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Research Article
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Effect of Divalent Metal Ions on Glucoamylase Activity of Glucoamylase Isolated from *Aspergillus niger*

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Abstract

This study investigates the production of glucoamylase from *Aspergillus niger* in a submerged fermentation process using amylopectin fractionated from guinea corn starch as the carbon source. This work also studies the effect of a few metal ions (Ca^{2+}, Zn^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+} and Pb^{2+}) concentration on the glucoamylase activity. A Fourteen day experimental study was carried out to determine the day of highest glucoamylase activity. Maximum glucoamylase activity was observed on day five of the submerged fermentation; hence, day five was mass produced. The specific activity for the crude enzyme was found to be 729.45 U/mg. The crude enzyme was purified to the level of gel filtration (using sephadex G-100) via ammonium sulphate precipitation. Ammonium sulphate saturation of 70% was found suitable to precipitate the enzyme. After ammonium sulphate precipitation and gel filtration, the specific activities were found to be 65.98 U/mg and 180.52 U/mg respectively. The glucoamylase activity was enhanced by 2 mM to 5 mM of Ca^{2+}, Co^{2+}, Fe^{2+}, $Mn^{2+}$and Zn^{2+} but Pb^{2+} had inhibitory effect on the enzyme. The Michaelis constant, Km and maximum velocity Vmax of the enzyme was obtained from the Lineweaver-Burk plot of initial velocity data at different substrate concentrations. They were found to be 770.75 mg/ml and 2500 µmol/min respectively, when using cassava starch as substrate. The enzyme glucoamylase is known to have useful applications in food processing industries and fermentation biotechnology.

Keywords Glucoamylase; Enzyme; Aspergillus; Metal ions; Activity

Introduction

Glucoamylase (exo-1, 4-α-D-glucan-glucanohydrolase, EC 3.2.1.3) belongs to the most important catalytically active proteins having broad possibilities of technical use. It is an exo acting enzyme that yields ß-D-glucose from the non-reducing chain ends of amylose and amylopectin by hydrolyzing α-1, 4 linkages in a stepwise manner [1]. It also hydrolyses α-1, 6 and the rare α-1, 3 linkages although at much slower rate [2]. Generally, amylases, (that is α- amylases, β-amylases and glucoamylase) can be produced either by submerged fermentation (SmF) or solid state fermentation (SSF) procedures; however, the convectional amylase production is carried out by submerged fermentation [3] Glucoamylase production from microbial sources especially from *Aspergillus niger* is generally extracellular, and the enzyme can be recovered from culture filtrates. However, the extensive utilization of glucoamylase is obtained by using the fungus *Aspergillus niger* in enzyme production industries. Enzymes may require metal ions for their maximal catalytic activity and are termed as holoenzymes. Metals are responsible for right orientation of active site of holoenzymes. Metal binding to enzyme is one of the factors responsible for protein stabilization [4,5]. As a result of the important role of glucoamylase, it is highly desirable to increase or enhance the glucoamylase activity in other to improve its useful applications and utilization.

Materials and methods

Chemicals

Bovine serum albumin (BSA), Sephadex G-100 was purchase from Sigma Chemical Company Limited (USA). Folin–Ciocalteau was purchased from Sigma-Aldrich (USA). Ammonium Sulphate and Tris HCL salt were purchased from British Drug House (BDH) Chemicals Limited (USA). All other chemicals used in this work were of analytical grade and were obtained from reputable sources.

Collection of plant material and processing of the starch

Plant material (guinea corn seed and cassava tuber) was purchased from the Ogige market in Nsukka, Enugu state, Nigeria, and its starch was processed as described by Agboola [6].

Fractionation of starch into amylopectin

The starch obtained from guinea corn was fractionated according to the method of Sobukola and Aboderin [7].

Isolation and identification of the glucoamylase producing fungi

Glucoamylase producing fungi was isolated and stored according to the method described by Martin et al. [8] and the identification was carried out using the method of Barnett and Hunter [9].

Fermentation broth for the enzyme production

Submerged fermentation (SmF) technique was employed using an Erlenmeyer flask containing 700 ml of sterile cultivation medium optimized for glucoamylase with 2.1 g $(NH_4)2SO_3$, 4.2 g KH_2PO_4, 0.7g $MgSO_4.7H_2O$, 0.07g $FeSO_4$ and 7 g amylopectin from guinea corn starch. From the 700 ml of the sterile cultivation medium optimized for glucoamylase, 50 ml each were poured into fourteen 250 ml cornical flask, labelled day one to day fourteen. These flasks were stopped with aluminium foil and autoclaved at 121°C for 15 min to ensure sterility. For inoculation, three days 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 medium was incubated for fourteen days (14 days) at room temperature. For each day of harvest, a flask is selected serially from the flasks labelled day one to day fourteen and then, the mycelia biomass is separated by filtration, using a muslin cloth. Each day, a flask was selected according to their labels and the filtrate analyzed for glucoamylase activity and extracellular protein concentration through the fourteen days of fermentation.

