tuber and cereal starches to produce sugars which will find applications in several industries such as the brewing industry.

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