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Effect of pH, various divalent metal ion and different substrates on glucoamylase activity obtained from *Aspergillus niger* using amylopectin from tiger nut starch as carbon source

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Glucoamylases were obtained from *Aspergillus niger* using amylopectin from tiger nut starch as carbon source on the 5th (GluAgTN5) and 12th day (GluAgTN12) of fermentation. The optimal pH for GluAgTN5 at 55°C were 6.5, 7.0, 6.0 while that for GluAgTN12 were 8.5, 6.0, 7.5 at 50°C using cassava, guinea corn and tiger nut starch as substrates, respectively. The enzyme activity in GluAgTN5 was enhanced by Ca^{2+} and Fe^{2+} while Zn^{2+} and Co^{2+} had inhibitory effects on the enzyme activity. $Mn^{2+}$and Pb^{2+}, however completely inactivated the enzyme. Enzyme activity in GluAgTN12 was enhanced by Ca^{2+} while $Co^{2+}$and Zn^{2+} had inhibitory effects, Fe^{2+}, Mn^{2+} and Pb^{2+} completely inactivated the enzyme. The Michealis-Menten constant, K_m and maximum velocity, V_{max} obtained from Line-Weaver-Burk plot of initial velocity data at different substrate concentrations were 222 mg/ml and 500 µmol/min, 291 mg/ml and 1000 µmol/min, 137.5 mg/ml and 500 µmol/min using cassava, guinea corn and tiger nut starch as substrate, respectively for GluAgTN5. While that for GluAgTN12 were 176.6 mg/ml and 100 µmol/min, 491 mg/ml and 1000 µmol/min, 131.5 mg/ml and 500 µmol/min using cassava, guinea corn and tiger nut starch as substrate, respectively.

Key words: Glucoamylase, pH, metal ions, *Aspergillus niger*, tiger nut starch, amylopectin.

INTRODUCTION

Glucoamylase (α-1, 4-glucan-glucohydrolases, EC 3.2.1.3) is an exoenzyme that hydrolyzes 1,4-alpha-glycosidic bonds from the non-reducing ends of starch and 1,6-alpha-glucsidic linkages in polysaccharides yielding glucose as the end-product, which serves as a feedstock for biological fermentations (Kumari et al., 2013). Glucoamylase from *Aspergillus niger* has been shown to be an acidic enzyme that shows highest activity within a pH range of 2.0 to 4.0 (Imran et al., 2012), whereas glucoamylase from *Aspergillus candidus* and *Rhizopus* lost their activities and conformation at this pH range (Shenoy et al., 1985). Glucoamylase from *Aspergillus oryzae* showed maximum activity at pH 5.0 (Parbat and Singhal, 2011), unlike α-amylase which

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exhibited maximum activity at neutral pH (pH 7.0) (Acharya et al., 2014). The pH value at which an enzyme exhibits highest activity is called “optimum pH” (Devasena, 2010). Changes in pH can result in the protonation or deprotonation of a specific side groups at the enzyme’s active site thereby changing its chemical features. For instance, the deprotonation of carboxyl termini could result in a potential loss of interaction with an adjacent subunit, changing the enzyme conformation which could cause a decrease in substrate affinity, or a complete loss of activity. Changes in pH can however, be utilized by enzymes for regulation or protein function. Variations in pH can influence the following characteristics of an enzyme; the binding of the substrate to the enzyme, the ionization states of the amino acid residues at the catalytic site of the enzyme, the ionization state of the substrate and variation in protein structure or complete denaturation of the enzyme (which occurs at extreme pH values) (Berg, 2007).

Glucoamylase obtained from *A. niger* in solid state fermentation was reported to be metalloenzyme and its activity has been shown to be increased by Mn^{2+} and Fe^{2+} (Selvakumar et al., 1996). Yusaku and Hiroshi (1996) reported that Ca^{2+} and Zn^{2+} ions increased glucoamylase activity from *Rhizopus sp*. Glucoamylase from *Aspergillus flavus* was activated by Mn^{2+}, Co^{2+} and Ba^{2+} and was inhibited by Hg^{2+}, Fe^{3+}, Zn^{2+} and Cu^{2+} (Koç, and Metin, 2010). Mn^{2+} and Mg^{2+} were shown to increase the activity of glucoamylase from *Rhizopus nigricans* while Fe^{2+}, Zn^{2+} and Cu^{2+} reduced glucoamylase activity (Jambhulkar, 2012). Metal ions affect enzyme catalysis by the formation of a nucleophilic hydroxide ion at neutral pH by activating a bound water molecule, thus stabilizing the negative charges that are formed which are highly susceptible to neucleophilic attack (Fersht, 1985). This study is aimed at establishing the effect of metal ion concentrations in relation to pH changes and different substrate concentrations on the activity ofglucoamylases obtained from *A. niger* using amylopectin from tiger not starch as carbon source.

MATERIALS AND METHODS

Collection of plant materials

Plant materials were obtained from Ogige main market in Nsukka Local Government Area of Enugu State, Nigeria.

