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Research article

Isolation, purification and characterization of cellulase produced by *Aspergillus niger* cultured on *Arachis hypogaea* shells

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ABSTRACT

Cellulases are enzymes that hydrolyse cellulose and related cellu-oligosaccharides derivatives. Its applications are enormous but high cost of production is the bottle-neck against the utilization of cellulase in industries. Therefore, this study investigated the isolation, purification and characterization of cellulase produced by *Aspergillus niger* cultured on *Arachis hypogaea* shells. The crude cellulase enzyme was produced by *A. niger* through submerged fermentation process using *A. hypogaea* shells as a carbon source. The optima fermentation conditions were determined by varying different parameters. The crude cellulase was purified through ammonium sulphate precipitation, dialysis and gel-filtration chromatography. The molecular weight was estimated using sodium dodecyl sulphate polyacrylamide gel electrophoresis. The effects of pH and temperature on the activity of the purified cellulase were investigated. The study revealed that the: optimal production of crude cellulase was achieved at incubation period of 120 h, pH 4, temperature 40 °C, and inoculum size of 13 × 10^5 CFU/ml. Cellulase was purified to 68.12-fold with a yield and specific activity of 3.87% and 484.3 U/mg respectively. The V_{max} for the cellulase was 9.26 U/ml while the K_m was 0.23 mg/ml. The molecular weight of the cellulase was approximately 13.5 kDa and the enzyme has higher specificity for CMC compared to other substrates. The optimum pH and temperature for the cellulase activity were 4 and 40 °C respectively. The present study has shown that *A. hypogaea* shells can be used as a carbon source by *A. niger* for the production of cellulase.

1. Introduction

The agricultural wastes are composed principally of cellulosic or lignocellulosic matter. These materials are considered to be the inexpensive basis for the production of different utilizable products throughout the world (Karmakar and Ray, 2011). Large amount of wastes is generated as a result of agricultural practice and industrial processing of agricultural materials, predominantly from industries such as breweries, paper and pulp, textile and timber. These wastes need to be managed and if left untreated, largely amass in the surroundings as environmental pollutants (Abu et al., 2000). A huge percentage of wastes generated either from wood product industries or as a result of agricultural practices is composed chiefly of lignocellulosic materials, which under normal circumstances are indigestible and as a result add no value to animal feed compositions. Efforts must be intensified by individuals, corporate bodies or Government to convert these wastes into useful products. Presently, the conversion of cellulosic materials to commodity chemicals and fermentable sugars offer important technical and economic challenges, and its accomplishment hang on the development of extremely resourceful and cost-effective enzymes for degradation of pretreated lignocellulosic substrates to fermentable sugars. Naturally, lignocellulosic materials can be fermented by microorganisms, which may result in a product whose activities will be very low and inefficient. Therefore, these residual components of lignocellulosic materials can serve as superior substrates for the growth of microorganisms that may produce enzymes such as lipase, glucoamylase, pectinase, xylanase and cellulase either through submerged fermentation or solid-state fermentation. Generally, microorganisms of the genera *Trichoderma* and *Aspergillus* are understood to have cellulase synthetic ability, and enzymes synthesized by this class of microorganisms are commercially available for industrial uses (Sukumaran et al., 2005; Kuhad et al., 2010). Cellulases are a group of complex enzymes, which catalyze the hydrolysis of cellulose and related cellu-oligosaccharide derivatives. The cellulase complex comprises three major components namely endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) (Kaur et al., 2007; Thongekkaew et al., 2008). Cellulases can be obtained

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from different sources. Cellulases from different sources possess distinctive features since they exhibit specific pH optima, solubility depending on the amino acid composition. Thermal stability and substrate specificity may also vary with the origin (Bhat, 2000; Parry et al., 2001). Cellulases have a wide range of applications in industries for biofuel production. Other applications of cellulase include, starch processing, animal feed production, grain alcohol fermentation, malting and brewing, extraction of fruit and vegetable juices, pulp and paper and textile industries. The major limitation in the use of cellulases in industry is the high cost of the production of enzyme. Therefore, this research work addressed the use of agrowastes especially *Arachis hypogaea* shells as a cheap and alternative source for cellulase production.

2. Materials and methods

2.1. Arachis hypogaea shells (Substrates)

A, hypogaea was bought from Oja-Tuntun, Ilorin, Kwara State, Nigeria. It was dehulled and the shells were removed. The shells were identified and authenticated at the Herbarium Unit of the Plant Biology Department, University of Ilorin, Ilorin, Nigeria, where a voucher specimen number (UILH/001/156) was deposited.

2.2. Test organism

Pure culture of *Aspergillus niger* was obtained from Microbial Culture Collection, Department of Microbiology, Faculty of Life Sciences, University of Ilorin, Ilorin, Nigeria and it was used throughout the study.

2.3. Chemicals and reagents

Potato dextrose agar (PDA) used for the cultivation of *A. niger* fungus stock was a product of Biotechnology Laboratory, United Kingdom. Carboxymethyl cellulose (CMC), Avicel and *p*-Nitrophenol- β-D-glucopyranoside (*p*-NPG) were products of BDA Chemicals Ltd., Poole, England. Sephadex G-100 was obtained from Superfine, India. 3,5-Dinitrosalicyclic acid (DNS) was obtained from Lab. Tech. Chemicals, Avighkar, India. Sodium hydroxide, sodium potassium tartarate (Rochelle salt), sodium dihydrogen phosphate, disodium hydrogen phosphate and Bovine Serum Albumin (BSA) and standard protein markers were products of Santa Crux Biotechnology (Germany). All reagents listed above were of analytical grade and prepared in all glass apparatus using distilled water and stored appropriately.

2.4. Preparation and pretreatment of A. hypogaea shell substrate

The *A. hypogaea* shells were first washed with distilled water in order to get rid of dust and other contaminants that may be attached to it. It was air-dried and later stored in a polypropylene bag until further use. The method described by Schell et al. (2003) and Solomon et al. (1999) were adopted for acid and alkaline pretreatment of *A. hypogaea* shells respectively. The proximate analysis of the treated *A. hypogaea* shells were carried out by following the method described by Gafar et al. (2011) for the determination of ash, moisture, crude fibre, crude proteins, lipid as well as carbohydrates contents. The quantity of cellulose present in each of the treated *A. hypogaea* was determined by following the method described by Updegraff (1969).

