PRODUCTION AND PURIFICATION OF CELLULASE FROM CULTURE OF S.CEREVISIAE UNDER OPTIMUM FERMENTATION CONDITIONS ON ONION BROWN SKIN

BY

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Abstract

An increase in the global population is currently accompanied by increased agricultural productivity. This concurrently causes the generation of enormous amounts of agricultural waste that puts the sustainability of the ecosystem at risk. This study aimed to produce cellulolytic enzymes using local fungi isolates through the solid-state fermentation of some underutilized agro wastes: roasted groundnut skin, onion-brown skin. The major population of the isolates was identified as saccharomyces cerevisiae. The maximum production of the cellulolytic enzymes (endoglucanase, exoglucanase, β-glucosidase) was observed at 96 h. The highest output of β-glucosidase and exoglucanase was at pH 4 while endoglucanase activity was highest at pH range 4 and 5. An increase in temperature increased the amount of cellulase production and the optimum temperature was observed at 40 °C. Activity of the cellulolytic enzymes follows Michaelis-menten hyperbolic curve using the three substrates. An increase in the inoculum size resulted in decreased activity of endoglucanase, exoglucanase on onion brown skin while β-glucosidase activity increased on the three substrate.

Introduction

The continuous increase in population of the whole world will lead to continuous increase in industrial or Agro and domestic wast, there is therefore the need to either clean up these waste produced or put into use to avoid the nuisance that may be created (Duque-Acevedo et al., 2020). As agricultural production is increasing, it is important to maintain balance between production and the preservation of nature, which is a major difficulty in the long-term sustainable management of natural resources (Velasco-Muñoz et al., 2021). However, among other methods, agrowastes can be valorized via fermentation (Sadh et al., 2018; Elegbede et al., 2021) and incorporated into circular bioeconomy. Cellulose is an important structural component of the primary cell wall of green plants and annually about 180 billion tons of cellulose are generated by plants, making this polysaccharide an enormous organic carbon reservoir on earth (Tabasomet al., 2015). This huge amount of biomass is biodegradable and can be converted into useful products such as biofuels, chemicals or cheap energy sources for fermentation (Tiwariet al., 2013). The key step in the utilization of cellulose is its hydrolysis into monomeric sugars and their eventual conversion into valuable chemicals and energy. The major limitation in the enzymatic conversion of this biomass into simple sugars and chemicals is the cost of cellulase enzymes (William, 2012).

Statement of Problem

Despite a worldwide and enormous utilization of natural cellulosic sources, there are still abundant quantities of cellulose-containing raw materials and waste products that are not exploited or which could be used more efficiently. These materials can be fermented by microorganisms to produce valuable hydrolytic enzymes, such as cellulase. For cost effectiveness, the best possible solution may be the utilization of the indigenous, cheaper and underutilized substances as substrate for the production of this valuable enzyme and then its further application in various food and industrial preparations. The availability of huge amount of less-economic cellulosic materials in Nigeria

underlines the need to explore the potentials of the natural decomposers of the plant cell-wall polymers for the transformation of these wastes into useful products using strains of viable organisms.

Justification

In Nigeria, large quantities of different types of agrowastes are dumped in the environment due to low nutritional qualities and presence of antinutritional factors. Thus, the agrowastes left behind for natural degradation can be utilized effectively to yield fermentable bioresources products that are mostly useful industrial, agricultural and domestic production of novel by-products which are economical advantages, and may also result to foreign exchange earnings and environmental pollution control. This research work will therefore focus on developing/synthesis of cellulolytic enzymes from underutilized agricultural wastes in the environments for purification and application in the agricultural poultry industries, that would be focus on reducing the cost of poultry products in the market and its performance.

Objectives of the study

The main aim of this study is to ”produce cellulolytic enzymes using local fungi isolates through the solid state fermentation of some underutilized agrowastes (Roasted groundnut skin and onion brown skin)” with application in poultry industries. The specific objective of the study are;-

i. To isolate cellulolytic filamentous fungi from aged Nigerian palmwine.
ii.To evaluate the fungal isolates for the production of cellulases, namely Carboxymethcellulase.
iii. To optimized the production of cellulase using some underutilized agrowastes, namely Roasted groundnut skin, onion brown skin and coconut shell.
iv. To isolate, purify and characterize the cellulases
v. To identify the fungal isolates using morphological and molecular techniques
vi To investigate the application of the enzyme as additive in animal feeds.

Materials and methods

SUBSRATE (Cellulosic materials)

Roasted groundnut skin, onion brown skin and coconut shell will be used as subsrates (carbon/energy sources) for cellulase production and characterization. Samples of the materials will be obtained from a local market. This was sun-dried, ground with a grinder (MarlexExcella Mixer grinder Mumbai, India) and passed through a sieve with fine mesh to obtain a very fine powder (40 mesh size) and subsequently treated with 1.5% NaOH before stored in clean plastic jars for subsequent use in the fermentation medium.