Processing of tiger nut and guinea corn starch

The tiger nut and guinea corn starch were processed using the method described by Agboola et al. (1990) with the following modifications. The seeds were sun dried and ground to fine flour. 300 g of the flours were suspended in 3 L of distilled water for 24 h. The suspended flour was sieved using muslin cloth. The extracted starch was allowed to sediment for 4 h at room temperature. The supernatant was decanted off and the starch washed with 3 L of distilled water twice and finally allowed to stand for 4 h. The supernatant was then decanted and the resulting wet starch was sun dried and then packaged in an air tight container and stored at room temperature.

Processing of cassava starch

Cassava starch was processed using the method described by Corbishley and Miller (1984) with the following modifications. Freshly harvested cassava tubers were peeled, washed clean and grated. The grated cassava (1.2 kg) was soaked in 4 L of distilled water for 1 h after which it was sieved (3 times) with muslin cloth. This was allowed to stand for 4 h and the supernatant decanted. The isolated wet starch was sun dried and packaged in plastic air tight container, labelled and kept in a cool, dry place.

Fractionation of tiger nut starch into amylose and amylopectin

Fractionation of amylose and amylopectin was carried out by following the general procedure of Sobukola and Aboderin (2012). This consists of heating and stirring starch dispersion (0.8%, w/v in water) in water bath at 100°C until starch is gelatinized. Starch solutions were filtered using filter paper to remove insoluble residues, and the pH adjusted to 6.3 using phosphate buffer. The solution was stirred in a boiling water bath for 2 h to disperse the starch molecules. Thereafter, n-Butyl alcohol was added (20%, v/v), and the solution was stirred at 100°C for 1 h, followed by cooling to room temperature over a period of 24 to 36 h. Amylose butyl alcohol complex crystals was formed and precipitated during cooling, and was separated by filtration. The amylopectin remaining in the supernatant was recovered by adding excess methyl alcohol.The percentage yield of amylopectin was calculated using:

$$\%\ \text{amylopecting} = \frac{\text{Amount of amylopectin}}{\text{ammount of starch}} \text{x}100$$

Isolation of glucoamylase producing fungi

Glucoamylase producing fungi were isolated by adopting the method of Martin et al. (2004) as modified by Okoye et al. (2013). Tiger nut starch was fractionated into amylose and amylopectin. The wet amylopectin was left open on shelve to allow micro-organisms to grow on it. A loop of each organism was streaked onto potato dextrose agar PDA under the flame of Bunsen burner. Streaks were made from each side of the plate, marking an initial point, with sterilization of the wire loop after each side has been completed. The plates were thereafter incubated at 35°C till visible colonies were observed. All morphological contrasting colonies were purified by repeated streaking and sub-culturing on separate plates. This process was continued till pure fungal culture was obtained. Pure fungal isolates were maintained on potato dextrose agar (PDA) slopes or slants as stock cultures. PDA media were prepared according to the manufacture’s description. In the description, 3.9 g of PDA powder was weighed and added in small volume of distilled water and made up to 100 ml. The medium was autoclaved at 121°C, 15 psi for 15 min. It was allowed to cool to about 45°C and then poured into Petri dishes and allowed to gel. The plates were then incubated in a B and T Trimline incubator at 37°C for 24 h to check for sterility. Three days old pure cultures were examined. The colour, texture, nature of mycelia or spores and growth patterns were also observed. The three day old pure cultures were used in preparing microscopic slides. A little bit of the mycelia was dropped on the slide and a drop of lactophenol blue was added to it. A cover slip was placed over it and examination was performed under the light microscope at X400 magnification.

Identification was carried out by relating features and the micro-graphs to “Atlas of mycology” by Barnett and Hunter (1972).

Glucoamylase production

Glucoamylase was produced by adopting the method described by Bagheri et al. (2014) with the following modifications. The 250 ml Erlenmeyer flask contained 100 ml of sterile cultivation medium optimized for glucoamylase with 0.3% ammonium sulphate $(NH4)_2SO_4$, potassium dihydrogen phosphate (0.6% KH_2PO_4), magnesium sulphate hepta hydrate0.1% ($MgSO_4.7H_2O$), 0.01% ferrous sulphate hepta hydrate ($FeSO_4.7H_2O$), 0.1 %calcium chloride ($CaCl_2$) and 1% amylopectin from tiger nut starch. The flask was stoppered with aluminum foil and autoclaved at 121°C, 15 psi for 15 min. From the PDA slants, fresh plates were prepared and three day old cultures were used to inoculate the flasks. In every sterile flask, two discs of the respective fungal isolates were added using a cork borer of diameter 10 mm and then plugged properly. The culture was incubated for 14 days on MK V orbital shaker (150 rpm) at room temperature (30°C). At each day of harvest, flasks were selected from the respective groups and mycelia biomass separated by filtration using filter paper. Each day, the filtrate was analyzed for glucoamylase activity and extracellular protein concentration till the 14^{th} day of fermentation. After the 14 days pilot SmF studies, 5^{th} and 12^{th} day of fermentation were chosen for mass production of enzymes coded GluAgTN5 and GluAgTN12, respectively.

