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Raw Starch Degrading, Acidic-Thermostable Glucoamylase from Aspergillus fumigatus CFU-01: Purification and Characterization for Biotechnological Application

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Research Article

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ABSTRACT

Improvements in the industrial process involved in starch degradation have led to the search for thermostable, salt-loving amylolytic enzymes especially glucoamylase, an enzyme that can completely hydrolyze starch to glucose. In this research, glucoamylase was optimally produced in liquid culture from *Aspergillus fumigatus* and purified to homogeneity by ammonium sulphate precipitation, ion-exchange chromatography and gel filtration, giving a yield of 8.19% with 20-fold purification. The 50 kDa glucoamylase, molecular weight estimated by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was highly stable over a wide pH range in the acidic region and also thermostable; retaining about 70% of initial activity after 60 min of incubation at 60ºC. The purified enzyme was active on amylose, dextran and different starches with K_m and V_{max} values of 1.59 mg/mL and 14.33 U/mg respectively, when raw cassava starch was used as substrate. Remarkably, this glucoamylase possesses high salt-tolerant property, with about 50% residual activity after 24 h incubation in 3.0 M NaCl solution. Some metal ions including K^+, Ca^{2+} and Mg^{2+} were activators of the enzyme while Cu^{2+}, Pb^{2+} and Hg^{2+} inhibited its activity. The unique biochemical characteristics of this glucoamylase qualify it for biotechnological use especially in food, pharmaceuticals and biofuel industries.

INTRODUCTION

In the face of challenging energy crises and global environmental health concerns associated with the use of fossil fuel, the development and use of alternative energy such as biofuel become highly imperative. Starch, a high molecular weight biopolymer and principal storage form of carbohydrate in plants, has been identified as a major feedstock for the generation of biofuel, a major renewable energy. Starch is one of the most abundantly distributed polysaccharides in nature and it is known together with cellulose, to be most abundant carbohydrate polymers on earth [1]. The starch molecules are first hydrolyzed by the action of bond-specific amylases into simple sugars which are in turn converted to ethanol by microorganisms, usually yeasts in a fermentation process. Economically, starch industry roughly accounts for 15–20 % of the total industrial consumption of enzymes [2].

Glucoamylase (EC 3.2.1.3) is one of the best known amylases but with unique starch hydrolyzing property among the amylase enzyme family in that it can completely hydrolyze starch and other 1,4-linked glucose-oligosaccharides by attacking both α-(1,4) and α-(1,6) glycosidic bonds in starch with direct formation of glucose [3]. In fact, glucose, the principal carbon source in many biotechnological processes, at present is mainly produced industrially by the enzymatic hydrolysis of starch replacing the acid hydrolysis method, since it is economically efficient and pure products are obtained in the final stages of the process [4,5].

Enzymes, when produced industrially by means of fermentation technology from microorganisms, are often contaminated with a number of other concomitantly produced enzymes that are active on the same substrate, thus, it is significant to develop economic processes for the optimum production of enzymes of interest. Efficient process is needed also for their purification to obtain pure enzymes with maximum specific activity [6] and to further understand the nature and mechanism of action of such

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enzymes. The production, purification, characterization and utilization of enzymes are unarguably very important in biotechnology and to meet the industrial requirements for starch processing, a glucoamylase with excellent thermostability, raw-starch degradation ability and high glucose yield is much needed [7]. In this paper appreciable biochemical characteristics of a purified glucoamylase produced from *Aspergillus fumigatus* CFU-01 are presented.

MATERIALS AND METHODS

Collection and Preparation of Soil Samples

Soil samples were collected randomly in clean and sterilized polyethylene plastic bags from the top soil layer, 10 cm deep from the soil environment of a cassava processing industry in Akure metropolis, Ondo State, Nigeria (coordinates 7°10'N 5°05'E) in August, 2014. One gram (1 g) soil was aseptically transferred to 9 mL sterile distilled water and the resulting mixture was well mixed, allowed to stand for a few minutes before decanting. The supernatant was then serially diluted and subjected to microbial analysis.

Isolation, Identification and Screening of Microorganisms for Amylolytic Activity

Microorganisms were isolated from the collected soil samples by serial dilution plate method and the identification of the fungi species was done according to the taxonomical methods of Gilman [8]. The soil fungi species were screened for amylolytic activity by inoculating pure culture of each identified organism onto freshly prepared starch agar medium. Plates were incubated at 37°C for 72 h, after which they were stained with a solution containing potassium iodide, KI (0.1% w/v)/ I_2 (0.1% w/v) in 1 M HCl for 15 min and later de-stained with distilled water. The plates were checked for the zone of clearance around the colonies which indicates the presence of an amylolytic enzyme.