PALMWINE

Fresh palm wine samples from Raphia palm trees (Raphiasudanica) will be collected from traditional palm wine tappers within 30 min of tapping. The freshly tapped samples will be collected using pre-sterilized 200 ml capacity sample bottle with perforated screw cap. The perforated screw caps will be plugged with sterile non-absorbent cotton-wool. The samples will be transported to the laboratory in a cooler equipped with packs of freezing mixture of salt and ice-block for analysis within 1 h of collection. Five milliliters of thoroughly mixed palmwine will be centrifuged in sterile centrifuge tubes at low speed for 5 minutes. This would serve as stock culture. Physiochemical and Mineral Elements Analysis of cellulytic (agrowastes) materials. Moisture, ash, crude protein, crude fat, crude fiber, carbohydrate content, energy value, lignin and mineral elements will be determined respectively using standard procedures of the Association of Official Analytical Chemists (AOAC., 2012).

Isolation of cellulytic fungi

Microfungi capable of degrading cellulose (and other cellulolytic materials) as sole carbon\energy source will be isolated from freshly tapped palmwine using a minimal salt agar medium (containing cellulosic materials as sole carbon/ energy souces) as described by Nwodo Chineduet al (2005) and Kastneret al, (1994) with modifications as listed in Table 1. The pH will be adjusted to 5.6 before autoclaving at 121 ºC for15 minutes. The minimal salt agar plates will be inoculated with 1ml sediment of stock culture and incubated at 30 ºC for 3–14 days.

Table 1: composition of minimal salt agar medium

No	Ingredients	Quantity (g/L)
1	Na_2HPO_4	2.13
2	KH_2PO_4	1.3
3	NH_4Cl	0.5
4	$MgSO_4.7H_2O$	0.2
5	Agar-agar	20.0
6	Trace element solution	1ml
7	Agrowaste	1.0

pH 5.6 and 37°C temperature

Purification and examination of fungi isolates of isolates.

The colonies of cellulolytic fungi that developed within 3-14 days of incubation period will be isolated and purified by continuous subculturing on potato dextrose agar supplemented with 0.5%(w/v) chlorophenicol until a pure culture is obtained. The pure cultures will be subculture on PDA+chloramphenicol slants, incubated for 24 h and refrigerated. The isolates will be maintained on the slant for further identification tests and other requirements.
Morphological characterization

Morphological characterization of fungal isolates will be achieved by macroscopic examination of the organisms based on colony differences in terms of colour, type and texture. Further identification will be done by microscopic examination of certain distinct features such as hypeae type, presence or absence conidiospores, chlamydospore, sporangiospores, and spore type in lactophenol cotton blue staining technique (Abrusci et al.,2005).

Biomolecular characterisation of fungi strains (Polymerase chain reaction).

DNA amplification will be done by PCR method using two primers (ITS 4 and 5) targeting the ITS region of the DNA template. Primers sequences were:

ITS 4-5'-TCCTCCGCTTATTGATATGC-3' as the sense primer and
ITS 5-3'-GGAAGTAAAAGTCGTAACAAGG-5' as the antisense primer.

Amplification will be carried out in a final PCR volume of 25 µl using manufactured master mix. The reaction mixture consisted of 1 µl of yeast DNA template, 1µl ITS 4 and 1 µI ITS 5 primers. The PCR conditions will be performed in 35 cycles: 96ºC for 5 min, 96ºC for 1 min. 55ºC for 1 min, 72oC for 2 min and 72ºC for 10 min. Each PCR amplicon will be resolved on 1.5% agarose high resolution, stained with 5 mg/ml ethidium bromide and visualized under UV light and photographed. A 100-bp DNA Ladder will be used as a maker (GIBCO BRL) to serve as the size standard.

Screening of cellulolytic isolates for production of cellulytic enzymes.

All strain isolate will be separately screened for endoglucanase (carboxymethycellulase) using 1% carboxymethyl cellulose (1% CMC). 1. Plate screnning: Plate screening of the isolated organisms for cellulase production will be carried out according to the method of Kumar and Thippe (2012). A point of inoculation the spores of each fungi will be grown on PDA supplemented with 1% (W/V) carboxymethylcellose (CMC) for screening of carboxymrthycellulase. The plate will be incubated at 29±10 °C for 48hr after which they will be stained with Cango red stain for 15min. Excess dye will be removed by washing with 1 N NaCl and the plate will be fixed with 1 N HCl. The production of cellulolytic enzymes by the organisms will be indicated by a zone of clearance around the colonies. Selection of enzyme- producing strains Isolates that shown maximum synthesis of respective cellulolytic enzymes; beta-glucosidase, endoglucanase (carboxymethycellase) and exoglucanase (cellobiohydrolase) for the production of beta-glucose, endoglucose and exoglucose respectively indicated by the highest zone of clearance around the colonies in screening phase will be selected for the respective enzyme production.

PRODUCTION

Production of enzymes using solid state fermentation method.

Each fungi strain will be sub-cultured on Petri-dishes containing minimum salt medium- cellulotic materials and incubated at 28 °C for 10 days. 10 ml of a sterile solution of 0.2%, Tween 80 will be added to a Petri dish containing the culture. The mycelium will be scraped from each Petri dish and the mycelia suspension will be recovered into a sterile vial using a sterile pipette. A 20 g of each cellulotic materials will be moistened to 50% and placed into 500 ml Erlenmeyer flask and then autoclaved at 121 °C for 20 min. 3mls of mycelial inoculum will be used to inoculate the substrate (8.25mg / g substrate). The substrate will be inoculated and incubated at 30 ℃ for 7 days (Nabila et al., 2011).Bioconverted samples will be withdrawn at intervals of two days interval. The activity of the enzyme cellulases will be assayed using fresh fermented sample without drying during the course of fermentation (Pothirajet al., 2006).