2.5. Extraction and concentration of cellulase

The method described by Baig and Saleem (2012) was adopted for cellulase extraction with slight modification. Culturing of the *A. niger* was performed in 250-ml Erlenmeyer flasks containing 100 ml of medium. The medium composition (in gl^{-1}) used for growth and enzyme induction was determined and it composed of *A. hypogaea* shells, 1–5%; $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 1; $MgSO_4{\cdot}7H_2O$, 0.6; $CaCl_2.2H_2O$, 0.4; $FeSO_4{\cdot}7H_2O$, 0.05; $MnSO_4.H_2O$, 0.1; $ZnSO_4.7H_2O$, 0.14; $CoCl_2.6H_2O$, 0.37 and protease peptone, 0.75. Inocula size was 10^5 spores' ml^{-1}. Flasks were shaken on an orbital shaker at 120 rpm for 7 days at 30 °C. The culture supernatant and pellet (mycelia mat) were separated by filtration. Supernatant was discarded and 1 g of pellet was re-suspended in 100 ml of 0.05M citrate buffer (pH 4.8) and homogenized with hand grinder and kept in an ice bath. This supernatant was taken as crude enzyme solution and concentrated to five-folds by citrate buffer. Optimum fermentation conditions of 120 h, pH of 4 ± 2, temperature of 40 ± 10 °C, substrate concentration of 1–5%, inoculum size of 10–13 × 10^5 CFU/ml and in the presence of protease peptone as nitrogen source.

2.6. Assay of cellulase activity

Endo-β-1, 4-glucanase and Exo-β-1, 4-glucanase assay was determined by the 3, 5 dinitrosalicylic acid (DNS) method as described by Iqbal et al. (2011) while β-glucosidase activity was determined by Parry et al. (2001). The concentration of protein was determined by following the method of Lowry et al. (1951) using Bovin Serum Albumin (BSA) as standard. The protein concentration was then estimated from a standard curve.

2.7. Optimization parameters for cellulase production

In order to determine the optimum conditions for cellulase production, various parameters were varied. These included: incubation period, substrate concentration, temperature, pH, inoculum sizes and nitrogen. The optimum incubation period was determined by following the method described by Bansal et al. (2012). The method described by Talekar et al. (2011) was adopted for the determination of optimum pH and temperature. The optimum substrate concentration and appropriate nitrogen source was determined using the method described by Vyas et al. (2005). The optimum inoculum size was determined following the method of Omojasola et al. (2008).

2.8. Purification of cellulase

The method described by Ibraheem et al. (2017) was adopted for the purification of cellulase with slight modification. The supernatant was subjected to 80 % ammonium sulphate precipitation by mixing a 480 ml of 100 % saturated ammonium sulphate solution with 120 ml of the supernatant. The precipitate obtained was allowed to settle, kept at 4 °C overnight and then centrifuged at 10,000 x g for 30 min. The pellet obtained was collected by gently slanting the container and decanting the supernatant, then re-dissolve with small amount of ice cold 0.05 M citrate buffer, pH 4.0. The partially purified cellulase obtained after precipitation was gently poured into a dialysis tube. The half-filled dialysis tube was then suspended in 1 L-beaker filled with ice-cold 0.05 M citrate buffer, pH 4.0. The suspension of the dialysis tube was achieved by attaching the ends of the tube to a glass rod and carefully placed across the beaker. The solution was then subjected to dialysis against the buffer for 24 h with continuous stirring using magnetic stirrer and intermittent replacement of the buffer at 3–4 h' interval. 10 ml of dialyzed enzyme was carefully layered on top of the well packed column with sephadex G-100 gel. Another cotton wool was then placed on top of the dialyzed enzyme and then eluted with mobile phase (0.05M citrate buffer (pH 4.0)). A flow rate of 0.1 ml/2 min was maintained. Twenty (20) fractions of 5 ml each were collected. Cellulase activity and protein concentration were determined in each of the fractions. The fractions with the highest cellulase activity were pooled together and used for electrophoresis (SDS-PAGE).

2.9. Characterization of cellulase

2.9.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) was carried out using the method described by Singh et al. (2012) on vertical slab-gel unit Mini Protean II electrophoretic cell (Bio Rad Laboratories). For enzyme separation, 12 % separating gel and 4 % stacking gel were prepared. 12 % separating gel was prepared by adding 3.35 ml distilled water, 2.5 ml of 1.5 M Tris (pH 8.8), 0.1 ml of 10 % SDS and 4.0 ml of 30 % acrylamide together. 50 μl of freshly prepared 10 % ammonium persulphate (APS) was then added and followed by 15 μl of tetramethylethylenediamine (TEMED). The total volume was 10 ml. The mixture was gently mixed and quickly introduced into the two short plates with in-built spacer mounted on the casting tray. 50 % n-butanol was carefully layered on top of the gel to make its surface smooth and left to stand until the gel solidified. After the solidification of the gel, the n-butanol was carefully removed by gently slanting the short plate upside down. The 4 % stackling gel which was formed by mixing 3.0 ml of distilled water, 1.25 ml of 0.5 M Tris (pH 6.8), 50 μl of 10 % SDS, 665 μl of 30 % acrylamide, 25 μl of 10 % APS and 10 μl TEMED together was layered on top of the separating gel. The wells were created on the short plates by inserting 10-tooth comb prior to the solidification of gel. The whole set-up was left for about 30 min until the gel solidified and after solidification of the gel, the comb was removed and the wells rinsed with buffer prior to loading of the samples. Electrophoresis was run for about 50 min at 150 V. The standard protein markers used are β-Galactosidase, 183 kDa; phosphorylase B, 36 kDa; albumin, 23 kDa; ovalbumin, 14 kDa; carbonic anhydrase, 3 kDa. Native molecular weight of the purified cellulase was estimated by the relative mobility (Rf values) of the protein bands compared with the standards.

2.9.2. Determination of substrate specificity of purified cellulase

The ability of purified cellulase to hydrolyze different substrates such as CMC, avicel, glucose, sucrose, $_{\rho}$-NPG and filter paper was determined. Briefly, 0.5 ml of 1 % substrate (CMC, avicel, glucose, sucrose, $_{\rho}$-NPG and filter paper) solution prepared in 0.5 M citrate buffer (pH 4.0) was added to 0.1 ml of purified cellulase in a test-tube and mixed well. One strip of filter paper was used as substrate. The mixture was incubated at 50 °C for 30 min. Then, 3 ml of 3,5-dinitrosalicyclic acid (DNS) solution was added and the mixture was placed in boiling water for 5 min. The mixture was then allowed to cool and 5 ml of distilled water was added. The absorbance was measured at 540 nm (Miller, 1959).

2.9.3. Effects of substrate concentration on the activity of purified cellulase

The Michaelis-Menten kinetic constants, K_m and V_{max} for purified cellulase were determined by varying concentration of carboxymethyl cellulose ranging from 0.01-5.0 mM. Lineweaver-Burke plot was also generated to determine the K_m and V_{max}. Briefly, 0.5 ml of varying concentrations of CMC (0.01–5.0 mM) in 0.5 M citrate buffer (pH 4.0) was added to 0.1 ml of purified cellulase in a test-tube and mixed well. The mixture was incubated at 50 °C for 30 min. 3 ml of 3,5-dinitrosalicyclic acid (DNS) solution was added to stop the reaction and the mixture was placed in boiling water for 5 min. The mixture was then allowed to cool and 5 ml of distilled water was added. The absorbance was measured at 540 nm. The double reciprocal transformation of the cellulase activity and substrate concentrations were determined (Miller, 1959).