Mass production of enzyme

After the fourteen days pilot submerged fermentation (SmF) studies, the day of peak glucoamylase activity was chosen for mass production of enzyme (that is, day five). Several Erlenmeyer flasks were used to produce two litres (that is, 2000 ml) of the enzyme. Harvesting was carried out on the day of peak glucoamylase enzyme activity. The medium composition that made up the 2000 ml was composed of 6.0 g $(NH_4)2SO_3$, 12 g KH_2PO_4, 2.0 g $MgSO_4.7H_2O$, 0.2 g $FeSO_4$ and 17.165 g amylopectin from guinea corn starch.

Assay of glucoamylase

Glucoamylase activity was assayed according to Miller [10]. It involves 0.5 ml of the enzyme incubated with 0.5 ml of 1 % starch dissolved in 20 mM sodium acetate buffer pH 5.0 at 55°C for 20 min. The released glucose was monitored by the addition of 1 ml of 3, 5-dinitrosalicyclic acid (DNSA), which also stopped the reaction, this was followed by boiling the mixture for 10 min. Thereafter, 1 ml of tartarate was added to stabilize the colour. The mixture was allowed to cool and the absorbance read spectrophotometrically at the wavelength of 540 nm. One enzyme activity unit (U) was defined as the amount of enzyme releasing 1 micro mole of glucose from the substrate in one minute under standard assay condition. The concentration of the released glucose was estimated using a glucose standard curve.

Assay of amylase

Amylase activity was determined by the dinitrosalicyclic acid (DNSA) method as described by Bernfeld [11]. The amylase activity was assayed by incubating 0.5 ml of the enzyme with 0.5 ml of 1% w/v starch dissolved in 20 mM sodium phosphate buffer pH 7.0, at 55°C for 60 min. The released reducing equivalent (maltose) was monitored by the addition of 1ml of 3, 5-dinitrosalicyclic acid (DNSA), which also stopped the reaction, this was then followed by boiling for 10 min, thereafter, 1 ml of tartarate was added to stabilize the colour, the mixture was then allowed to cool and the absorbance read spectrophotometrically at a wavelength of 600 nm against a blank, using UV-VIS (JENWAY 6405) spectrophotometer. The blank was made up of 0.5 ml of the 1 % starch dissolved in 20 mM sodium phosphate buffer pH 7.0, 1 ml of 3, 5-dinitrosalicyclic acid and 1 ml of tartarate. The same procedures used for the test solutions were used for the blank except that the blank lacked the enzyme solution. One unit of amylase activity was expressed as the amount of enzyme that releases one micro mole (μmole) of the reducing equivalent (maltose) per minute under assay conditions. Amylase activity was estimated by the amount of reducing equivalent (maltose) released during the hydrolysis of the starch.

Protein determination

Protein content of the enzyme was determined by the method of Lowry et al. [12], using Bovine Serum Albumin (BSA) as standard.

Enzyme purification

The crude glucoamylase was purified by using the following and important purification techniques

i) Ammonium Sulphate Precipitation.

ii) Gel Filtration

For the partial purification of the enzyme, the crude enzyme was submitted to fractionation by ammonium sulphate precipitation (70% saturation). The precipitate was recovered by centrifugation at 4000 rpm for 10 min and the pellet dissolved in 20 mM sodium acetate buffer pH 5.5. The suspension was further subjected to gel chromatography on sephadex G-100.

The active fractions were pooled. Enzyme activity and protein concentration were determined in the eluted solution at 540 nm and 280 nm respectively.

Enzyme characterization

The partially purified glucoamylase was subjected to characterization through kinetic studies by studying the effect of pH, effect of temperature and effect of metalion (Ca^{2+}, Zn^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+} and Pb^{2+}) concentration on the enzyme activity as described by Vijayaraghavan et al. [13].