Enzyme extraction.

A 200ml of distilled water will be mixed with 10g of the fermented substrate, the pH of the mixture will be measured and the mixture will be centrifuged at 1000 rpm at 20 °C for 20 min. The supernatant contained a crude enzyme extract. The supernatants will be carefully collected and stored aseptically under refrigerated conditions to prevent contamination. The crude enzyme thus obtained will be subjected to purification after enzyme assay (Li et al., 2003).

Assay for enzyme activity

Endoglucanase (carboxymethycellulase) activity will be measured as described by Ghose (1987) using a reaction containing 1ml of 1% CMC in 0.05M Sodium acetate buffer pH5.0 and aliquots of suitably diluted filtrate (Fungi). The reaction mixture will also be determined by DNS method. Enzyme activity will be read from a glucose standard curve. Purification (Ammonium sulphate precipitation) The method of De-Moraeset al., (1999) with modifications will be followed for purification of cellulytic enzymes. Different levels of ammonium sulphate (30, 60 and 80%) in citrate buffer (pH 4.8) will be used for the enzyme precipitation. The respective amounts will be added to 5 mL of crude enzyme solution and placed at 4°C for two hours with continuous stirring before centrifuged at 4000rpm for 20 minutes in macro centrifuge. The pellets will be collected carefully and the supernatant will be discarded. The pellets would then be dissolved in distilled water at a rate of 0.1g/mL. The dissolved pellets will be assayed for enzyme activities. The activity of cellulose would reflected the best level of $(NH_4)_2SO_4$ concentration (gram) for purification. Dialysis After ammonium sulphate precipitation the enzyme salt solution will be dialyzed against the citrate buffer for 24 hours at 4°C to remove ammonium salt (Peshinand Mathur 1999).

Gel filtration

The dialyzed fraction (5.0 ml) will be subjected to the gel filtration on Sephadex G- 75 column (1.6 cm×60 cm), pre-equilibrated with 0.05mol/L citrate buffer (pH 4.8) at a flow rate of 1.0 ml/min. Fractions of 5.0 ml will be collected; examined at 280 nm and assayed for respective cellulase activity. The active fractions containing cellulase activities from the column will be pooled and dialyzed against the citrate buffer for further analysis (Peshin and Mathur 1999).

Polyacrylamide disc gel electrophoresis

Polyacrylamide disc gel electrophoresis will be performed in 7.5% polyacrylamide gel at 28℃, pH 8.5 as described by Most and Nural (2009) for molecular weight determination. Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) will be performed on a 5% stacking and a 12% separating gel according to the method of Laemmli (1970) to determine the molecular weight of purified cellulase. To 100 µL of protein sample, 50 µL of sample buffer (0.05% bromophenol blue, 5% β-mercap- toethanol, 10% glycerol, and 1% SDS in 0.25 M Tris– HCl buffer; pH 6.8) will be added and boiled in boiling water bath for 5 min, cooled at room temperature and loaded onto the gel. Electrophoresis will be performed at room temperature for 2.5 h with a 120 Volt and then gel will be placed in fixing solution for 20 minutes followed by washing with three changes of distal water over 30 minutes time period. The protein bands will be visualized by staining with Coomassie Brilliant Blue G (Sigma) and destaining will be done again with distal water and kept for overnight in water at room temperature. The molecular weight of the purified cellulase will be determined in com-parison to marker protein (standard protein marker, 21- 116 kDa; Sigma, USA) after documentation.

Protein determination

Protein concentrations in the enzyme preparations will be determined using the method of Bradford (1976) by comparing the results with a standard curve for bovine serum albumin. Determination of shelf life of purified cellulase Shelf life or storage is an important parameter for commercial utilization of industrial products. The purified cellulase will be stored at room temperature (28oC-30°C) for 20 - 30 days to check the effect of storage on the enzyme activities of purified cellulase (Hafiz et al., 2011).

Physicochemical characterization

Characterization of the purified enzyme will be carried out according to the procedure of Coral et al., (2002).

Optimum temperature

For estimation of the optimum temperature of the enzyme, the activity will be determined by carrying out the assay at several temperatures between 30 and 90°C.

Optimum pH

The pH profile of the enzymes will be evaluated by incubating the purified enzymes for 10 min at 50oC in appropriate buffers: 50 mM sodium acetate (pH 3–4.5), 50 mM sodium citrate (pH 5–5.5) and 50 mM sodium phosphate buffer (pH 6–9).

Heat stability

The thermostability of cellulase will be studied by heating it at different temperatures (40-90°C) for 15 min. Percentage of the original activity retained after heat treatment at 90°C for 15 min will be calculated.