2.9.4. Effect of pH on the activity of purified cellulase

The activity of purified cellulase was measured by varying the pH of 50mM citrate buffer. The pH was varied between 3.0 - 6.0, after which cellulase activity was determined. 0.5 ml of 1 % CMC prepared in 0.5 M citrate buffer of varying pH (3.0–6.0) was added to 0.1 ml of purified cellulase in a test-tube and mixed well. The mixture was incubated at 50 °C for 30 min. Then 3 ml of 3, 5-dinitrosalicyclic acid (DNS) solution was added to stop the reaction and the mixture was placed in boiling water for 5 min. The mixture was then allowed to cool and 5 ml of distilled water was added. The absorbance was measured at 540 nm (Miller, 1959).

2.9.5. Effect of temperature on the activity of purified cellulase

The purified cellulase was incubated with substrate at different temperatures of 30, 40, 50 and 60 °C. The activity of cellulase was determined by following the method described by Miller (1959). 0.5 ml of 1 % CMC prepared in 0.5 M citrate buffer (pH 4.0) was added to 0.1 ml of purified cellulase in a test-tube and mixed well. The mixture was incubated at various temperatures of 30, 40, 50 and 60 °C for 30 min. Then 3 ml of 3,5-dinitrosalicyclic acid (DNS) solution was added to terminate the reaction and the mixture was placed in boiling water for 5 min. The mixture was then allowed to cool and 5 ml of distilled water was added. The absorbance was measured at 540 nm (Miller, 1959).

2.10. Statistical analysis

All experiments and enzyme assays were performed in triplicates and the results were expressed as mean ± SEM. Data obtained were subjected to one-way analysis of variance and means found to be significantly different at $p < 0.05$ were separated by Duncan Multiple Range Test. Graphpad prism version 6.02 was used to plot all the graphs.

3. Results and discussion

3.1. Proximate composition of Arachis hypogaea shells

The proximate composition of *A. hypogaea* shells (untreated and treated) used as substrate is presented in Table 1. The crude fibre content of the alkaline pretreated substrates had the highest percentage of crude fibre with an increase of about 6.09 % when compared to acid pretreated and untreated *A. hypogaea* shell (control). Also, there was a significant increase ($p < 0.05$) in the content of crude protein following the treatment of *A. hypogaea* shell especially with alkaline pretreatment, compared to the untreated substrate with about 4 folds increase. There was about 19 % reduction in the content of carbohydrates, upon treating the *A. hypogaea* shell with alkali compared with the untreated *A. hypogaea* shell. The untreated *A. hypogaea* substrate had the highest percentage of

Table 1. Proximate analysis of *A. hypogaea* shell substrate.

Composition	Untreated (% w/w)	Acid Pretreatment (% w/w)	Alkaline Pretreatment (% w/w)
Moisture	4.0 ± 0.005^{b}	2.0 ± 0.005^{a}	1.8 ± 0.005^{a}
Ash	3.0 ± 0.005^{b}	2.5 ± 0.005^{a}	2.2 ± 0.005^{a}
Lipid	8.4 ± 0.005^{b}	8.0 ± 0.005^{b}	6.38 ± 0.005^{a}
Crude Fibre	82.0 ± 0.005^{a}	85.5 ± 0.005^{ab}	87.0 ± 0.005^{b}
Protein	0.16 ± 0.005^{a}	0.17 ± 0.005^{a}	0.65 ± 0.005^{b}
Carbohydrate	2.44 ± 0.005^{b}	1.83 ± 0.005^{a}	1.97 ± 0.005^{a}

Each value is expressed as mean ± SE of three different determinations. Values with different superscripts along a row are significantly different ($p < 0.05$) from one another.

Table 2. Cellulose content of untreated and treated *A. hypogaea* shells.

Pretreatment	Percentage (w/w)
Unpretreated	23.7 ± 2.8^{a}
Acid	39.1 ± 3.3^{b}
Alkaline	52.5 ± 2.5^{c}

Each value is expressed as mean ± SE of three different determinations. Values with different superscripts along a column are significantly different ($p < 0.05$) from one another.

moisture content when compared to acid and alkaline pretreated substrate. There was no significant difference ($p > 0.05$) in the ash content of untreated, acid and alkaline pretreated *A. hypogaea* shell. There was about 24 % reduction in the lipid content of alkali pretreated substrate when compared to untreated and acid pretreated *A. hypogaea* shell. The content of cellulose present in untreated and treated *A. hypogaea* shells is shown in Table 2. The alkaline pretreated substrates had the highest percentage of cellulose with 2.2-fold increase when compared to untreated substrate. Also, the percentage of cellulose in the acid pretreated substrate was higher with a 1.7-fold increase, compared to untreated *A. hypogaea* shell.

3.2. Optimization of cellulase production

3.2.1. Effect of incubation period on cellulase production

The effect of incubation period on the production of cellulase is presented in Figure 1. The endoglucanase, exoglucanase and β-glucosidase showed maximum activity at 120 h. Cellulase production increased as the time of incubation increased and reached its maximum production at 120 h followed by gradual decrease up till 168 h. There was about 4 folds' increase in activities of endoglucanase and β-glucosidase while that of exoglucanase was about 14 folds at 120 h with respect to 0 h.

3.2.2. Effect of pH on cellulase production

The effect of pH on cellulase production is shown in Figure 2. Cellulase production increased proportionately with increase in pH.

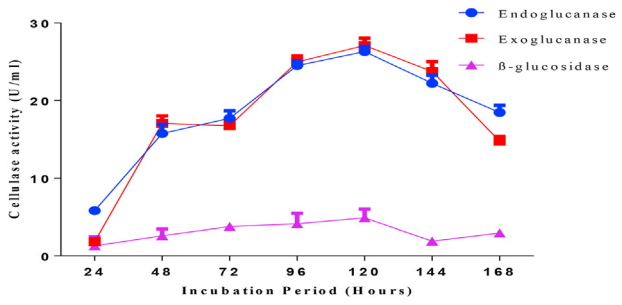

Figure 1. Cellulase activity of *A. niger* cultured on *A. hypogaea* shell at varying incubation times. Each value is expressed as mean ± SE of three different determinations.

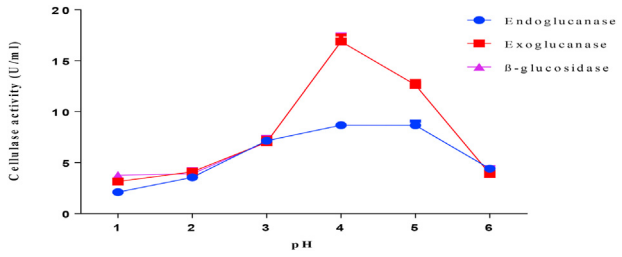

Figure 2. Cellulase activity of *A. niger* cultured on *A. hypogaea* shell at varying pH. Each value is expressed as mean ± SE of three different determinations.