Optimum pH

The optimum pH for enzyme activity was determined using 0.02 M sodium acetate buffer pH 3.5 to 5.5, phosphate buffer pH 6.0 to 7.5 and Tris-HCl buffer pH 8.0 to 10.0 at intervals of 0.5, then, 0.5 ml of gelatinized starch solution (1%) was equilibrated with 1 ml of the buffers (20 mM) of respective pHs for 5 min at 37°C. 0.5 ml of the enzyme was added and the reaction mixture was mixed properly and allowed to stand for 20 min at 50°C. The glucoamylase activity was assayed as described above using starch as substrate

Optimum temperature

The optimum temperature for glucoamylase activity was determined by incubating the enzyme with gelatinized starch solution (1%) at 30 to 80°C for 20 min at the predetermined optimal pH. Glucoamylase activity was assayed as described above using starch as substrate.

Effect of metal ions on glucoamylase activity

Different concentrations (2 mM, 3 mM, 4 mM and 5 mM) of metal salts ($CaCl_2$, $MgCl_2$, $MnCl_2$, $FeCl_2$, $ZnCl_2$ and $PbCl_2$) were prepared in

20mM sodium acetate. Each of the reaction mixtures contained 0.5 ml of enzyme solution, 0.5ml of 1% starch solution and 1 ml of metal ion solutions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+} and Pb^{2+}). The mixtures were incubated for 20 min at the predetermined optimal pH and optimal temperatures. The control was carried out without metal ions.

Results and Disscussion

Amylopectin yield

Amylopectin fractionated from guinea corn starch was used as the carbon source for the extraction of glucoamylase from Aspergillus niger. Table 1 summarizes that amylopectin fractionated from the guinea corn starch gave a yield of 62.4% at temperature of 100°C and extraction time of 60 min, using the method described by Sobukola and Aboderin [7]. Mahsa et al. [14] discovered during the partial fractionation of wheat starch using zonal ultracentrifugation that in cereal starches, they is approximately 25% amylose and 75% amylopectin. Balole and Legwaila [15] studied the starch components of guinea corn and discovered that the guinea corn starch has approximately 70–80% amylopectin content. Lawal et al. [16,17], in the study on the functional properties of amylopectin and amylose fractions isolated from bambarra groundnut starch, discovered that the amylose and amylopectin content of the bambarra groundnut starch were 75% and 11% respectively.

Weight of the Guinea corn starch (g)	Weight of the dried amylopectin (g)	Percentage yield (%)
20	12.48	62.4

Table 1: Percentage yield of the amylopectin from guinea corn starch

This high amylopectin content could be responsible for easy gelatinization, as the high amylopectin content of starch aids starch gelatinization Okporie et al. [16].

Preliminary studies

Figure 1 shows the glucoamylase activities during the experimental study of fourteen (14) days (1-14 days). High glucoamylase activity were obtained on the fifth (5th) day (319.059 μmol/min) and on the twelfth (12th) day (299.5626 μmol/min), when cassava starch was used as the substrate, while the least activity was on the fourteen (14th) day (12.7551 μmol/min). Glucoamylase activity increased from day 3 to day 5 and from day 11 to day 12, with maximum levels of glucoamylase activity obtained at day five (5) and day twelve (12) of submerged fermentation. Similar observations were reported by Fabiana et al. [18], in their report; the maximum glucoamylase activity was achieved on day 3 of the submerged fermentation. Also, the decrease in glucoamylase production from day 6 to day 10 and from day 12 to day 14 could be attributed to high glucose concentration obtained by the microorganism, Christiane et al. [19], in their research, reported that glucoamylase production by *Aspergillus niger* is repressed due to high glucose concentration in the microorganism, nevertheless, when glucose concentration decreases in the microorganism, the microorganism (Aspergillus niger) then starts to produce glucoamylase again, to hydrolyze the substrate (in this case, amylopectin from guinea corn starch) to produce more glucose due to increased production of glucoamylase, as observed from the peaks obtained.

Figure 1 also shows α -amylase activity during the pilot study of fourteen (14) days (1-14 days) when cassava starch was used as substrate. High α-amylase activities were obtained on the fifth (5th) day (103.290 μmol/min) and on the twelfth (12th) day (166.666 μmol/min), while the least activity was on the fourteen (14th) day (75.431 μmol/min). Kshipra et al. [20], in their work, reported that other amylolytic enzymes particularly α-amylase is most likely to be concomitantly produced with glucoamylase. Figure 1 also shows, protein concentration of the filtrate for an incubation period of fourteen days (14) days (1-14 days). The highest protein concentration was obtained on the fifth (5th) day (13.2826 mg/ml) while the least protein concentration (0.7391 mg/ml) was obtained on the first (1st) day. The protein production observed in this study could be due to an array of proteinous metabolites generated during the growth and metabolism of the microorganism. The day of highest glucoamylase activity (that is, day five) was then mass produced.