Inoculum Preparation and Enzyme Production

The production of glucoamylase was carried out under optimized conditions of submerged fermentation [9]. The liquid mineral medium consist of soluble starch; 1%, yeast extract; 0.67%, ammonium sulphate; 0.333%, magnesium sulphate heptahydrate; 0.05%, iron(II)sulphate; 0.01%, calcium chloride dihydrate; 0.01%, potassium dihydrogenphosphate; 0.02% with pH adjustment to 5.0. The fermentation process in a set of shake-flasks containing five Erlenmeyer flasks (250 mL) was allowed to go on for 72 h in an orbital shaker (a product of Stuart, Model S150) set at 150 rpm and 30°C. The production media was centrifuged at 10,000 x g for 20 min at 4°C to obtain a cell-free supernatant herein referred to as the crude enzyme.

Enzyme Activity Assay and Protein Concentration Determination

Glucoamylase activity was determined by a standard assay procedure earlier described by Cereia et al. [10] Enzyme solution (1 mL) was added to a test tube containing 1 mL of 1% (w/v) soluble starch buffered with 0.05 M acetate buffer, pH 5.5 and incubated at 60°C in a water bath for 10 min. The amount of reducing sugar (glucose) released was estimated by the method of Miller [11]. One unit of enzyme activity was defined as the amount of enzyme required to liberate one micromole of reducing sugar per minute under standard assay conditions.

The total protein concentration of the solutions was determined by the method of Bradford [12], using Bovine Serum Albumin (BSA) as the standard protein.

Purification of Enzyme

The purification of glucoamylase from the culture filtrate was done in a 3-step process; the crude enzyme was gradually brought to 60% saturation with solid ammonium sulphate on cold bath and centrifuged at 10, 000 x g for 20 min. The precipitated fraction (pellet) containing glucoamylase was re-dissolved in sodium acetate buffer (50 mM, pH 5.5) and was dialyzed against same buffer at 4ºC extensively. The dialysate (32 mL) was loaded onto a CM–Cellulose column (25 mm diameter and 40 cm height product of Pharmacia) previously equilibrated with the same buffer; fractions of 5.0 mL each were collected at a flow rate of 60 mL/h and the adsorbed protein was eluted with increasing salt gradient to a final concentration of 1.0 M sodium chloride. The presence of protein in the eluted fractions was monitored by measuring the absorbance at 280 nm and glucoamylase activity assay was carried out on the eluted fractions using the described standard assay procedures. The active fractions were pooled and concentrated using an ultrafiltration system before loading onto a Sephadex G-100 gel filtration column (25 mm diameter and 75 cm height - product of Pharmacia) previously equilibrated with 50 mM acetate buffer (pH 5.5) and adjusted to a final flow rate of 15 mL/h.

Determination of Molecular Weight by SDS-PAGE

The molecular weight of the purified glucoamylase was determined under denaturing condition by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (10% SDS-PAGE) as described by Laemmli [13] using the Bio-Rad electrophoresis system (Bio-Rad, UK).

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Physicochemical Characterization

Effect of ph on enzyme activity and enzyme stability: To determine the optimum pH for glucoamylase activity, the substrate was prepared in different buffers: 50 mM Glycine-HCl buffer (pH 2.0–3.0), 50 mM sodium acetate buffer (pH 4.0 – 5.5), 50 mM potassium phosphate buffer (pH 6.0 - 7.5) and 50 mM Tris-HCl buffer (pH 8.0 - 9.0) and the glucoamylase activity was determined under standard assay conditions. To determine the pH stability, the enzyme was pre-incubated in various solutions of pH 4.0 - 7.0 at room temperature for 2 h with periodic sampling every 20 min. The residual activity of the enzyme was thereafter measured under standard assay conditions.

Effect of temperature on enzyme activity and enzyme stability*:* The optimal temperature for the activity of the purified glucoamylase was determined by carrying out the standard assay at different temperatures (30–100°C) in 50 mM sodium acetate buffer solution, pH 5.5. The thermal stability of the purified enzyme was determined by pre-incubating the diluted enzyme solution with sodium acetate buffer (50 mM, pH 5.5) at different temperatures between 30 and 90°C for 2 h with periodic withdrawal of aliquot of enzyme every 20 min. The aliquots were first cooled on ice before measuring the residual enzyme activity under standard assay conditions.

Effect of metallic ions and edta on enzyme activity: The effects of metal ions (Na^+, K^+, Mg^{2+}, Ca^{2+}, Al^{3+}, Cu^{2+} and Hg^{2+}) and EDTA on the activity of the purified glucoamylase were determined by performing the enzyme activity assay in the presence of the chloride salts of the metal ions and EDTA at varied concentrations of 1, 5, 10, 20 and 30 mM in 50 mM sodium acetate buffer (pH 5.5) using soluble starch as substrate. The relative enzyme activity was measured under standard assay conditions.