Glucoamylase assay

Glucoamylase activity was assayed by the method of Parbat and Singhal (2011) with the following modifications. 0.5 ml of the enzyme was added into a clean test tube followed by 0.5 ml 1% soluble starch solution in 50 mM acetate buffer (pH 5.5) at 50°C and was allowed to stand for 20 min. 1 ml of 3, 5-dinitrosalicyclic acid (DNSA) reagent was added and boiled for 10 min to stop the reaction. 1 ml of sodium potassium tartarate was added to stabilize the red colour produced. The mixture was then allowed to cool and the glucose released was measured using a JENWAY 6405 UV/VIS spectrophotometer (Beckman/Instruments, Inc., Huston Texas) at 540 nm. Absorbance values were converted to glucose concentrations by extrapolation from the glucose standard curve. Glucoamylase activity unit (U) was expressed as the amount of enzyme releasing one µ mole of glucose equivalent per minute per ml.

α-amylase assay

The α-amylase activity was assayed by adopting the method of Bernfield (1955). The reaction mixture contained 0.5 ml of the enzyme preparation and 0.5 ml of 1% w/v starch solution in 20 mM sodium phosphate buffer (pH 7.0). The reaction mixture was incubated at 55°C for 60 min after which the reaction was stopped by addition of 1 ml of 3, 5-dinitrosalicyclic acid (DNSA) reagent and boiling for 10 min. 1 ml of sodium potassium tartarate was added to stabilize the red colour produced. The mixture was then allowed to cool and the glucose released was measured using a JENWAY 6405 UV/VIS spectrophotometer (Beckman/Instruments, Inc., Huston Texas) at 600 nm. α-Amylase activity unit (U) was expressed as the amount of enzyme releasing 1 µ mole of reducing end groups (maltose) per minute under assay conditions.

Protein determination

Protein content of the enzyme was determined by the method of Lowry et al. (1951), using Bovine Serum Albumin as standard.

Purification of crude glucoamylase

Ammonium sulphate saturations of 20 and 70% were found suitable to precipitate protein with highest glucoamylase activity in GluAgTN5 and GluAgTN12, respectively.The crude enzyme preparation was made up to 20 and 70% ammonium sulphate saturation with solid $(NH_4)_2SO_4$ for GluAgTN5 and GluAgTN12, respectively. This was kept at 4°C for 30 h, thereafter it was centrifuged with Cole-palmer VS-13000 micro centrifuge at 4000 rpm for 30 min. The precipitate was collected and redisolved in 20 mM acetate buffer pH 5.5. The glucoamylase activity and protein were determined as described above.

Gel filtration

A volume (20 ml) of the precipitated enzyme was introduced into a (50 × 2.5 cm) gel chromatographic column and subjected to gel filtration using sephadex G-100 pre-equilibrated with 0.02 M sodium acetate buffer pH 5.5. Fractions were collected at a flow rate of 5 ml/20 min. The protein concentration of each fraction was monitored using a JENWAY 6405 UV/VIS spectrophotometer (Beckman/Instruments, Inc. Huston Texas) at 280 nm. The glucoamylase activity of each fraction was assayed with the active fractions pooled together and stored at -10˚C.

Effect of pH

The optimum pH for enzyme activity was determined using 0.02 M sodium acetate buffer (pH 3.5 - 5.5), phosphate buffer (pH 6.0 - 7.5) and Tris-HCl buffer (pH 8.0 - 10.0) at intervals of 0.5. The reaction mixture contains 0.5 ml of starch solution (1%) and 0.5 ml of enzyme solution and the enzyme activity was assayed as described above.

Effect metal ions concentration

The concentrations, 20, 30, 40 and 50 mM of metal salts ($ZnCl_2$, $CoCl_2$, $MnCl_2$, $FeCl_2$, $PbCl_2$ and $CaCl_2$) were prepared in 20 mM sodium acetate. Each of the reaction mixtures contains 0.5 ml of enzyme solution, 0.5 ml of starch solution (1%) and 1 ml of metal ion solutions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, $Co^{2+}$and Zn^{2+}). The mixtures were incubated for 20 min at the predetermined optimal pH and temperatures. To study the effect of metal ionson glucoamylase activity/stability, the reaction was carried out with and without metal ions. In all the above experiments, the enzyme activity was calculated as the average of three independent sets of experiments and the standard deviation in all cases was negligible.

Determination of kinetic parameters

The effect of substrate concentration on glucoamylase activity was determined by incubating 0.5 ml of enzyme with 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mg/ml of starch solution for 20 min at the respective predetermined optimal pHs and temperatures. The glucoamylase activity was assayed as described above using starch from cassava, guinea corn and tiger nut starch as substrate. The V_{max} and K_m values of the enzyme were determined using the Line-Weaver-Burk plot of initial velocity data.