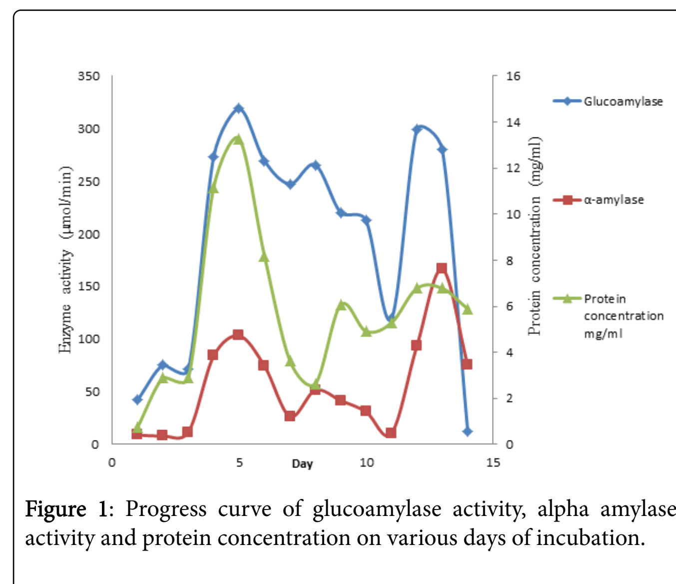

Figure 1: Progress curve of glucoamylase activity, alpha amylase activity and protein concentration on various days of incubation.

Purification studies

The partial purification of glucoamylase harvested on day five of the submerged fermentation is summarized in Table 2.

Enzyme	Volume (ml)	Protein (mg/ml)	Total Protien	Activity (μmol/min)	Total activity	Specific (U/mg)	Purification Fold	% Yield
Crude	1000	0.455	455	331.9	331900	729.45	1	100
(NH4)2SO4	280	1.662	465.36	109.67	30707.6	65.98	0.1	9.25

Gel filtration	30	0.134	4.02	24.19	725.7	180.52	2.7	2.36

Table 2: Purification table for glucoamylase harvested on day five of the submerged fermentation

In this study, maximum glucoamylase activity was obtained with 70% of ammonium sulphate $(NH_4)2SO_4$ concentration and the enzyme activity was found to be 109.67 µmole/min and a specific activity of 65.98 U/mg. After ammonium sulphate purification, gel filtration was carried out to purify more glucoamylase. From the elution profile of the gel chromatography on sephadex G-100 (Figure 2), peaks showing glucoamylase activity were obtained, these peaks were pooled together and enzyme activity as well as protein concentration was assayed. In this study, after the gel filtration, glucoamylase activity was obtained to be 24.19 µmole/min, with a specific activity of 180.52 U/mg. The decrease in the enzyme activity after each purification step could be attributed to the removal of impurities such as other proteins which enhances the enzyme activity as the enzyme might have lost some cofactors or ions that it needs for its activity. In similar manner, decrease in specific activity of the enzymes after ammonium sulphate precipitation may be attributed to the precipitation of other unwanted proteins at different percentages of the ammonium salt during the salting out process. Sangeeta et al., [21] reported similar result during the extraction and purification of glucoamylase and protease produced by Aspergillus awamori in Single-Stage fermentation. Also, the increase in specific activities for both enzymes after gel filtration could be ascribed to the fact that for a purification procedure to be successful, the specific activity of the desired enzyme must be greater after the purification procedure than as it was before Lukong et al., [22]. The percentage yield of the enzyme was 100%, 9.25% and 2.36% for crude, precipitate and gel filtration chromatography respectively.