AGRICULTURAL APPLICATION

Source of Bird

A total of 25 cockerel chick birds will be purchased from Affcom Nig. Limited, kulende , Ilorin, Kwara State Nigeria. Bird Diet Feeding Twenty five, one day old, broiler chicks will be weighed individually and randomly distributed into five dietary groups, 5 in each. All chicks would receive vaccination against Newcastle and infectious bursal diseases. The feeding rations will be fed ad libitum to chicks for five weeks. During first two weeks, the birds were fed on basal feed and later (three weeks), on the feeds containing incorporated different concentrations of purified cellulase as shown in table below.

TABLE 3: Different concentration of enzymes used in the broilers' feed Treatment

Cellulase concentration	iu/bird/day
T1	100
T2	250
T3	500
T4	1000

Assessment of body performance and feed efficiency

Dietary effect on body performance (body weight, carcass weight, chest muscle, body fat, liver, heart and spleen), feed efficiency (weight gain, feed intake and feed conversion ratio) will be determined according to the standard methods (Ukoet al., 2006) Collection and preparation of blood sample After the termination of the feeding trials (5 weeks), the birds will be deprived of feed for 6 hours before blood collection. Each bird will be slaughtered using sterile equipment and under sterile condition. Blood will be collected in two sets of tubes containing heparin (0.2 mg/mL blood) and EDTA (1.0 mg/mL) for serological and hematological parameters, respectively. The blood will be centrifuged immediately after collection using the centrifuge and serum will be stored at -20°C till further analysis. Chemical-pathological analysis the serum samples will be analysed for liver function tests: Bilirubin total, bilirubin direct, bilirubin indirect and serum glutamate pyruvate transaminase), serum proteins (total protein and albumin), renal function tests (urea, creatinine and uric acid), electrolytes (sodium, potassium, chloride and bicarbonates) and lipid profile (cholesterol, triglycerides and high density lipoproteins) using commercial assay kits.

Haematological studies

The blood will be analysed for red blood cells index (hemoglobin, hematocrit, total red blood cells, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and platelets count) and white blood cells index (total white blood cells count).

Statistical analysis

All experiments and enzyme assays will be performed in triplicates; Complete Randomized Design (CRD) will be be applied and to determine the level of significance, the data obtained for each parameter would be subjected to Analysis of Variance technique. Duncan's Multiple Range Test (DMR) will be used to compare the means at 5% level of probability using CoStat Statistical Software, (2003).

Result and Discussion

Proximate composition of the Agrowastes

The proximate composition of onion brown skin, roasted groundnut, and coconut shell used as substrate were presented in Table 1. The Ash content of the alkaline-treated substrates increased significantly ($p < 0.05$). There was a recorded higher value of ash in coconut shells than in other substrates. Also, there was a significant increase ($p < 0.05$) in the content of crude fiber and carbohydrates in coconut treated shell compared to onion brown skin and roasted groundnut skin substrates. The moisture content of onion brown skin increased significantly ($p < 0.05$) compared to other substrates and the lipid and crude protein content of the roasted groundnut skin had a significant increase ($p < 0.05$) compared to other substrates.

Table 9 Physicochemical properties of the Agrowastes

Composition	Onion brown skin
Ash	0.8 ± 0.001^a
Moisture	0.5 ± 0.001^a
Crude Fiber	82.5 ± 0.003^a
Protein	0.25 ± 0.001^a
Carbohydrates	0.78 ± 0.003^a
Lipid	3.46 ± 0.005^a

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

Effect of inoculum size on cellulase production

The activities of endoglucanase, exoglucanase and β-glucosidase as a measure of cellulase production on onion brown skin, roasted groundnut skin, and coconut shell are shown in Figure 13, 14, and 15, respectively. An increase in the size of inoculum resulted in decreased activities of exoglucanase and endiglucanase on onion brown skin while β-glucosidase activity increases on the three substrates. The optimum inoculum size was $10x10^3$ CFU/ml for exoglucanase and endoglucanase in the three substrates while for β-glucosidase, optimum inoculum size was $13x10x^5$ CFU/ml.