Effect of some protein denaturants on enzyme activity: The purified enzyme (5 mL) was incubated at 40°C for 30 min in 50 mM sodium acetate buffer (pH 5.5) containing the denaturants (Urea and SDS) at final concentrations of 1, 5 and 10 mM. The relative enzyme activity was measured under standard assay conditions.

Salt tolerance test: The purified glucoamylase (5 mL) was incubated in a solution of sodium chloride (NaCl) at different concentrations between 0.5 and 5.0 M for 24 h at 4°C after which the relative enzyme activity was measured under standard assay conditions to determine the tolerance of the glucoamylase to high salinity.

Kinetic studies

Substrate specificity studies: The relative hydrolysis rate of various substrates (soluble starch, amylose, soluble potato starch, dextran, raw cassava starch, raw cornstarch and sucrose) by the purified glucoamylase was determined under standard assay conditions. The substrate solutions were prepared at a concentration of 1% in 50 mM sodium acetate buffer (pH 5.5) and the relative activity of the enzyme with each substrate was measured with respect to soluble starch.

Determination of kinetic parameters: The kinetic parameters (K_m and V_{max}) of the purified glucoamylase were determined by measuring the initial reaction rates with soluble starch, raw corn starch, potato starch, raw cassava starch, dextran, amylose and sucrose at various substrate concentrations (0.1-10 mg/mL). The accurate values of the apparent kinetic parameters were obtained from double reciprocal plot by Lineweaver and Burk [14].

RESULTS

Isolation, Identification and Screening of Microorganisms for Amylolytic Activity

From the collected soil sample, the isolated and identified *Aspergillus species; A. flavus, A. fumigatus* and *A. parasiticus*, were observed to be very good producers of amylases with zones of clearance of 3.31 cm, 3.34 cm and 3.18 cm respectively **(Table 1)**. A clear halo zone around the colonies indicated amylolytic activity. The isolate designated *Aspergillus fumigatus* CFU-01 was thereafter chosen for glucoamylase production under submerged fermentation.

Table 1. Plate (qualitative) screening of isolated soil fungi species for amylolytic potential

Isolate code	Halo-zone diameter (cm)	Amylolytic potential	Name of Isolate Identified
CNP-01	2.66	+ +	*Penicillium notatum*
CFA-01	3.31	+ + +	*Aspergillus flavus*
CCE-01	2.38	+ +	*Trichoderma viride*
CFU-01	3.34	+ + +	*Aspergillus fumigatus*
CNP-02	1.38	+	*Penicillium citrinium*
CSP-01	3.18	+ +	*Aspergillus parasiticus*
CGA-01	0.67	-	*Geotricum albidum*
Remark: + + + very good producer + + good producer - Poor producer + weak producer			

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Enzyme Purification

The crude enzyme solution (650 mL) from *Aspergillus fumigatus* CFU-01 had a total glucoamylase activity, total protein and specific activity of 16178.5 U, 2900.95 mg and 5.58 U/mg proteins respectively. After elution from the CM-Cellulose ion-exchange column, one main peak with total glucoamylase activity of 1485.12 U and total protein content of 20.502 mg were obtained **(Figure 1)**. The elution profile of the glucoamylase from the Sephadex G-100 gel filtration column also showed one major peak of glucoamylase activity **(Figure 2)**. Each of the purification steps resulted in enhanced specific activity as summarized in **(Table 2)**.

Table 2. Summary of Purification of glucoamylase from *Aspergillus fumigatus* CFU-01

Purification Step	Volume (mL)	Enzyme Activity (U/mL)	Protein Concentration (mg/mL)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Purification Fold
Crude filtrate	650	24.89	4.463	16178.5	2900.95	5.58	100	1
Amm. Sulf. Ppt (60%)	32	52.694	3.214	1686.208	102.848	16.4	10.42	2.94
Ion-exchange Chromatography on CM-Cellulose	51	29.12	0.402	1485.12	20.502	72.44	9.18	12.99
Gel filtration on Sephadex G-100	56	23.66	0.212	1324.96	11.872	111.6	8.19	20.01
All Purification steps were carried out at 4 °C								

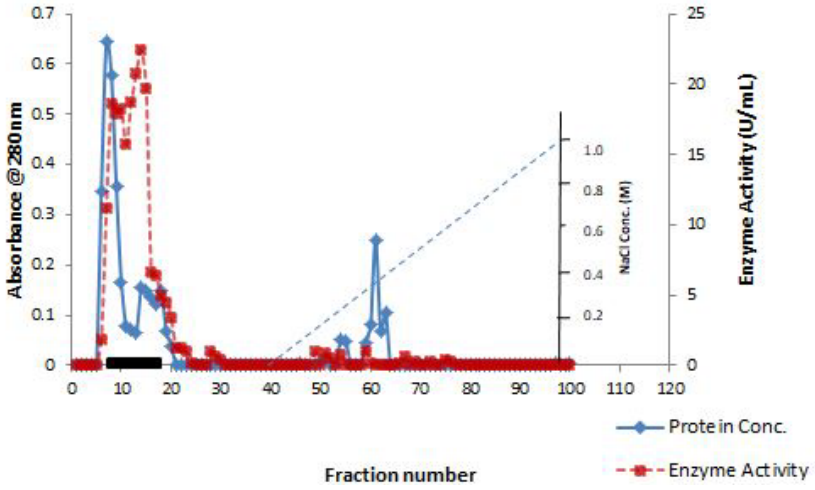

Figure 1. Elution profile of glucoamylase from *Aspergillus fumigatus* CFU-01 using CM-cellulose cationic exchanger.