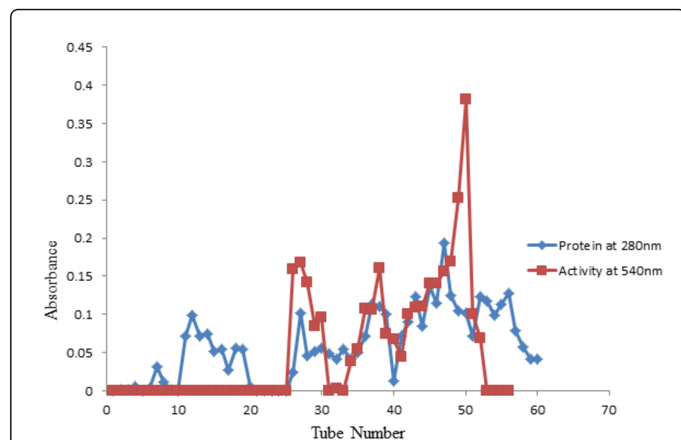

Figure 2: Elution profile of glucoamylase (harvested on day five of submerged fermentation) during gel filtration on a 120 ml volume of sephadex G-100 packed into a column (75 by 2.0 cm) and equilibrated with 50 mM sodium acetate buffer, pH 5.0. A volume of 5 ml of 60 fractions were collected at an elution rate of 5 ml per 20 min. Glucoamylase activity and protein determination was assayed in each of the fractions collected.

Characterization of glucoamylase

Optimum pH: The effect of pH on the glucoamylase activity is as shown in Figure 3. As the pH increased, the activity of glucoamylase also increased until optimum pH was obtained at 7.5 with an enzyme activity of 67.416 µmol /min when cassava starch was used as the substrate, then the activity of the enzyme decreased till pH 11. James and Lee [23] reported that the range of glucoamylase pH is between 3.7 and 7.4. Nahar et al., [24] reported that the optimum pH range of glucoamylase varies from 4.5 to 5.5 with stability at pH 7.0. Changes in pH can change the shape of the active site in an enzyme. Extremely high or low pH concentrations usually result in complete loss of enzyme activity due to denaturation Helms et al. [25].

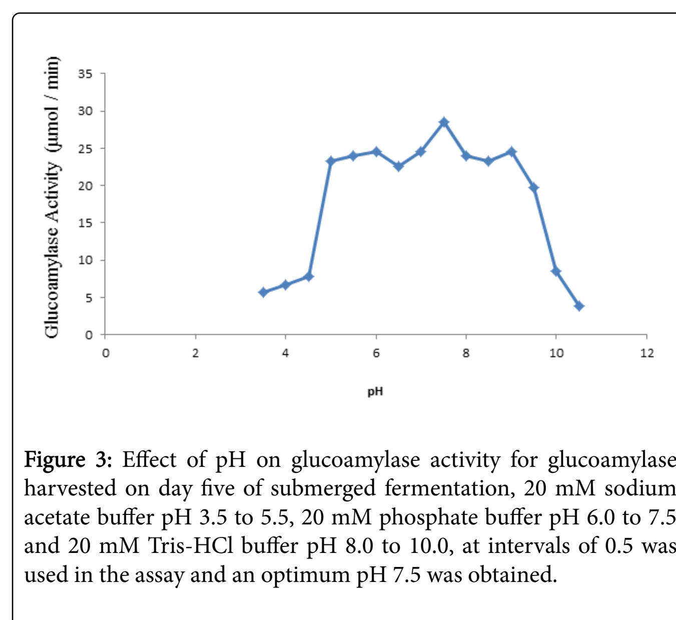

Figure 3: Effect of pH on glucoamylase activity for glucoamylase harvested on day five of submerged fermentation, 20 mM sodium acetate buffer pH 3.5 to 5.5, 20 mM phosphate buffer pH 6.0 to 7.5 and 20 mM Tris-HCl buffer pH 8.0 to 10.0, at intervals of 0.5 was used in the assay and an optimum pH 7.5 was obtained.

Optimum temperature

The optimum temperature for glucoamylase harvested on day five was 50°C, with glucoamylase activity of 67.055 µmole/min as shown in Figure 4; this was in accordance with Siddhartha et al., [26] who reported that temperature optima for glucoamylases are generally in the range of 45°C to 60°C. Sarojin et al., (2012) also reported that optimum glucoamylase activity was obtained at 45°C. All enzymes have an optimal temperature at which reaction rates go fastest without denaturation of the enzyme Campbell and Reece, [27].

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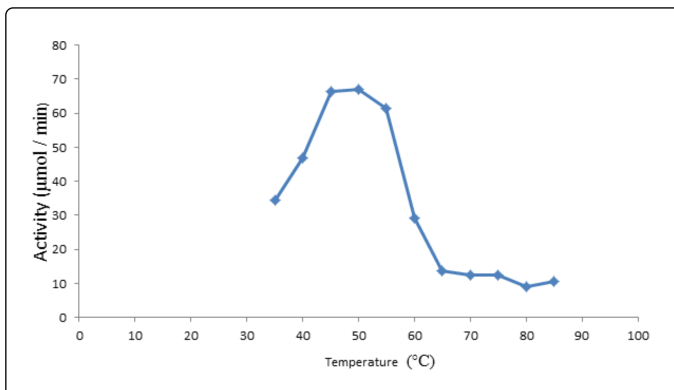

Figure 4: Effect of temperature on glucoamylase activity for glucoamylase harvested on day five of submerged fermentation. Activity was assayed in 20 mM phosphate buffer pH 7.5 from 30pbel°C to 90°C after incubation for 20 min and coupling with DNS (3, 5- dinitrosalicyclic acid). Optimum temperature of 50 °C was obtained.