RESULTS AND DISCUSSION

The percentage yield of amylopectin fractionated from

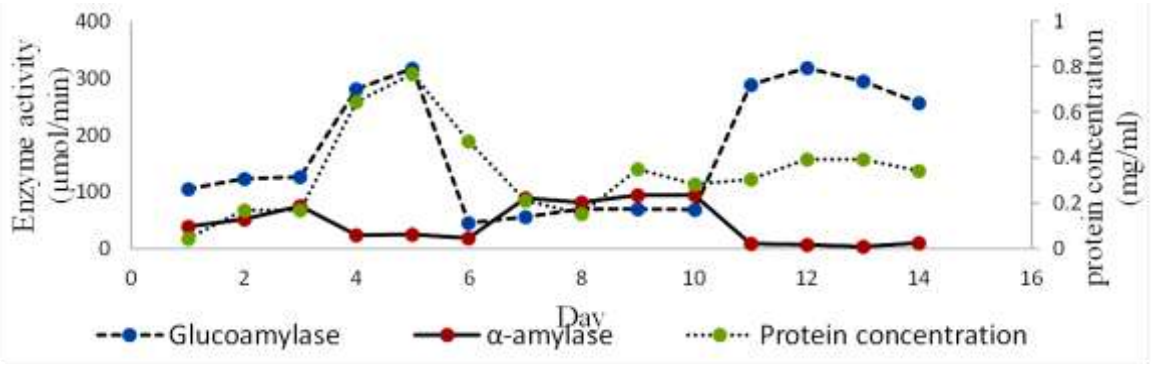

Figure 1. Monitoring the day of highest glucoamylase production in liquid broth using amylopectin from tiger nut starch as the only carbon source (using cassava starch was used as substrate).

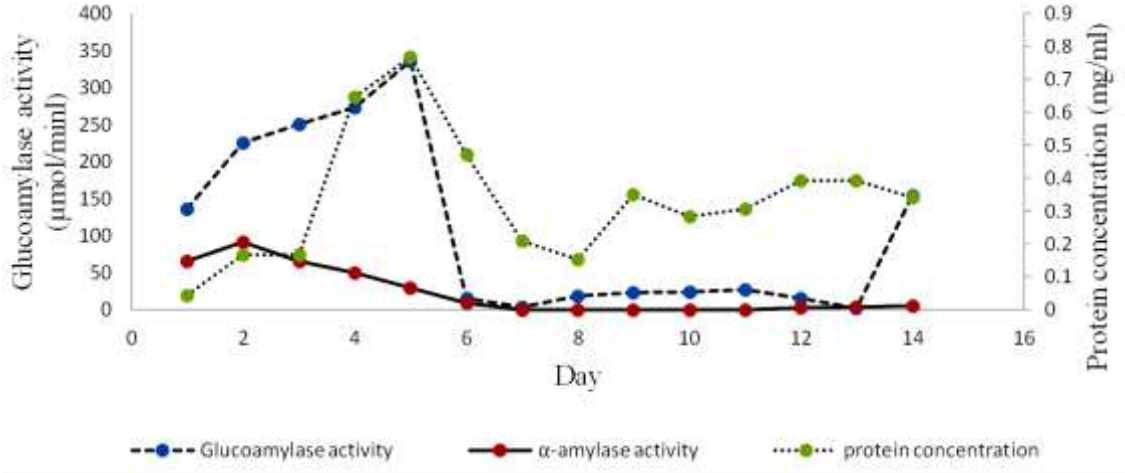

Figure 2. Monitoring the day of highest glucoamylase production in liquid broth using amylopectin from tiger nut starch as the only carbon source (using guinea corn starch as substrate).

tiger nut starch was 60%. A fourteen day pilot study was carried out to determine the day of highest protein production, α-amylase activity and glucoamylase activity in submerged fermentation using amylopectin obtained from tiger nut starch as carbon source. Enzyme activities were assayed using cassava, guinea corn and tiger nut starch as substrate (Figures 1 to 3). Two major peaks were obtained on day 5 and 12 with activities 316.51 and 318.15 µmol/min, respectively using cassava starch as substrate. When guinea corn starch was used as substrate, a major peak was obtained on day 5 with glucoamylase activity of 336.00 µmol/min. More so, the major peaks were observed on the 5th and 12th day with glucoamylase activities of 242.16 and 158.53 µmol/min using tiger nut starch as substrate. Therefore, day 5 and 12 were chosen for mass production of the enzyme. The steady increase in glucoamylase activity observed between days 1 and 5 may have occurred due to multiple branched points in amylopectin from tiger nut. Since glucoamylase is an inducible enzyme produced by the organism in high amounts in the presence of highly branched amylopectin, at the initial stage of fermentation, the organism is prompted to express increasing amounts of glucoamylase to enable the utilization of the amylopectin in the medium for glucose primarily needed for production of energy and other biomolecules required by the organism for growth and replication. When the amylose and glucose level of the broth increase as a consequence of the debranching activity of glucoamylase, the mechanism for the production of glucoamylase is turned off and later turned on when the amylose content in the fermentation broth decreases. This result correlates with the findings of Nahid et al. (2012) and Imran et al. (2012) that reported maximum glucoamylase activity on day 4 of fermentation from *Aspergillus niger* using different carbon sources. Lin et al. (2013) reported maximum glucoamylase production on day 10 of fermentation from *Aspergillus awamori* using pastry wastes as carbon source. The decrease in glucoamylase activity could be due to depletion in the level of branch