Exoglucanase exhibited highest activity at a pH of 4.0 while endoglucanase and β-glucosidase had optimum pH in the range of 4.0 and 5.0. There was about 4, 5 and 4.5 folds increase in the activities of endoglucanase, exoglucanase and β-glucosidase respectively. An increase in pH by a factor of 2 resulted in over 49 %, 76.9 % and 77 % loss of endoglucanase, exoglucanase and β-glucosidase activities respectively.

3.2.3. Effect of temperature on cellulase production

The effect of temperature on cellulase production is presented in Figure 3. Increase in temperature increased the amount of cellulase production. Endoglucanase and β-glucosidase had an optimum temperature of 40 °C while exoglucanase had an optimum temperature of 50 °C. This result represented about 7 fold increase in activity of endoglucanase and exoglucanase while there was just 1.5 fold increase in the activity of β-glucosidase. After optimum temperature, there was a stepwise decreased in the activities of three components of cellulase even with increased in temperature.

3.2.4. Effect of substrate concentration on cellulase production

The activity of endoglucanase, exoglucanase and β-glucosidase increased with increase in substrate concentration (Figure 4). The activity of the three components measured followed Michaelis-menten hyperbolic curve with the substrate concentrations investigated. The activities of endoglucanase and β-glucosidase increased and reached maximum at a concentration between 1 – 5 % while exoglucanase activity dropped after reaching its maximum at 4 % substrate concentration.

3.2.5. Effect of inoculum size on cellulase production

The activities of endoglucanase, exoglucanase and β-glucosidase as a measure of cellulase production are shown in Figure 5. Increase in the size of inoculum resulted in increased activities of the three components of cellulase. The optimum innoculum size was between 10 to 13 × 10^5 cfu/ml for exoglucanase and endoglucanase while for β-glucosidase, optimum inoculum size was 13 × 10^5 CFU/ml.

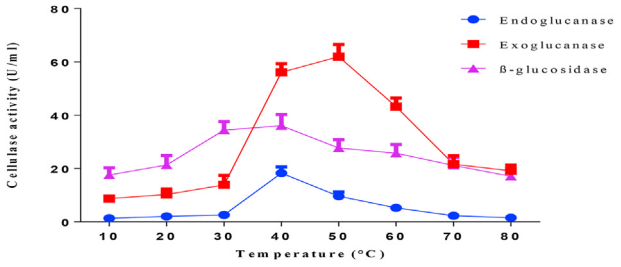

Figure 3. Cellulase activity of *A. niger* cultured on *A. hypogaea* shell at varying temperatures. Each value is expressed as mean ± SE of three different determinations.

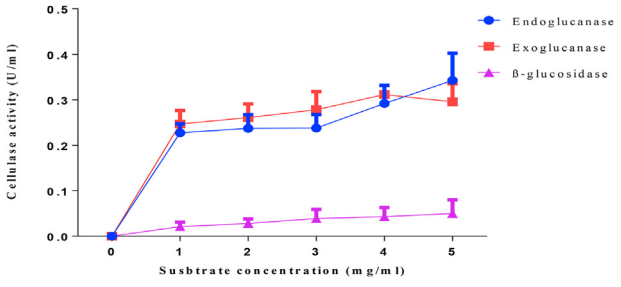

Figure 4. Cellulase activity of *A. niger* cultured on *A. hypogaea* shell at varying substrate concentrations. Each value is expressed as mean ± SE of three different determinations.

3.2.6. Effect of nitrogen source on cellulase production

Figure 6 shows the activities of the three components of cellulase varied with different nitrogen source. Maximum endoglucanase and β-glucosidase activities were obtained in the presence of protease peptone as nitrogen source while exoglucanase showed maximum activity when yeast extract was used.

3.3. Purification of cellulase produced by Aspergillus niger cultured on Arachis hypogaea shell

The summary of purification processes of cellulase produced by culturing *A. niger* on *A. hypogaea* shell is presented in Table 3. *A. niger* was inoculated in the fermentation medium and only the endo-ß-1,4-glucanase activity was determined under optimum fermentation conditions of 120 h, pH of 4–5, temperature of 40 °C, substrate concentration of 5%, inoculum size of 10–13 × 10^5 cfu/ml and in the presence of protease peptone as nitrogen source. However, the activities of other 2 enzymes, exoglucanase and β-glucosidase were not expressed during purification. The crude cellulase had a total activity and specific activity of 87.69 U/ml and 7.11 U/mg respectively. As crude enzyme was subjected to each step of purification process, there was reduction in the total activity of the enzyme, which was accompanied by corresponding increase in the specific activity. Partially purified enzymes obtained by precipitation with 80 % saturation of ammonium sulphate had a specific activity of 14.68 U/mg and 2.06-fold

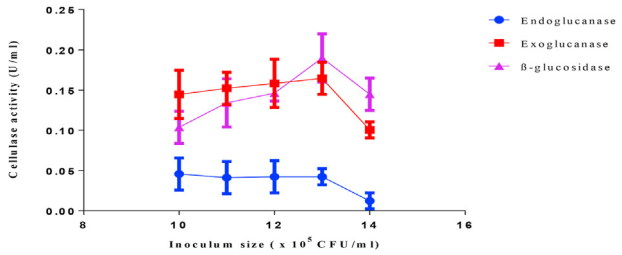

Figure 5. Cellulase activities produced by *A. niger* cultured on *A. hypogaea* shell at varying inoculum size. Each value is expressed as mean ± SE of three different determinations.

purification. Upon dialysis with three changes of buffer, the further partially purified cellulase gave specific activity of 222 U/mg and 31.22 folds purification. The purified cellulase obtained after sephadex G-100 gel filtration gave 68.12 folds purification and specific activity of 484.3 U/mg respectively (Table 3). Percentage yield of cellulase decreased stepwisely from 100 % to 3.87 %. The elution profile diagram of *A. hypogaea* shell cellulase on sephadex G-100 is presented in Figure 7.

Optimum fermentation conditions of 120 h, pH of 4–5, temperature of 40 °C, substrate concentration of 1–5%, inoculum size of 10–13 × 10^5 CFU/ml and in the presence of protease peptone as nitrogen source.

3.4. Substrate specificity of purified cellulase produced by Aspergillus niger cultured on Arachis hypogaea shell

The purified cellulase showed highest relative activity with carboxylmethylcellulose as substrate and the least relative activity with filter

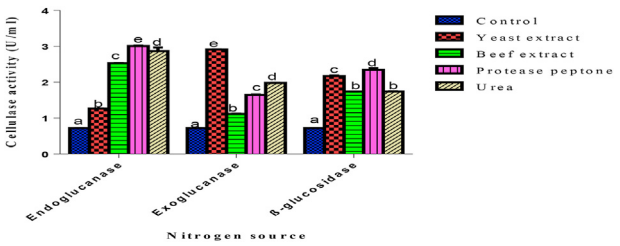

Figure 6. Cellulase activities produced by *A. niger* cultured on *A. hypogaea* shell using different nitrogen source. Each value is expressed as mean ± SE of three different determinations. Bars with different superscripts for the parameter are significantly different ($p < 0.05$).

paper (Table 4). The relative activity of purified cellulase was also low with other substrates like avicel, glucose, sucrose and $_p$-NPG.