..... Indicates NaCl concentration gradient.

▬ Indicates the pooled fractions

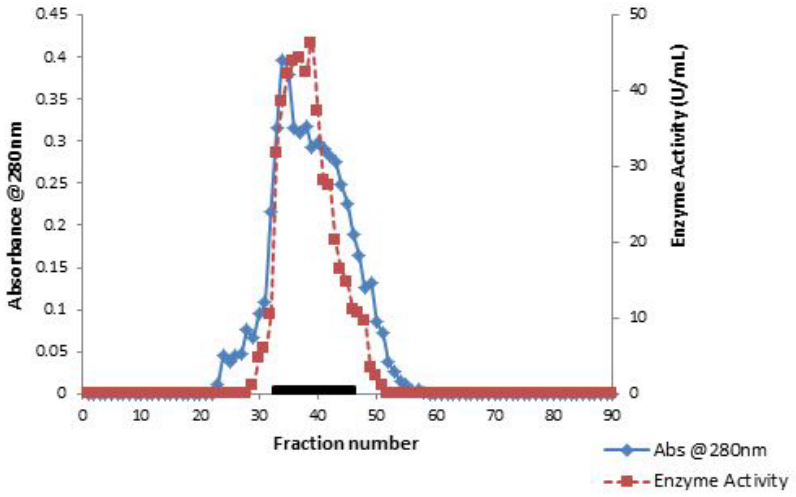

Figure 2. Elution profile of glucoamylase produced from *Aspergillus fumigatus* CFU-01 separated on Sephadex G-100 column chromatography.

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The gel filtration chromatogram showed one major peak with glucoamylase activity which is

[redacted] Indicates the pooled fractions

Molecular Weight of Glucoamylase

The molecular weight of the purified glucoamylase from Aspergillus fumigatus CFU-01 as estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was 50 kDa (approximate) **(Figure 3)**.

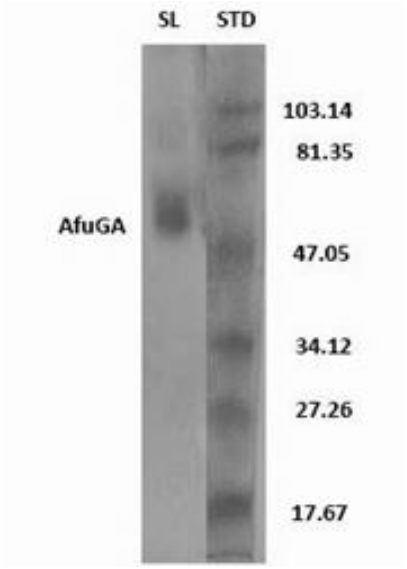

Figure 3. SDS-PAGE of purified glucoamylase from *Aspergillus fumigatus* CFU-01 after gel filtration on Sephadex G-100. Lane SL: purified Aspergillus Fumigatus CFU-01 Glucoamylase (AfuGA). Lane STD: Standard molecular weight markers.

Physicochemical Characterization of Purified Glucoamylase

Effect of pH on the Activity and Stability of Purified Glucoamylase: The purified glucoamylase was very active between the pH of 4 and 6.5 with pH optimum observed at pH 5.5 with activity of 22.96 U/mL **(Figure 4)**. The activity of the purified glucoamylase only reduced to about 80% of the initial activity after incubation for 1 h at pH 5.5 and to about 60% at pH 7.0. The residual activity of the purified enzyme was 55% after 2 h of incubation at pH 5.0 **(Figure 5)**.