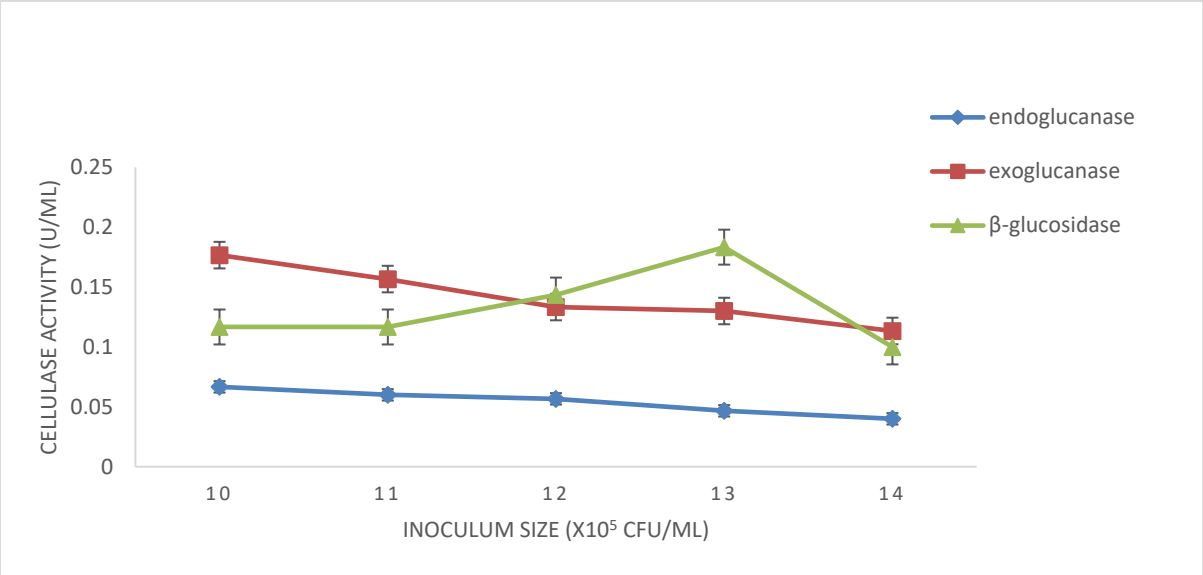

Figure 1 Cellulase activity of S. cerevisiae cultured on onion brown skin at varying substrate concentration.

Each value is expressed as mean ± SE of three different determination

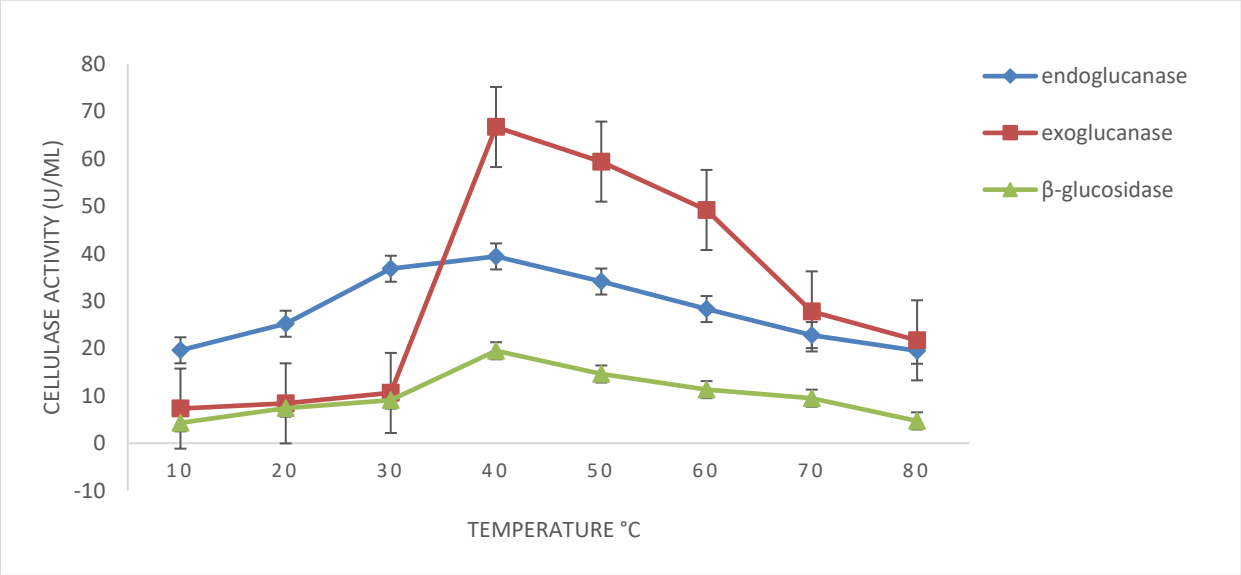

Figure 2 Cellulase activity of S. cerevisiae cultured on roasted groundnut skin at varying temperatures.

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

Effect of pH on cellulase production

The effect of pH on cellulase production on different substrates are shown in Figure 4-6. Cellulase production increased proportionately with increase in pH. β-glucosidase and Exoglucanase exhibited highest activity at a pH of 4.0 while endoglucanase had optimum pH in the range of 4.0 and 5.0. There was about 5 folds increase in the β-glucosidase and exoglucanase activity and 2 folds increase in the activities of endoglucanase on onion brown skin. An increase in pH after the optimum resulted in the loss of endoglucanase, exoglucanase and β-glucosidase activities on all the substrates. The endoglucanase activity on the roasted groundnut skin substrate increased compared to the other substrates.