3.5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) profile of purified cellulase produced by Aspergillus niger cultured on Arachis hypogaea shell

SDS-PAGE profile of cellulase produced by *A. niger* is shown in Figure 8. Lane A is the crude enzyme; B is the dialysed enzyme; C is the ammonium sulphate fraction; D and E are the fractions obtained after gel filteration chromatography. A clear single band was observed on lane E while more than one bands were seen on lanes A - D. The purified cellulase observed in lane E has an estimated molecular weight of 13,500 Da based on the relative movement of the protein when compared to the standard marker on the gel from estimation on the calibration curve of the gel filtration on Sephadex G-100. Using SDS-PAGE, the purified cellulase showed a single band.

3.6. Kinetic analysis of purified cellulase

A plot of cellulase activity against concentration of substrate yielded a hyperbolic curve which showed that the purified cellulase obeyed Michaelis-Menten type kinetics (Figure 9). From the Lineweaver-Burk plot (Figure 10), K_m and V_{max} values of purified cellulase from *A. niger* were calculated to be 0.23 mg/ml and 9.26 U/ml respectively. The turnover number, K_{cat}, for purified cellulase was extrapolated from the graph and it was found to be 0.08 per second.

Table 3. Summary of purification of cellulase produced from culture of *Aspergillus niger* under optimum fermentation conditions on *Arachis hypogaea* treated shells.

S/N	Purification Steps	Total Volume (ml)	Endoglucanase Activity (U/ml)	Total Protein (mg/ml)	Specific Activity (U/mg)	Purification Fold	Percentage Yield (%)
1	Crude	120	87.69	12.330	7.11	1	100
`2	$(NH_4)_2SO_4$ Precipitation	30	25.99	1.770	14.68	2.06	29.64
3	Dialysis	10	6.66	0.030	222.00	31.22	7.59
4	Gel Filtration	5	3.39	0.007	484.30	68.12	3.87

Each value is expressed as mean ± SE of three different determinations.

Table 4. Substrate specificity of purified cellulase (endoglucanase).

Substrates	Relative Activity (%)
CMC	100
Avicel	32.60
Glucose	3.30
Sucrose	3.22
$_p$-NPG	3.16
Filter Paper	1.16

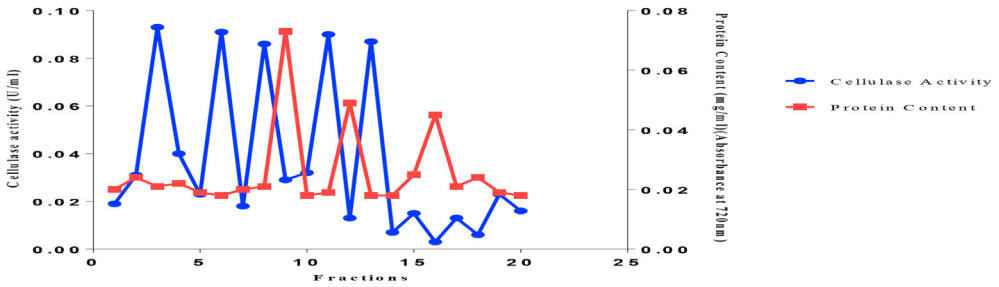

Figure 7. Elution Profile of *A. hypogaea* shell cellulase on sephadex G-100 chromatography.

3.7. Effect of pH on the activity of purified cellulase

The endoglucanase activity of the purified cellulase was optimal at pH 4 with an activity of 48.78 U/mL (Figure 11). There was a drastic decrease in the activity of endoglucanase activity of purified cellulase above or below the pH 4 (Figure 11). A change in pH by a factor of 1 resulted in over 50% loss of activity.

3.8. Effect of temperature on the activity of purified cellulase

The endoglucanase activity of the purified cellulase was optimal at temperature of 40 °C. There was a dire decrease in the activity of endoglucanase activity of purified cellulase above or below the temperature of 40 °C (Figure 12). A 5 °C increase or decrease in temperature resulted in about 64 % fall in the activity, while a 10 °C increase in temperature resulted in about 158 % decrease in activity.

4. Discussion

The substantial amounts of waste materials such as shells, tatters, trunks, peels and seeds are engendered as a result of agricultural practice. Large quantities of these agrowastes obtained are from heavy consumption of agricultural products. These wastes particularly groundnut shells are copious because once the nuts have been removed, the shells are always discarded. Accumulation of these shells constitutes what is called "wastes" and consequently lead to environmental pollution.

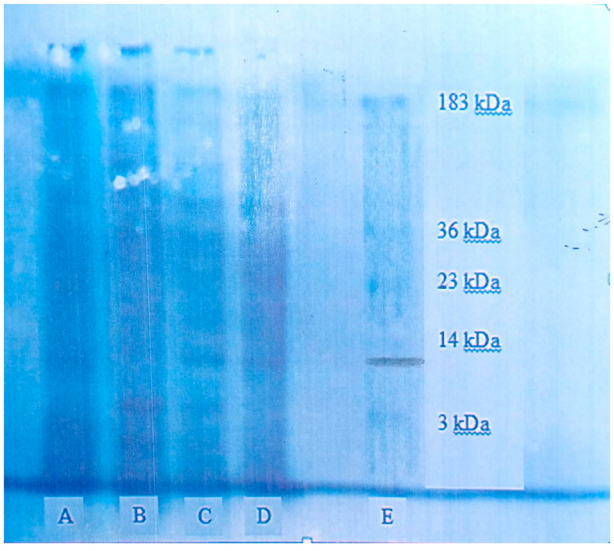

Figure 8. SDS-PAGE profile of cellulase produced by *A. niger* cultured on treated *A. hypogaea* shell. Lane A, crude cellulase; B, ammonium sulphate precipitation; C, dialyzed enzymes; D, partially and E, purified enzyme (13.5 kDa) and beside E are the molecular weights in kDa of standard marker (standard protein markers are β-Galactosidase, 183 kDa; Phosphorylase B, 36 kDa; albumin, 23 kDa; ovalbumin, 14 kDa; carbonic anhydrase, 3 kDa).

Transformation of these wastes to expedient products will not only combat environmental pollution arising from unnecessary discarding of shell but also boast the economy of our country. The uses of different agricultural wastes such as corn cob, rice bran, bagasses, wheat bran, banana trunk for production and characterization of cellulase have been previously studied in the past (Yang and Wyman, 2008). Therefore, this study addressed the possible use of *Arachis hypogaea* shells as a substrate for cellulase production from *Aspergillus niger*.