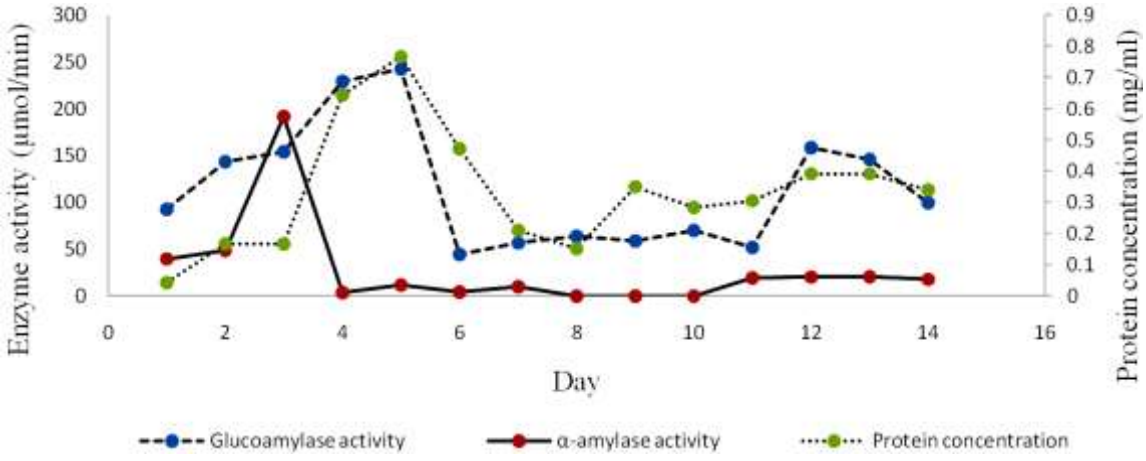

Figure 3. Monitoring the day of highest glucoamylase production in liquid broth using amylopectin from tiger nut starch as the only carbon source (using tiger nut starch as substrate)

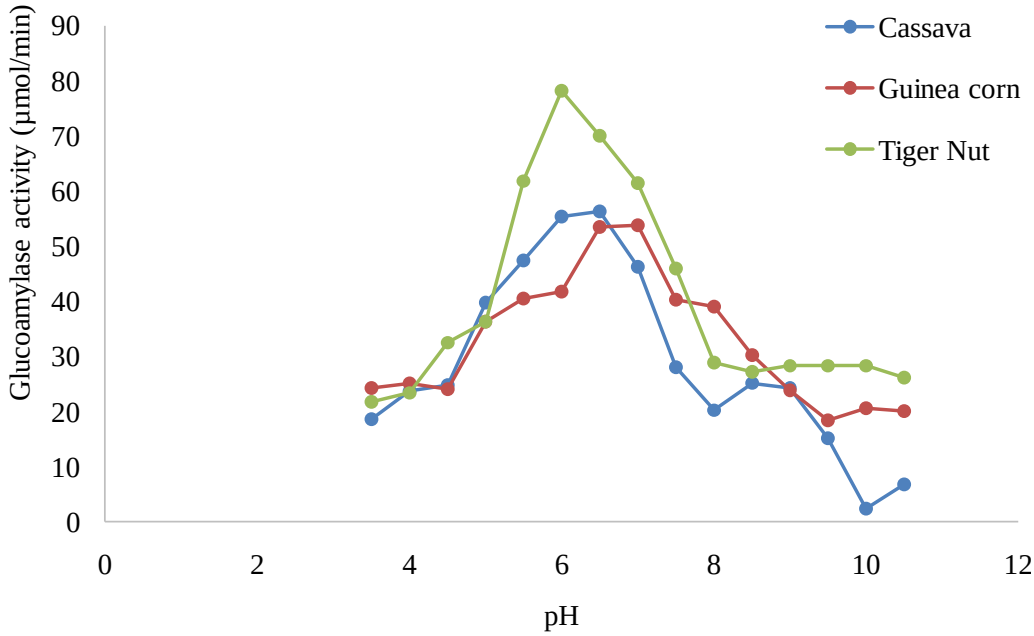

Figure 4. Effect of pH on glucoamylase activity obtained on the 5th day of fermentation (GluAgTN5).

points in the carbon sources, product inhibition as well as depletion in growth supplement in the broth (Nahar et al., 2008).

The optimum pH required for maximum activity of the glucoamylase harvested on days 5 and 12 varied when the activities were assayed using cassava, guinea corn and tiger nut starch as substrates. Glucoamylase harvested on the 5th day was found to have optimum activity at a slightly acidic pH range of 6.0 to 7.0, while glucoamylase harvested on the 12th day was found to have an optimum pH within a basic range of 7.5 to 8.5 when cassava and tiger nut starch were used as substrates, respectively (Figures 4 and 5). Differences in optimum pH obtained may be attributed to the ionic state of the amino acid residues as well as that of substrate due to protonation or deprotonation of specific side groups at the active site of the enzyme thereby changing its chemical conformation (Lee and Paetzel, 2011). At the active site of glucoamylase, deprotonation of carboxyl termini of glutamate or aspartate could result in a potential loss of interaction with an adjacent subunit, changing the enzyme conformation leading to a decrease or complete loss of activity with a change in pH (Berg, 2007). Imran et al. (2012) reported an optimum pH between the range of 4.5 to 6.0, while Lin et al. (2013) reported an optimum pH of 5.5.