Effect of metal ion concentration

Figure 5 summarizes the effect of metal ion concentration on glucoamylase activity. Ca^{2+}, Zn^{2+}, Co^{2+}, Fe^{2+} and Mn^{2+} ions all enhanced the activity of glucoamylase, while Pb^{2+} inhibited the glucoamylase activity.

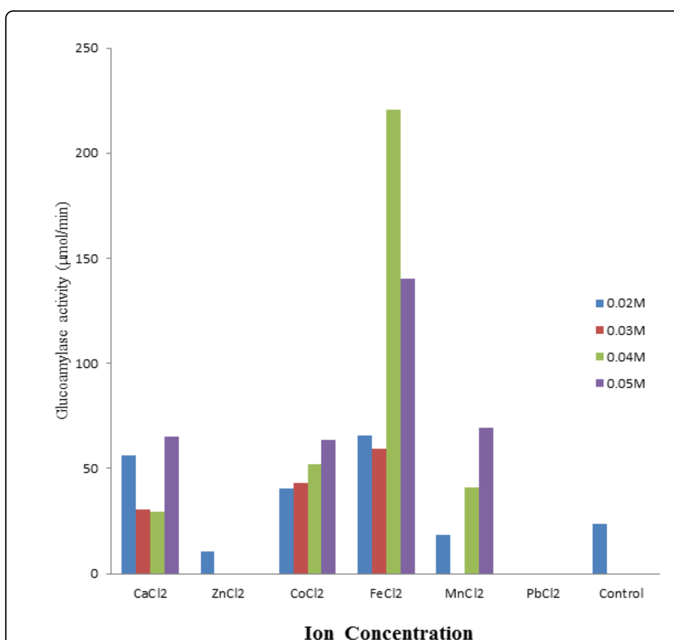

Figure 5: Effect of various ion concentrations (2 mM, 3 mM, 4 mM and 5 mM) of metal salts ($CaCl_2$, $MgCl_2$, $MnCl_2$, $FeCl_2$, $ZnCl_2$ and $PbCl_2$) on glucoamylase activity for glucoamylase harvested on day five of submerged fermentation. The enzyme activity was enhanced by Ca^{2+}, Co^{2+}, Fe^{2+} and Mn^{2+} while Zn^{2+} and Pb^{2+} had inhibitory effect on the glucoamylase activity.

The concentrations of the Fe^{2+} solutions gave the highest activity of glucoamylase as 0.02 M, 0.03 M, 0.04 M and 0.05 M showed an activity of 65.597, 59.584, 220.84 and 140.488 µmol/min respectively. All the concentrations of the lead ion used complete inhibition of the enzyme. Vivian et al., [28] reports the activation of glucoamylase from Aspergillus phoenicis by manganese (Mn^{2+}) and calcium (Ca^{2+}) ions. The increase in glucoamylase activity caused by these metal ions (Ca^{2+}, Zn^{2+}, Co^{2+}, Fe^{2+} and Mn^{2+} ions) could be attributed to the ability of these metals ions to serve as an electron donor or Lewis acid as they participate directly in the catalytic mechanism of the enzyme. Similarly, the decrease in the enzyme activity caused by the lead ion could be due to the interaction of the lead ion with the amino acid residues of the catalytic sites that also serves as electron donors in the catalytic site of the enzyme, as most heavy metals interfere strongly with amino acid residues of the catalytic site of enzymes [29].

Conclusion

In the light of above result, it therefore suggests that the enzyme glucoamylase can be enhanced by the use of some metal ions (such as Ca^{2+}, Co^{2+}, Fe^{2+} and Mn^{2+}) to bring about an improved use of the enzyme. Glucoamylase is found useful in industries where starch hydrolysis is required, such as, the food processing, textile industries, brewery industries and in fermentation biotechnology

Contributions

Prosper secured the samples used in this research work, carried out the research work jointly and together with Arinze, under the supervision of Chilaka and Eze, while Kenneth read and arranged the manuscript of this work.

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