4.1. Proximate analysis of pretreated A. hypogaea shells

The potential usefulness of agricultural wastes depends on pretreatment approaches as well as chemical composition. An efficient pretreatment distorts cell wall physical obstructions as well as cellulose crystallinity and connotation with lignin so that biomass can be amenable to hydrolytic enzymes (Wyman et al., 2005). The pretreatment process has become an important step, limiting the progress of cellulosic ethanol (Himmel et al., 2007; Yang and Wyman, 2008). Several approaches have been employed for the treatment of lignocellulosic biomass, but only few of them gave encouraging results. These pretreatment methods include dilute acid pretreatment, alkaline

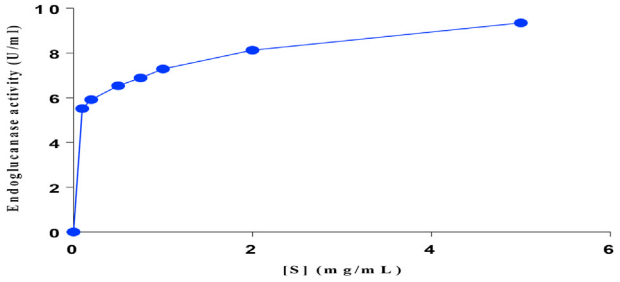

Figure 9. Substrate kinetics of purified cellulase produced by *A. niger*. Each value is expressed as mean ± SE of three different determinations.

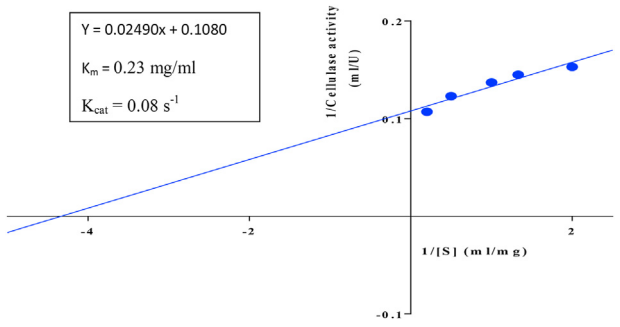

Figure 10. Lineweaver-Burk plot of cellulase hydrolyzing carboxylmethylcellulose. Each value is expressed as mean ± SE of three different determinations.

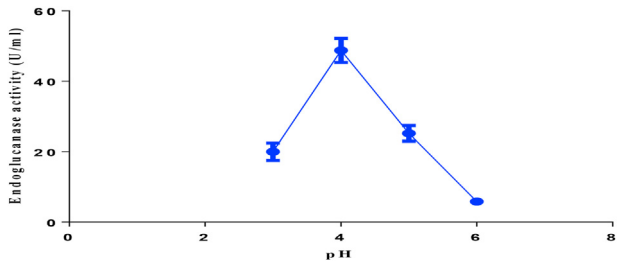

Figure 11. Effect of pH on endoglucanase activity of purified cellulase produced by *A. niger*. Each value is expressed as mean ± SE of three different determinations.

pretreatment, steam explosion (CO_2 explosion), pH-controlled water pretreatment, ammonia fiber expansion, ammonia recycle percolation (ARP), and lime pretreatment (Mosier et al., 2005; Wyman et al., 2005; Yang and Wyman, 2008).

In this study, chemical pretreatment involving acid and alkaline were employed to choose the most suitable pretreated substrates that will serve as inducer of cellulase from *A. niger*. The results obtained showed that alkaline pretreatment produced the highest percentage of crude fibre as well as highest percentage of cellulose, an indication that it may serve as the best substrate for cellulase production from *A. niger* (Table 1). The alkaline solutions used in treating *A. hypogaea* shells employed in this study might have removed some considerable amounts of lignins present in the *A. hypogaea* shells and increased it digestibility. This agreed with the findings of Beukes and Pletschke (2011) who reported that alkali removes lignins and make agrowaste more digestible. One of the benefits of employing alkaline as a pretreatment strategy is the flexibility of the process. Mosier et al. (2005) and Chang (2007) also reported that alkali pretreatments solubilized lignin and increase the approachability of the lignocellulose surface by the removal of acetyl and uronic acid substituents on hemicellulose.

The proximate analysis of the treated and untreated *A. hypogaea* was carried out in order to determine the percentage crude fiber as well as cellulose composition in each of the pretreated *A. hypogaea* shells. The results obtained from this study revealed treated, and untreated substrates have high percentage of crude fiber (Table 1). Crude fiber is a measure of the quantity of undigested carbohydrates such as cellulose, pentosans, lignin, pectins and other components of this type present in foods (Van Soest and Robertson, 1979). It is the residue of plant materials remaining after solvent extraction followed by hydrolysis with dilute acid and alkali. The undigested or dietary fibers are a complex mixture of different materials. The major ones are cellulose, the glucose polymer that is the predominant material of plant cells; hemicellulose, a shorter version of cellulose: pectin, the glue that binds plant cells together with cellulose from the woody cell walls of plants (Gidenne, 2003). The high crude fibre content obtained from this study for untreated, pretreated shells is an indication that *A. hypogaea* shell is very rich in cellulose and can serve as an inducer for cellulase production (Table 1). However,

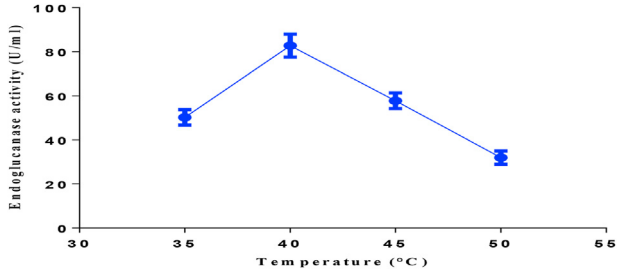

Figure 12. Effect of temperature on endoglucanase activity of purified cellulase produced by *A. niger*. Each value is expressed as mean ± SE of three different determinations.

alkaline pretreatment gave the highest percentage of cellulose (52.5 %) compared to acid pretreatment (39.1 %) and unpretreated substrate (23.7 %) (Table 2). This observation agreed with findings of Gimba et al. (2010) who reported the value of 50–60 % cellulose from *A. hypogaea* shells. It can therefore, be said that alkaline treated *A. hypogaea* may be the preferred substrate for cellulase production from *A. niger*.

4.2. Optimization of cellulase production

Incubation period plays an important role in the production of enzymes. Enzyme production by microorganisms' especially *A. niger* is based on the two factors that are very significant during fermentation processes; these are the specific growth rate of microorganisms as well as synthetic ability of the organisms (Kunamneni et al., 2005). In this present study, cellulase activity as a measure of cellulase production increased steadily and reached maximum at 120 h of incubation under submerged fermentation (SmF) (Figure 1). Further extension of incubation time beyond 120 h resulted in loss of cellulase activity, which might be as a result of reduction in enzyme production. This reduction in cellulase activity might be due to the exhaustion of carbon source in the fermentation medium with the lapse in time, which stressed the fungal physiological response to enzyme production resulting in the decrease in the secretory ability of microorganisms (Nochure et al., 1993). The potentials of microorganisms to produce enzymes in a relatively short incubation period have been reported (Sonjoy et al., 1995). The result obtained from this study is similar to that reported by Abu et al. (2000) who reported 120 h of incubation period for cellulase production but differs from that of Devanathan et al. (2007) and Acharya et al. (2008) who reported 96 h of incubation period for cellulase production. For maximum cellulase production from *A. niger* cultured on *A. hypogaea* shell, 120 h of incubation is required.