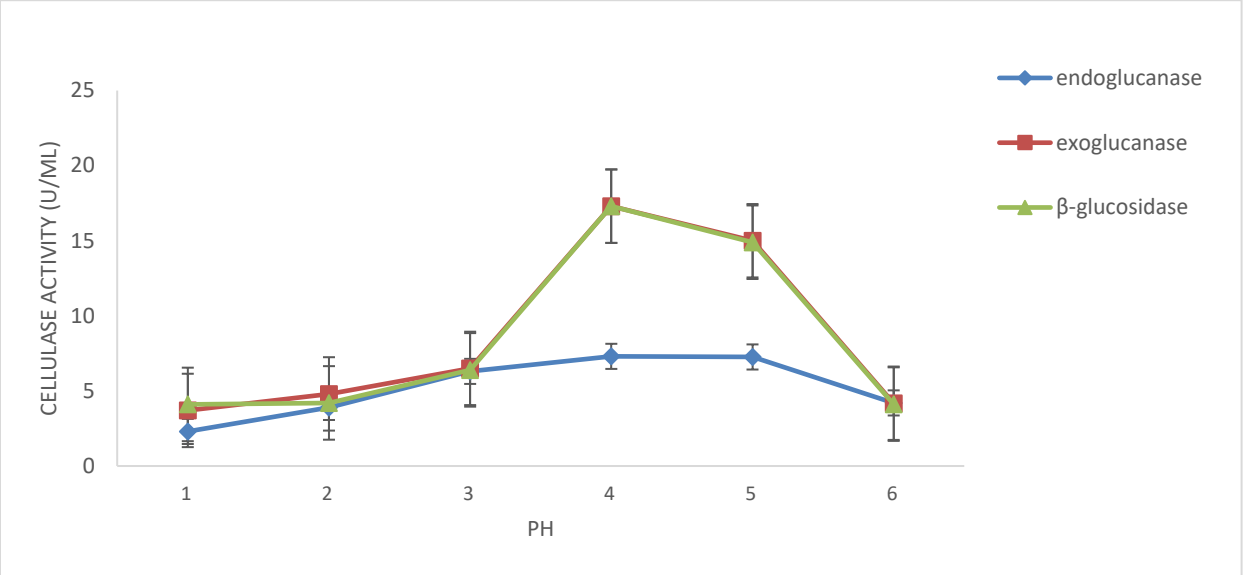

Figure 3 Cellulase activity of S. cerevisiae cultured on Onion brown skin at varying pH.

Each value is expressed as mean ± SE of three different determination

Effect of incubation period on cellulase production

The effect of incubation period on the production of cellulose in different substrates are presented in Figure 1-3. The endoglucanase, exoglucanase and β-glucosidase showed maximum activity at 96 h for each of the substrates. Cellulase production increased as the time of incubation increased and reached its maximum production at 96 h followed by gradual decrease up till 168 h. There was about 20 folds' increase in activities of endoglucanase and exoglucanase while that of β-glucosidase was about 3 folds at 96 h with respect to 0 h. There was a significant increase in the cellulase activity of β-glucosidase at the maximum incubation period (96 h) in onion brown skin compared to roasted groundnut skin and coconut shell

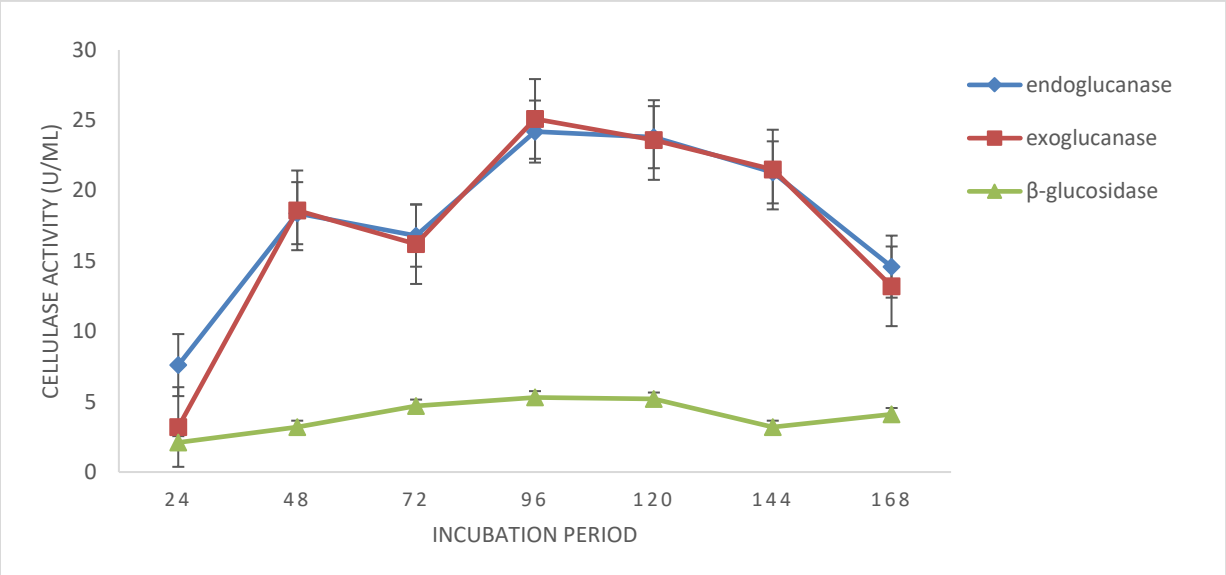

Table 2 Summary of purification of cellulase produced from culture of S. cerevisiae under optimum fermentation conditions on Onion brown skin.