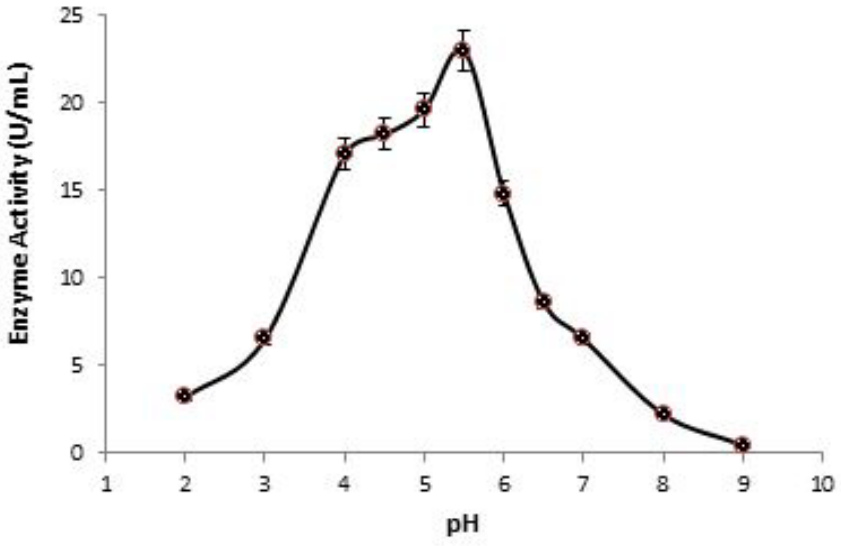

Figure 4. Effect of pH on the activity of glucoamylase from *Aspergillus fumigatus* CFU-01

(Error bar indicates mean ± standard deviation)

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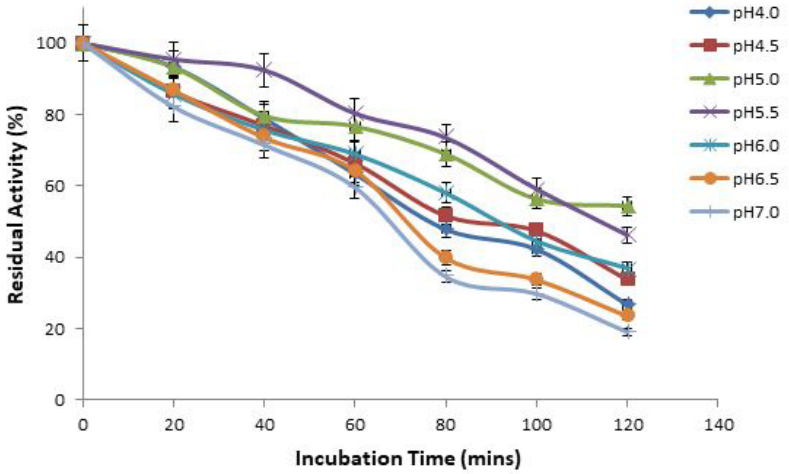

Figure 5. Effect of pH on the stability of glucoamylase from *Aspergillus fumigatus* CFU-01

(Error bar indicates mean ± standard deviation)

Effect of temperature on the activity and stability of the purified glucoamylase: High enzyme activity was observed at temperatures between 40 and 70°C **(Figure 6)**. The purified glucoamylase retained about 70 % of initial activity after 60 min of incubation at 60°C while the enzyme activity remained relatively unchanged after 2 h of incubation at 30 and 40°C retaining about 90 and 85% of initial activity respectively **(Figure 7)**.

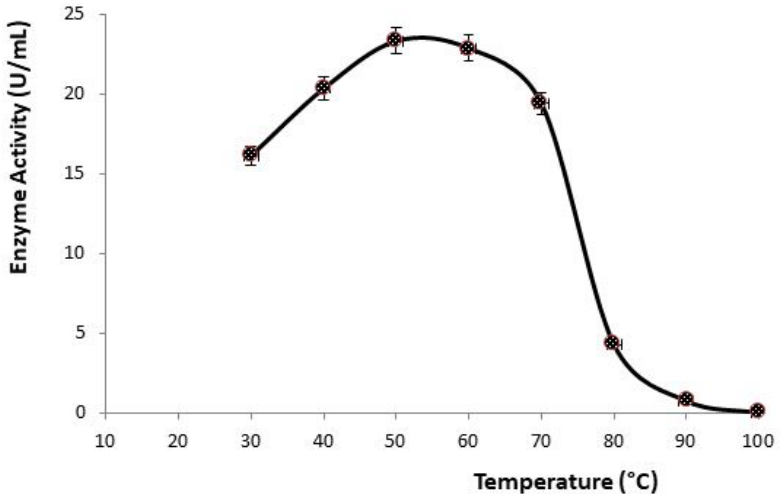

Figure 6. Effect of temperature on the activity of *Aspergillus fumigatus* CFU-01 glucoamylase

(Error bar indicates mean ± standard deviation)