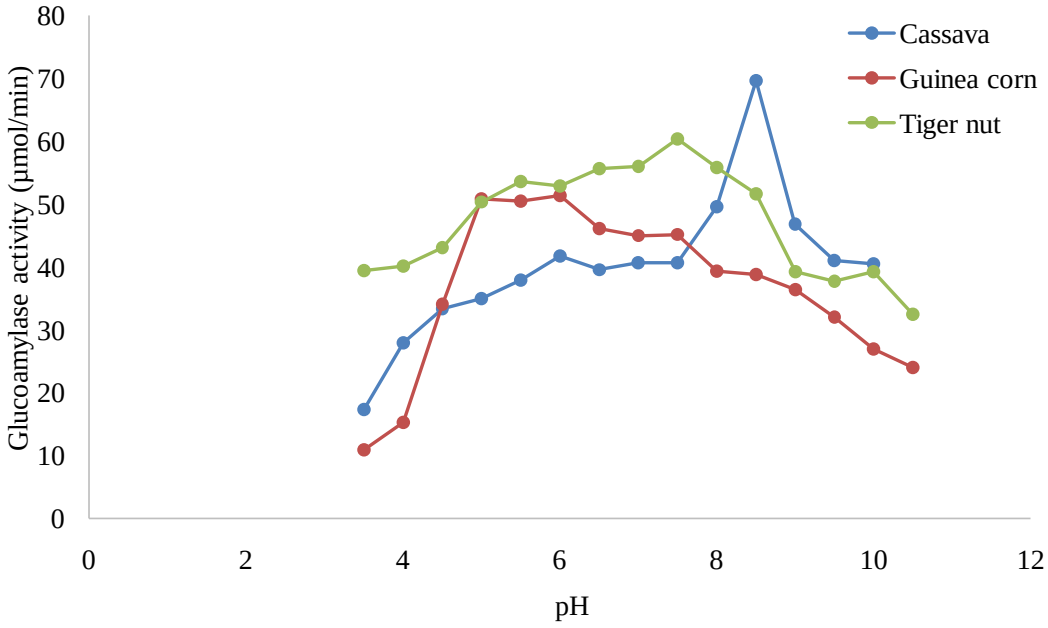

Figure 5. Effect of pH on glucoamylase activityobtained on the 12th day of fermentation (GluAgTN12).

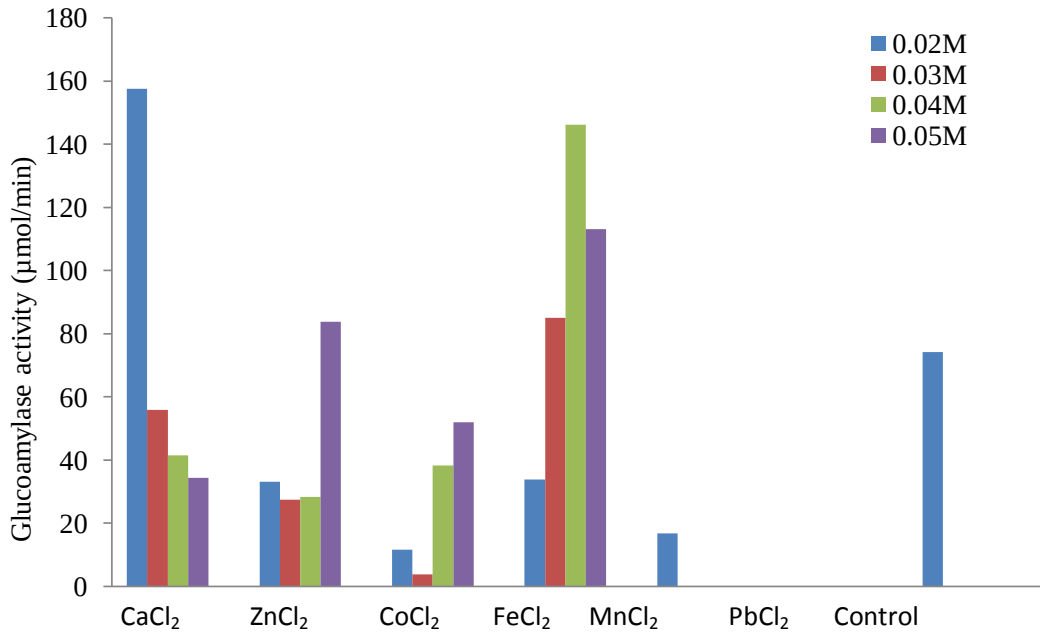

Figure 6. Effect of metal ion concentrations on the activity of glucoamylase harvested on day 5 of fermentation (GluAgTN5).

The effect of several metal ion concentrations on the activity of glucoamylase was monitored and it was observed that low concentration of Ca^{2+}increased the activity of GluAgTN5 and GluAgTN12. Whereas Zn^{2+} and Fe^{2+}caused a slight increase in the activity of gluco-amylase (GluAgTN5). Co^{2+} and Mn^{2+} were observed to have inhibitory effect on glucoamylase activity while Pb^{2+} totally inhibited the enzyme. Zn^{2+} and Co^{2+} had inhibitory effect on GluAgTN12) while Mn^{2+}, Fe^{2+} and Pb^{2+} completely inactivated the enzyme (Figures 6 and 7). This result is in accordance with the report of Jambhulkar (2012) which reported a complete inactivation of glucoamylase produced from *R. nigricans* by Pb^{2+}. Since the glucoamylase harvested on day 5 (GluAgTN5) was stable at neutral pH 7.0, it is more likely to readily form neucleophilichydroxide ions with metals by activating a