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	87.46	13.65	8.31	1	100
2	NH_4SO_4 precipitation	25	22.55	2.230	15.52	2.65	30.37
3	Dialysis	15	8.35	0.072	228.14	33.47	8.32
4	Gel Filtration	5	2.46	0.010	489.36	69.25	3.15

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

Discusision of Findings

The substantial amounts of waste materials such as shells, skins, 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. 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 onion brown skin, roasted groundnut skin, and coconut shells as a substrate for cellulase production from yeasts cultured from Palm wine. The alkaline solutions applied to the substrates in this research may have eliminated significant amounts of lignin, enhancing their digestibility. This supports the observations made by Beukes and Pletschke (2011), who stated that alkali can remove lignins and improve the digestibility of agricultural waste. One advantage of using alkaline treatment as a pretreatment method is the versatility of the process. Mosier *et al*. (2005) and Chang (2007) also noted that alkali pretreatments dissolved lignin and improved the accessibility of the lignocellulosic surface by removing acetyl and uronic acid substituents from hemicellulose. A proximate analysis of the treated substrates was performed to assess the percentage of crude fiber and cellulose content. Crude fiber indicates the amount of undigested carbohydrates, including cellulose, pentosans, lignin, pectins, and other similar components found in food (Van Soest and Robertson, 1979). It represents the remaining residues of plant materials after solvent extraction and subsequent hydrolysis with dilute acid and alkali. The undigested or dietary fibers are a diverse mixture of various substances. The primary components include cellulose, a glucose polymer that is the main constituent of plant cells; hemicellulose, which is a shorter form of cellulose; and pectin, which acts as a binding agent for plant cells along with cellulose from the woody cell walls (Gidenne, 2003).

The Ash content of the alkaline-treated substrates increased significantly ($p < 0.05$). There was a recorded higher value of ash in coconut shells than in other substrates. Also, there was a significant increase ($p < 0.05$) in the content of crude fiber and carbohydrates in coconut treated shell compared to onion brown skin and roasted groundnut skin substrates. The moisture content of onion brown skin increased significantly ($p < 0.05$) compared to other substrates and the lipid and crude protein content of the roasted groundnut skin had a significant increase ($p < 0.05$) compared to other substrates (Table 1). The elevated crude fiber levels found in this study for treated onion brown skin, roasted groundnut skin, and coconut shell indicate that these three substrates are particularly rich in cellulose, making them suitable as a substrate for cellulase production (Table 1). Two different yeasts were identified in the palm wine. Of all the isolates, three were identified as *Saccharomyces cerevisiae* and one as *Geotrichum candidum* (Table 2). This makes *Saccharomyces cerevisiae* the dominant microorganism present in palm wine during fermentation, this is in agreement with previous studies carried out (Djeni *et al*., 2020; Amoa-Awua *et al*., 2007; Amoikon *et al*., 2018). The non- Saccharomyces present in the palm wine was much lower. The presence of *Geotrichum candidum* in palm wine have also been reported previously (Amoikon *et al*., 2018), which was stated could be that the area the sample was collected is a niche for the growth of the specie. However, it has been involved in other food product like cheese during the ripening (Perkins *et al*., 2020).

The optimal pH range for yeast growth can vary from pH 4.0 to 6.0 depending on temperature,the presence of oxygen and the strain of yeast. The pH of the fermentation medium is a crucial factor for cellulase production, as it influences the morphological characteristics of microbes and the enzyme output (Mrudula and Murugammal, 2011). According to Gupta *et al*. (2003), pH serves as a significant factor that impacts enzyme production during the fermentation process. Changes in pH during fungal growth also affect the stability of the products in the medium (Gupta *et al*., 2003). The optimal pH for achieving maximum cellulase production found in this study was between pH 4.0 and 5.0 under submerged fermentation (SmF) conditions (Figure 4, 5 and 6). Cellulase production increased proportionately with increase in pH. β-glucosidase and Exoglucanase exhibited highest activity at a pH of 4.0 while

endoglucanase had optimum pH in the range of 4.0 and 5.0. There was about 5 folds increase in the β-glucosidase and exoglucanase activity and 2 folds increase in the activities of endoglucanase on onion brown skin. An increase in pH after the optimum resulted in the loss of endoglucanase, exoglucanase and β-glucosidase activities on all the substrates. In addition, the endoglucanase activity on the roasted groundnut skin substrate increased compared to the other substrates. This findings align with the research by Fakruddin *et al*., 2012 who noted that a pH of 4 was optimal for maximum ethanol production when *S. cerevisiae* was grown on molasses. Additionally, Abubakar and Oloyede (2013), who noted that a pH of 4 was optimal for maximum cellulase production when A. niger was grown on rice bran and orange peel. Omojasola *et al*. (2008) also observed an optimal pH of 3.5 for cellulase production from A. niger cultured on pineapple waste. Despite the difference in organism used, the optimum pH is still maintained.Conversely, the results from this research differ from the findings regarding cellulase production from A. terreus QTC 828 in SmF by Ali *et al*. (1991) and from Trichoderma reesei in solid state fermentation (SSF) by Doppelbauer *et al*. (1987), both of which reported a pH of 6.0 for cellulase production. Furthermore, Krishna (1999) documented a pH of 7.0 for cellulase synthesis by bacteria using banana peel as the substrate in solid state fermentation. The discrepancies between the results from this study and previous reports may stem from the use of different microorganisms and varying agricultural waste materials.