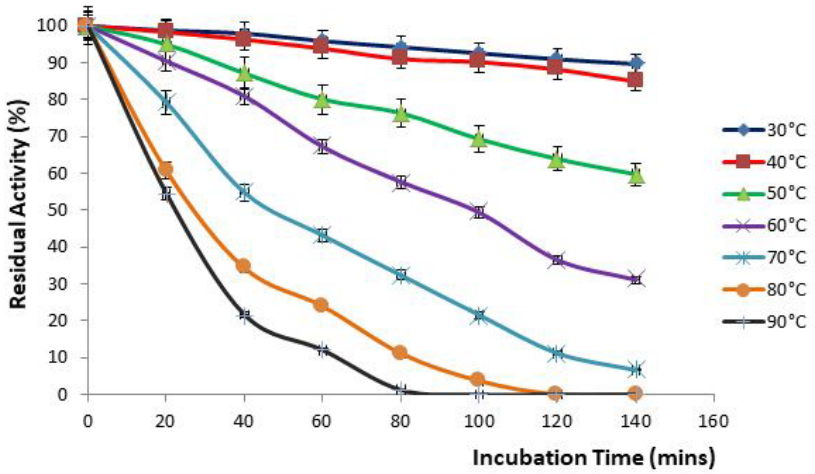

Figure 7. Effect of temperature on the stability of *Aspergillus fumigatus* CFU-01 glucoamylase

(Error bar indicates mean ± standard deviation)

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Effect of metallic ions on the activity of the purified glucoamylase: The activity of glucoamylase was stimulated by K^+ and Ca^{2+} with increasing concentration from 1 mM to 30 mM. However, Mg^{2+} was observed to stimulate the activity of the enzyme up to a final concentration of 10 mM. The activity of the purified enzyme was inhibited by Al^{3+}, Pb^{2+}, Cu^{2+} and Hg^{2+} at all salt concentrations tested **(Figure 8)**.

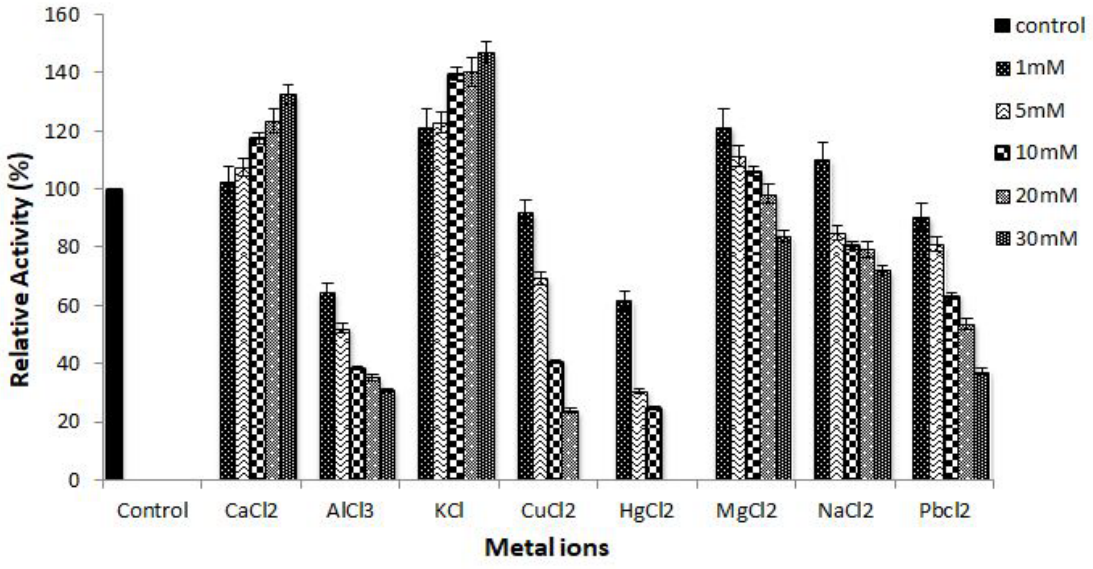

Figure 8. Effect of different Metallic Chlorides at varied concentration on the activity of glucoamylase from *Aspergillus fumigatus* CFU-01.

(Error bar indicates mean ± standard deviation of triplicates)

Effect of some chemical agents on the activity of the purified glucoamylase: The chelating agent, EDTA, has little inhibitory effect on the activity of the purified fungal glucoamylase as the relative activity stood at 92% and 83% respectively at 1 mM and 5 mM concentrations of EDTA. Urea also at these concentrations had a denaturing effect on the activity of the purified enzyme, with a relative activity of 91% at 1 mM and 86% at 5 mM. However, SDS reduced the enzyme activity relatively from 64% at 1 mM to 30% at 10 mM **(Figure 9)**.