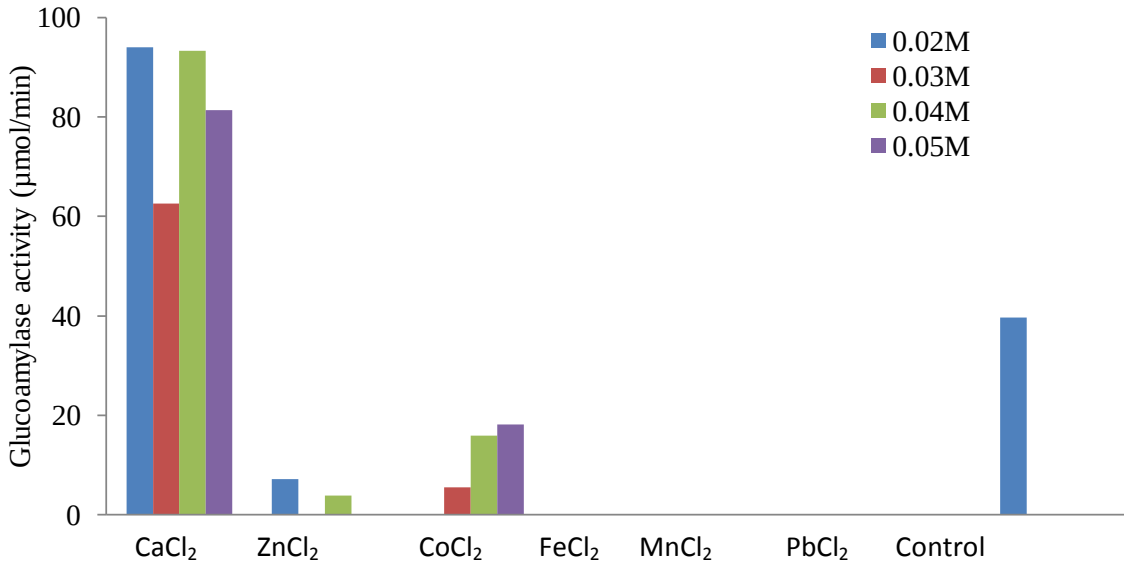

Figure 7. Effect of metal ion concentrations on the activity of glucoamylase harvested on day 12 of fermentation (GluAgTN12).

bound water molecule (Fersht, 1985); this stabilizes the negative charges that are formed at the active sites allowing more weak interactions to hold the substrates in proper orientation at the active site of the enzyme. Thus, the presence of Fe^{2+} and Ca^{2+} will be needed by GluAgTN5 to hold the substrates in close proximity for proper binding by the weak interactions involving the nucleophilic hydroxide ions created by the metal ions at the active site. Since glucoamylase harvested on the day 12 was not stable at neutral pH 7, it could be suggested that less neucleophilic hydroxide ions were formed by metal ions at the active site of GluAgTN12. Hence, low concentration of metal ions will be required at the active site for stability to enable substrate binding to improve catalytic activity. Enhancement of glucoamylase activity such as the ones observed with Ca^{2+}, Zn^{2+} and Fe^{2+} ions could be based on its ability to interact with negatively charged amino acid residues, such as aspartic and glutamic acid by stabilizing the negative charges established at the enzyme active site. This could be observed in other starch degrading enzymes (Carvalho et al., 2014).

High level of hydrogen ions may compete with the cations at the ion binding site of the enzyme. Thus an increase in hydrogen ion concentration as a result of acidic pH will lead to a simultaneous decrease in the concentration of bound metal ions. On the other hand, decreasing the hydrogen ion concentration by increasing the pH may lead to an increase in hydroxyl ion concentration. This may compete with the enzymes for divalent cations, leading to formation of hydroxides.

The Michaelis constant (K_m) and maximum velocity (V_{max}) obtained from the Lineweaver-Burk plot of initial velocity data at different substrate concentration were 222 mg/ml and 500 µmol/min, 291 mg/ml and 1000 µmol/min, 137.5 mg/ml and 500 µmol/min using cassava, guinea corn and tiger nut starch as substrates, respectively for GluAgTN5 while 176.6 mg/ml and 100 µmol/min, 491 mg/ml and 1000 µmol/min, 131.5 mg/ml and 500 µmol/min, were obtained for GluAgTN12 using cassava, guinea corn and tiger nut starch substrate, respectively (Figure 8 and 9). Thus the enzymes, GluAgTN5 and GluAgTN12, had high affinity for cassava and tiger nut starch and low affinity for guinea corn starch. This results suggests that a small amount of the enzyme should be efficient in hydrolyzing large amount of starch especially that from tiger nut and cassava. The charge distribution on the substrates is also affected by changes in the pH of the medium. This can lead to a reduction or an increase in affinity of the substrate and glucoamylase since enzyme-substrate interaction at the enzyme active site occurs through non-covalent interactions of the side chains of amino acids with charged groups on the substrate. Hence, changes in the pH in the medium influences the substrate binding to the enzyme, reducing the catalytic activity of glucoamylase.