Microorganisms generally have been shown to be sensitive to the concentration of hydrogen ions present in the fermentation medium. Among optimization parameters for cellulase production, pH of the fermentation medium plays a significant role by inducing morphological changes in microbes and in enzyme production (Mrudula and Murugammal, 2011). Gupta et al. (2003) reported that pH is a key machinery that affects enzyme production during fermentation. The pH change observed during the growth of microbes also affects product stability in the medium (Gupta et al., 2003). Optimum pH for maximum production of cellulase obtained from this study was 4.0 under SmF (Figure 2). The result obtained from this study agreed with the findings of Abubakar and Oloyede (2013) who reported a pH 4 for maximum cellulase production when *A. niger* was cultured on rice bran and orange peel. Omojasola et al. (2008) also reported a pH 3.5 for cellulase production from *A. niger* cultured on pineapple waste. The result obtained from this study was contrary to the observation which was made for cellulase production from *A. terreus* QTC 828 in SmF by Ali et al. (1991) and *Trichoderma reesei* in SSF by Doppelbauer et al. (1987) who both reported a pH of 6.0 for cellulase production. Also, Krishna (1999) reported a pH of 7.0 for cellulase produced by bacteria using banana peel as a substrate in solid state fermentation. The optimum pH for producing cellulase from *A. niger* cultured on *A. hypogaea* is 4.0. The differences in the results obtained from this study and the earlier reported work may be as results of using different microorganisms as well as different agricultural wastes.

Incubation temperature, one of the factors that affect the growth of microorganisms plays a central role in the metabolic activities of a microorganism and should be determined for each set of conditions (Bhanja et al., 2007). As incubation temperature increases, the rate of collision increases and subsequently increases the rate of reaction as observed in many chemical reactions. However, the stability of the enzyme decreases due to thermal degradation. Holding enzyme at a high enough temperature may denature the enzyme. The results obtained from this study revealed that optimum temperature for maximum enzyme production was recorded at 40 °C for endoglucanase and β-glucosidase whereas the optimum temperature for exoglucanase was

recorded at 50 °C (Figure 3). The results obtained from this study agrees with the findings of Ali et al. (1991) who reported maximum yield of cellulase from *A. niger* Z10 strain and *A. terreus* at 40 °C, respectively in SSF. Loss of cellulase activity observed beyond 40 °C and 50 °C may be due to thermal degradation of enzymes. The optimum temperature for producing cellulase from *A. niger* when cultured on *A. hypogaea* shell is 40–50 °C.

Substrate concentration is an important factor that affects the activity of enzyme and subsequently enzyme production. Enzymes are not passive surfaces on which reactions take place but rather are complex machines that operate through a great diversity of chemical mechanisms (Scott, 1996). In an enzyme catalyzed reaction, the activity of enzyme generally increased as substrate concentration increases (Scott, 1996). Though, at a point, a further increase in substrate concentration will have little or no effect on the activity of enzyme, at that point the enzyme is said to be saturated with its substrate (Scott, 1996). The results obtained in this study revealed that maximum cellulase activity was achieved at the maximum concentrations investigated (Figure 4).

The size of the inoculum is the number of microorganisms introduced into the fermentation medium. In this present study there was an increase in the production of cellulase as the size of inoculum increases. Maximum enzyme production was obtained at inoculum size of 13×10^5 CFU/ml (Figure 5). When a single inoculum is employed for enzyme production during fermentation, it may take a longer time before enzymes are secreted and the quantity of enzymes that will be produced will be very small compared to when the innocula is double or even thrice. At the inoculum size above 13×10^5 CFU/ml, a sharp reduction in cellulase production was observed. This reduction in cellulase production with further increase in inoculum size might be as a result of clumping of cells which could have reduced the macro and micronutrients present in the fermentation medium (Srivastava et al., 1987). This result is in line with earlier work reported by Kunamneni et al. (2005) who explained that further increase in inoculum size resulted in decreasing enzyme production owing to nutrients constraint.

Nitrogen is the main component of protoplasm and building block of proteins. In this study, all the nitrogen sources enhanced cellulase production when compared to control. Among them peptone supported maximum enzyme production of endoglucanase and β-glucosidase while yeast extract supported maximum exoglucanase production. Similar reports on stimulation of cellulase by protease peptone had earlier been reported (Kathiresan and Manivannan, 2006; Devanathan et al., 2007). Sun et al. (1999) reported that good cellulase yield can be obtained with ammonium compound as the nitrogen source.

4.3. Purification of cellulase produced by A. niger cultured on A. hypogaea shell

Enzymes generally can be obtained from three different sources namely plants, animals and microorganisms. These enzymes are usually confined to a diverse compartment in the sources mentioned above; some are localized in the cytosol while others in mitochondria and other organelles (Ibraheem et al., 2017). In order to carry out structural elucidation of a particular enzyme and to characterize it for analytical purpose, the crude enzymes must first be obtained after which it will be subjected to series of purification procedures (Ibraheem et al., 2017). Cellulase isolated from *A. niger* when cultured on *A. hypogaea* shells used as substrate gave a specific activity and percentage yields of 484.30 U/mg and 3.87 % (Table 3) respectively after gel filtration chromatography. The percentage yields after gel filtration chromatography was far less than the value obtained for crude enzyme (100 %). Crude enzyme contains total proteins which include desired and undesirable proteins. Therefore, percentage yield is expressing how much of the desired enzyme was actually recovered. The reduction in percentage yields obtained from this study may be as a result of removal of some undesirable proteins at each purification stage or may as well be due to denaturation of unwanted proteins during purification steps. The percentage yield (3.87 %) (Table 3) obtained from this study was more than 2.11 % reported by Iqbal et al. (2011) who obtained cellulase from *Trichoderma viride* cultured on wheat straw under SSF. Thus, it can be inferred that *A. hypogaea* may be a better inducer of cellulase than wheat straw. Also, Olama et al. (1993) reported cellulase purification from *T. viride* and recorded a 99.8 % loss of protein with the specific activity increased to about 22.8 folds. Sultana (1997) reported 13.71 U/mg specific activities which were increased by about 32 folds from *Aspergillus* sp. Po-Jui et al. (2004) also observed that the specific activity of 38.22 U/ml increased by about 9.04 folds from *Sinorhizobium fredee* by DEAE Sepharose anion-exchange column and followed by Phenyl-Sepharose column purification. The increase in the specific activity of cellulase from crude (7.11 U/mg) to sephadex G-100 column chromatography (484.30 U/mg) as well as a corresponding increase in purification fold from crude (1.0) to sephadex G-100 column chromatography (68.12) obtained from this study (Table 3) may be due to removal of some endogenous inhibitors that may be present in the crude enzyme (Adeleke et al., 2012).