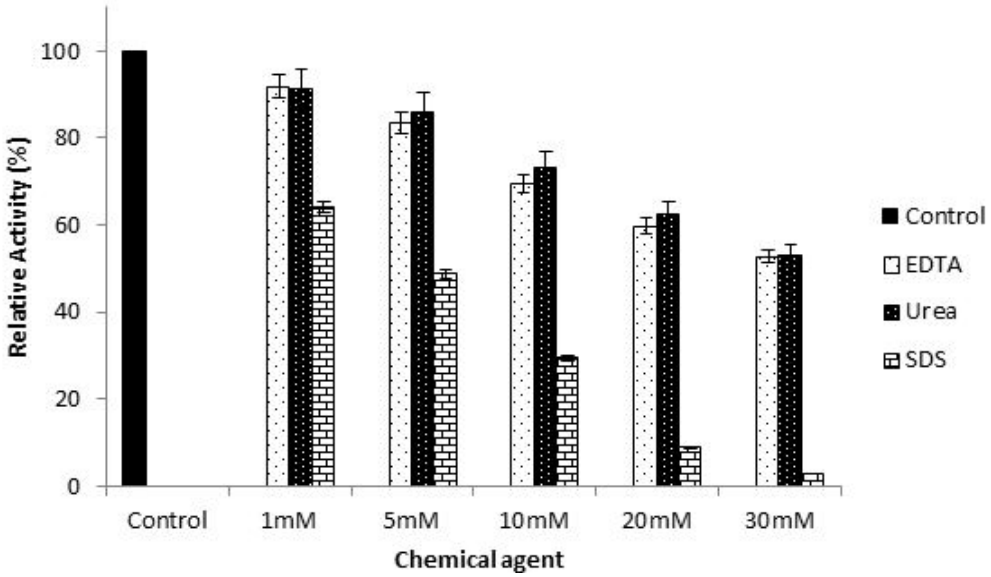

Figure 9. Effect of different chemical agents at varied concentration on the activity of glucoamylase from *Aspergillus fumigatus* CFU-01

(Error bar indicates mean ± standard deviation of three replicates)

Salt tolerance of the purified glucoamylase: The purified glucoamylase from Aspergillus fumigatus CFU-01 maintained about 90%, 67% and 50% of its initial activity after 24 hours of incubation in 1, 2 and 3 M NaCl solution, respectively **(Figure 10)**.

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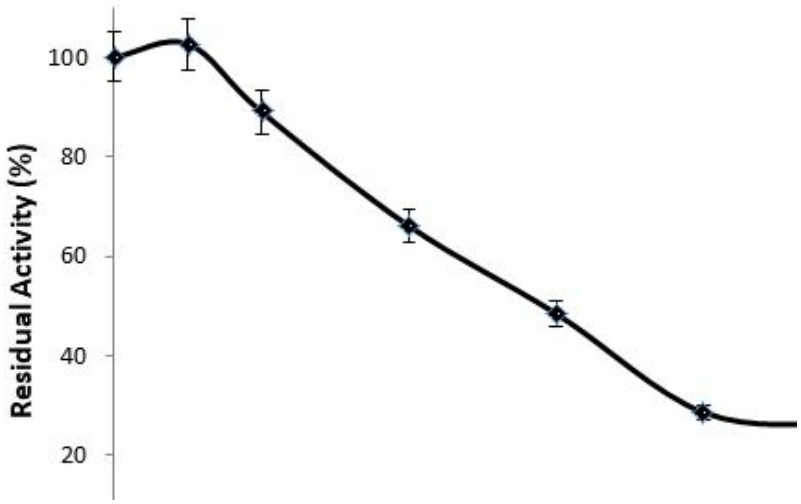

Figure 10. Salt tolerance of the glucoamylase from *Aspergillus fumigatus* CFU-01 using different concentrations of NaCl (Error bar indicates mean ± standard deviation of three replicates).

Kinetic Studies

Substrate specificity: The purified glucoamylase from Aspergillus fumigatus CFU-01 was active on long chain substrates (polysaccharides) including soluble starch, dextran, amylose, potato starch and raw starches (corn and cassava) but exhibited low activity with sucrose, a disaccharide **(Table 3)**.

Table 3. Substrate specificity of purified glucoamylase from *Aspergillus fumigatus* CFU-01

Substrate	Relative Activity (%)
Soluble Starch	100
Amylose	81 ± 3.56
Potato Starch	82 ± 4.48
Dextran	90 ± 2.16
Raw Corn Starch	86 ± 3.34
Raw Cassava Starch	69 ± 1.64
Sucrose	40 ± 1.02

Kinetic Parameters (K_m and V_{max}): The kinetic constants (K_m) and maximum velocities (V_{max}) of the enzyme for soluble starch, dextran, amylose, raw corn starch, potato starch, raw cassava starch and sucrose are summarized in **(Table 4)**.

Table 4. Michaelis-Menten Constants (K_m) and Maximun Velocities (V_{max}) of glucoamylase produced from *Aspergillus fumigatus* CFU-01 with different substrates.

Substrate	K_m (mg/mL)	V_{max} (U/mg)
Soluble Starch	1.64	20.96
Dextran	1.43	16.26
Amylose	1.57	16.67
Raw Corn Starch	1.62	19.42
Potato Starch	1.71	18.48
Raw Cassava Starch	1.59	14.33
Sucrose	1.57	8.5
Reactions carried out at pH 5.5 and 60 °C.		