Conclusion

Glucoamylase harvested on the 5th day was found to operate at acidic pH range of 6.0 to 7.0, 55°C while glucoamylase harvested on the 12th day was found to operate at basic pHrange of 7.5 and 8.5, 55°C. The addition of divalent metal ions showed significant increase in enzyme activity suggesting its suitability for industrial application especially in starch processing, high glucose syrup production and other biotechnological applications.

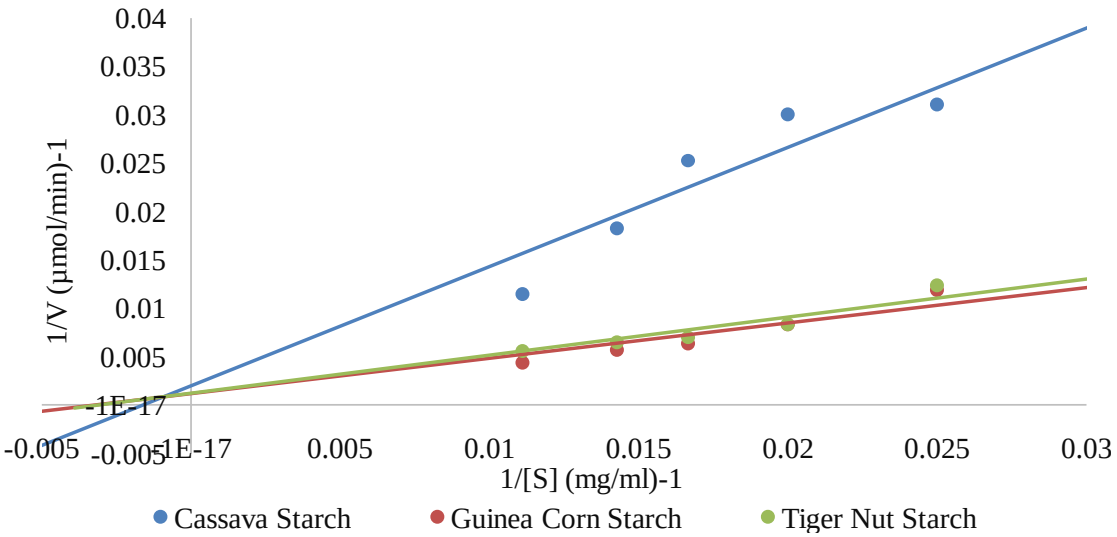

Figure 8. Line-Weaver-Burk plot of initial velocity data at different substrate concentration for glucoamylase harvested after five days of fermentation monitored using different substrates.

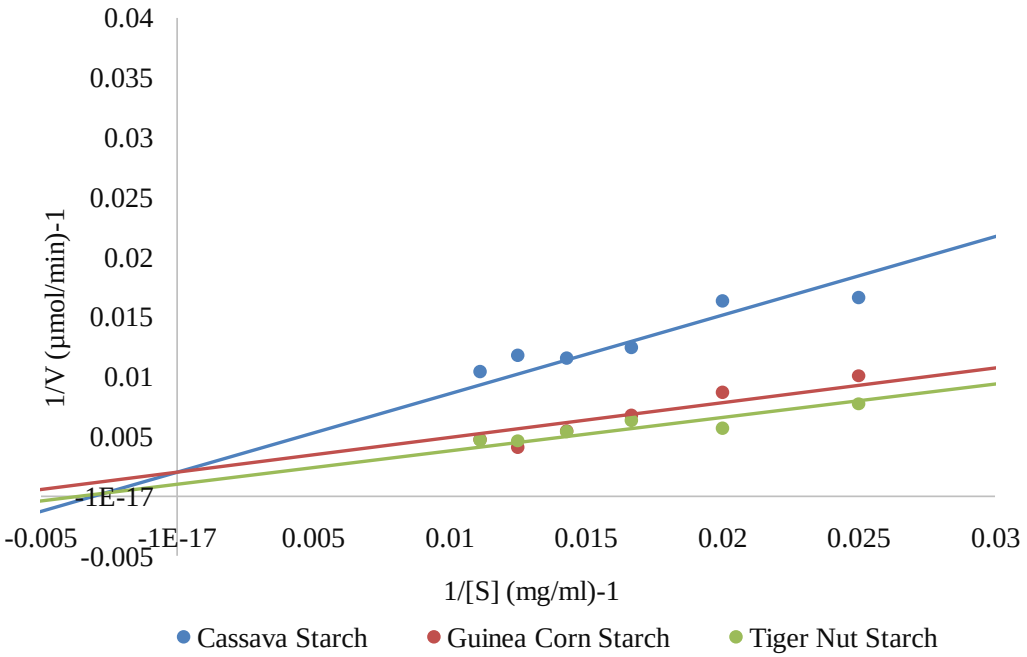

Figure 9. Line-Weaver-Burk plot of initial velocity data at different substrate concentration for glucoamylase harvested after 12 days of fermentation monitored using different substrates.

The results suggest that the affinity of the enzyme for substrate is affected by the pH and divalent metal ion concentration which could be the reason while optimum pH varied with different substrates used.

Conflict of Interests

The authors have not declared any conflict of interest.

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