The elution profile of *A. hypogaea* shell cellulase on sephadex G-100 chromatography produced 5 distinct peaks. This implies that cellulase enzyme was localized in those 5 peaks produced. This observation was contrary to the findings of Adeleke et al. (2012) who reported 3 peaks obtaining from elution profile of rerun of cellulase obtained from *Bacillus coagulans Co4* on CM Sepharose CL-6B.

4.4. Substrate specificity of purified cellulase produced by Aspergillus niger cultured on Arachis hypogaea shell

Substrate specificity refers to the ability of an enzyme to select the precise substrate from sets of chemical compounds. Specificity is a molecular recognition mechanism and operates through structural and conformational complementarity between the enzyme and substrate (Fersht, 1999). Enzymes show different degree of specificity towards their substrates. The results obtained from this study revealed that the purified cellulase was able to hydrolyze CMC and shows absolute specificity for CMC (Table 4). The purified enzyme displayed little hydrolytic activity against other substrates tested. This finding agreed with the work of Yin et al. (2010) who reported 100 % relative activity for cellulase against CMC.

4.5. Estimation of Molecular Weight of Purified Cellulase Produced by A. niger Cultured on A. hypogaea Shell

The electrophoretic pattern obtained from this study give an indication that to some extent, certain degree of purification was attained. This was evidenced with a decreased in the number of bands formed after each purification step. The molecular weight of purified cellulase obtained from this study was found to be 13.5 KDa as determined by SDS polyacrylamide gel electrophoresis (Figure 8). Reports of molecular weight of purified cellulase from different microorganisms include (i) cellulase purified from *T. viride*, with a molecular weight of 38–54 KDa reported by Ogawa (1989), (ii) cellulase from *T. viride,* with a molecular weight of 58 KDa reported by Olama et al. (1993), (iii) cellulase from *Aspergillus sp,* with a molecular weight of 31.2 KDa reported by Sultana (1997) and (iv) Shaojun et al. (2001) purified cellulase from *V. volvaceae* and obtained a molecular weight of 42 KDa. In addition, Saha (2004) obtained a molecular weight of 27 kDa from *Mucor circinelloides*, Lucas et al. (2001) obtained 35 kDa from *Chalara paradoxa*, Mawadza et al. (2000) obtained 40 kDa from *Bacillus* strains, and Akiba et al. (1995) also obtained a molecular weight of 40 kDa from *A. niger.* The differences in the molecular weight of purified cellulase obtained from this study and those earlier reported may be as a result of different species of microorganisms as well as agricultural wastes used as substrate in this study.

4.6. Kinetic analysis of purified cellulase

K_m is a measure of affinity a particular enzyme has for its substrates. It can also be expressed as the concentrations of the substrate when the

velocity of the enzyme catalyzed reaction is half maximum. V_{max} on the other hand is the maximum velocity of an enzyme catalyzed reaction. These two parameters are very important in enzyme study. Usually, when the K_m value is low, it implies that a particular enzyme has a very strong affinity for its substrate and to achieve maximum rate of reaction, little amounts of substrates will be required. The value obtained from this study for K_m and V_{max} are 0.23 mg/ml and 9.26 U/ml respectively (Figure 10), differ from K_m and V_{max} for different microorganisms that have been reported. Ekperigin (2007) reported K_m values of 0.32 and 2.54 mg/ml for *A. anitratus* and *Branhamella* sp. respectively using cellobiose as substrate whereas 4.97 and 7.90 mg/ml were reported using CMC as substrate. Also, Bakare et al. (2005) and Cascalheira and Queiroz (1999) reported K_m value of 3.6 mg/ml for *P. fluorescens* and 1.1 *m*M for *T. reesei* respectively. The variation in the value of K_m obtained from this study and other reported studies may be due to genetic variation among different species of microorganisms employed (Iqbal et al. 2011). K_{cat} measures the number of substrate molecule each enzyme site converts to product per unit time. The higher the Kcat, the more substrates get turned over to products in one second and vice-versa. The value obtained for K_{cat}, from this study is 0.08 s^{-1} (Figure 10) which implies that more enzyme will be needed to convert substrate to product.

4.7. Effect of pH on endoglucanase activity of purified cellulase

The effect of pH on the activity of purified cellulase was presented in Figure 11. Cellulase activity was found to be affected by change in the concentration of hydrogen ions present in the solution. The optimum enzyme activity was observed at pH 4.0 in this study. This finding agreed with Kim (1995) who isolated cellulase from *M. circinelloides* and reported a pH value between 4.0 - 7.0. Fungal cellulases with pH values of 4.5–6.0 have been reported and have been obtained from *T. viridie* (Gupta and Gupta, 1979); *A. niger* and *A. terreus* (Goma et al., 1982); *N. crassa* (Macris et al., 1987); *A. aureolus* and *A. clavatus* (Mishra, 1988); *R. oryzae* (Amadioha, 1993); *V. diplasia* (Bhadauria et al., 1997); *T. reesei* QM 9414 (Wang, 1999). From the results obtained from this study, it can be suggested that purified cellulase is moderately active in acidic solution. A sharp decrease in cellulase activity at pH 4 approaching neutral and alkaline regions (Figure 11) may be due to destruction of active site as well as changes in secondary or tertiary structure of cellulase.

4.8. Effect of temperature on endoglucanase activity of purified cellulase

The finding of optimum temperature for the purified cellulase (40 °C) (Figure 12) in this study differs from that reported in several other studies. The results obtained in this study are in close agreement with the findings of Thongekkaew et al. (2008) who reported between 40 – 50 °C as optimum temperature during the characterization of CMCase produced from *Cryptococcus sp.* S-2 as against Fadel (2000) who found 55 °C as the best temperature at which the enzyme was most active and stable. Saha (2004) also reported the same temperature of 55 °C as optimum for CMCase activity. Loss of activity observed after the optimum temperature may be due destruction of weak bonds such as Van der Waals, and hydrogen bonds that help in maintaining the three-dimensional structure of the enzymes. Temperature optimum for purified cellulase was observed at 40 °C. For temperatures higher than 40 °C, enzyme starts to lose its activity rapidly as the denaturation of the enzymic protein occurs at elevated temperatures (Figure 12). For a variety of industrial applications, relatively high thermostability is an attractive and desirable characteristic of an enzyme. Therefore, cellulase obtained from *A. niger* cultured on *A. hypogaea*, the optimum temperature for the maximum activity is 40 °C.

5. Conclusion

The present work was carried out to optimize parameters to improve cellulase production by the cellulose producing fungi. From this present study, the result showed that cellulolytic fungi can grow at optimized conditions of 120 h, pH of 4–5, temperature of 40 °C, substrate concentration of 1–5%, inoculum size of 10–13 $\times$ 10^5 CFU/ml and in the presence of protease peptone as nitrogen source. The properties of this cellulase from *A. niger* makes it a model candidate for the bioconversion of biomass in second generation biofuel production.

Declarations

Author contribution statement

A. O. Sulyman: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

A. Igunnu: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

S. O. Malomo: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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