DISCUSSION

The analysis of enzyme activity in crude culture filtrates or crude extracts does not accurately provide the characteristics of an enzyme as it does not indicate either an isolated enzymatic action or the presence of a multi-enzymic system working in synergy on the degradation of the substrate [15]. As such, an extensive and high yielding purification process is much needed to provide pure and homogenous glucoamylase to be studied for industrial use. In this study, the purification to homogeneity of the extracellular

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glucoamylase produced by *Aspergillus fumigatus* CFU-01 under submerged fermentation was achieved through the combination of ammonium sulphate precipitation, ion-exchange chromatography on CM-Cellulose and gel filtration on Sephadex G-100 giving a 20-fold increase in specific activity with 8.19 % recovery. Glucoamylase has been partially purified by ammonium sulphate precipitation in recent times [16,17]. Manera et al. [18] had earlier obtained a purification fold of 9.9 for glucoamylase from *Aspergillus niger* with a recovery of 47.6% using DEAE–cellulose ion exchange chromatography. Also, the combination of ammonium sulphate precipitation, ion-exchange chromatography and gel filtration chromatography for the purification of glucoamylases from different species has been reported earlier [19,20]. The 50 kDa molecular weight of the glucoamylase from *A. fumigatus* CFU-01 falls within the 25 - 112 kDa average molecular weights range of fungal glucoamylases earlier reported [21-23].

The optimum pH for maximal enzyme activity for the purified enzyme was observed at pH 5.5 which agrees with an earlier report that fungal glucoamylases from *Aspergillus strains* are usually active at acidic pH (3.5–7.0) depending on the strains and amino acid sequences [24]. Lam et al. [3] had earlier reported this same value for the glucoamylase produced from *Aspergillus awamori*. The purified glucoamylase from *Aspergillus fumigatus* CFU-01 is very thermostable. The optimal reaction temperature of 50 - 60ºC obtained for the purified enzyme is in agreement with optimal reaction temperatures usually found for glucoamylase from Aspergillus species [2,24]. The hydrolysis of starch involves two high energy demanding steps; liquefaction and saccharification, for which the pH and the temperature must be well determined in order to minimize cost and avoid the formation of undesirable by-products [25]. Interest in thermostable amylases has increased tremendously, since resistance to thermal inactivation has become a desirable property in many industrial applications [26].

Metallic ions have either inhibitory or stimulating effect on the activity of enzymes depending on the concentration of the metallic salt solution. This inhibitory effect of Al^{3+}, Cu^{2+} and Hg^{2+} was also reported for the glucoamylases produced by some strains of *A. flavus* earlier characterized [27,28] and also for the glucoamylases from other species [22,29]. The inhibitory effect of EDTA was not profound suggesting that metallic ions might not be required for activity in the active sites of these amylolytic enzymes. The purified glucoamylase was observed to appreciably tolerate high salinity compared to a previous study done on the α-amylase which reports about 60% loss of activity at high salt concentrations [30,31]. The salt tolerance test is important in saccharification of starch and in treatment of effluent with high salinity containing starch or cellulose residues in pollution control mechanism [28,31].

A unique characteristic also observed in this glucoamylase, is its high affinity for α-1,6-bonds exclusively present in dextran as well as for the α-1,4-bonds in amylose, hence, this glucoamylase could completely hydrolyze starch molecules especially raw starches whose molecular structure is a complex of these two types of bond. This is in agreement with earlier reports on the glucoamylases characterized from *A. flavus* [28,29].

The value of the kinetic constant (K_m), 1.64 mg/mL, obtained from the double reciprocal plot when soluble starch was used in the enzyme-substrate reaction carried out at pH 5.5 and 50ºC is comparable to the 1.67 mg/mL obtained by Akinmosun et al. [32] and also to the 1.90 mg/mL for the glucoamylase from *Fusarium solani* obtained by Bhatti et al. [33]. The kinetic constant, K_m can provide a lot of biochemical and physiological information about an enzyme [34]. These kinetic parameters are also fundamental to choosing the enzymes for large scale industrial applications [9] since lower K_m values allow for faster and easier industrial processes.

CONCLUSION

The results from this study affirm that *Aspergillus fumigatus* CFU-01 isolated from the soil of a cassava processing site is a good producer of the amylolytic enzyme, glucoamylase, under submerged fermentation. The purified enzyme possesses good biochemical characteristics with incredible ability to utilize and completely hydrolyze varieties of starch including raw starches. Thus, this novel glucoamylase can be employed in the different starch-utilizing industries for possible biotechnological applications, especially in the efficient conversion of starch to glucose needed for ethanol production in biofuel industries.

CONFLICT OF INTEREST

The authors declare no conflict of interest regarding the publication of this article.

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