As the incubation temperature rises, the frequency of collisions increases, leading to a higher reaction rate as seen in numerous chemical reactions. Nevertheless, the enzyme's stability diminishes due to thermal degradation. Keeping an enzyme at elevated temperatures for extended periods can result in denaturation. The finding from this research indicated an increase in temperature increased the amount of cellulase production in onion brown skin, roasted groundnut skin, and coconut shell. In the coconut shell substrates, it was observed that endoglucanase, exoglucanase, and β-glucosidase had an optimum temperature of 40 °C while the optimum temperature on the onion brown skin for endoglucanase, exoglucanase, and β-glucosidase is 30 °C, likewise β-glucosidase of the roasted groundnut skin. The findings from this research indicated that the optimal temperature for maximal enzyme production range within 30 °C-40 °C (Figure 7, 8, and 9). These results are consistent with the observations made by Ali *et al*. (1991) and Sulyman etc al., 2020, who noted the highest cellulase yield from the A. niger and A. terreus at 40°C, respectively, in solid-state fermentation. The loss of cellulase activity observed above 30°C and 40°C is likely attributed to the thermal degradation of enzymes.

Based on the findings gathered, along with those from other studies, the ideal inoculum density for enzyme production in solid-state fermentation can differ significantly, depending on the growth rate of the specific isolate being used and the substrate intended for colonization and utilization. An inoculum size that falls short of the optimal level can prolong the time required for cells to multiply, colonize the substrate, and generate the desired products (Ramachandran *et al*., 2004). On the other hand, a higher inoculum density exceeding the optimal point has typically been found to negatively impact enzyme production (Ibrahim *et al*., 2012). An increase in the size of inoculum resulted in decreased activities of exoglucanase and endiglucanase on onion brown skin while β-glucosidase activity increases on the three substrates. The optimum inoculum size was $10x10^3$ CFU/ml for exoglucanase and endoglucanase in the three substrates while for β-glucosidase, optimum inoculum size was $13x10^5$ CFU/ml (Figure 13, 14, and 15).

Substrate concentration plays a crucial role in influencing enzyme activity and, in turn, enzyme production. Enzymes function as intricate systems rather than mere surfaces where reactions occur, employing a wide range of chemical mechanisms (Scott, 1996). In reactions facilitated by enzymes, an increase in substrate concentration typically leads to enhanced enzyme activity (Scott, 1996). However, beyond a certain concentration, additional substrate will hardly affect enzyme activity, as the enzyme becomes saturated with its substrate at that point (Scott, 1996). The findings of this study indicated that the highest cellulase activity was reached at the highest concentrations tested. In this study, the activity of endoglucanase, exoglucanase and β-glucosidase increased with increase in the three substrates concentration (Figure 10-12). The activity of the three components measured followed Michaelis-menten hyperbolic curve with the substrate concentrations investigated. The activities of endoglucanase, β-glucosidase, and exoglucanase increased and reached maximum at a concentration of 4% in the

substrates and the activity of the cellulase dropped after reaching its maximum at 4 % substrate concentration in the three substrates.

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 *S. cerevisiae* when cultured on onion brown skin, roasted groundnut skin, and coconut shells used as substrate gave a percentage yield of cellulase that decreased stepwisely from 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 crude cellulase in the onion brown skin, roasted groundnut, and coconut shell had a total activity of 87.46 U/ml, 85.86 U/ml, and 86.16 U/ml while the specific activity was 8.31 U/mg, 8.73 U/mg, and 8.13 U/mg respectively. As crude enzyme was subjected to each step of purification process, there was reduction in the total activity of the enzyme across the three substrates used, which was accompanied by corresponding increase in the specific activity (Table 3,4 and 5). The percentage yield reported by this study was within 2.85-3.15 which is more than 2.11% reported by Iqbal *et al*., (2011) but less than 3.87 reported by Sulyman *et al*., (2020).

All hematological parameters apart from WBC in treatment groups receiving exogenous enzyme-supplemented diets (T_1, T_2, T_3, and T_4), were significantly ($P<0.05$) higher than the non-enzyme-treated diet groups (T_0) (Table 7). This suggests that the commercial feed enzyme-supplemented diets provided a better blood-building capacity than the non-enzyme-treated diets. This is in agreement with the reports of Ukorebi and John (2023) which posited that the inclusion of exogenous enzymes in the diets of animals also results in reductions in pathogenic microflora and the improvement in the health and welfare of birds. Indeed, hematological indices such as RBC, WBC, PCV, and hemoglobin concentration, have been found useful in disease prognosis and therapeutic regimen, as well as feed stress monitoring (Ukorebi and John, 2023). The RBC for instance functions primarily in the transport of oxygen from the lungs to body tissues, and the removal of carbon dioxide from the tissues to the lungs through hemoglobin, in the course of the biological oxidation of nutrients known as tissue respiration. This process is crucial to the maintenance of other biological functions in vertebrate organisms (Ukorebi, 2022).

Conclusions and Recommendations

The present work was carried out to optimize parameters to improve cellulase production by the cellulose producing fungi obtained from palm wine and determine the effect of different environmental factors on the production of cellulase. Additionally, this study also compare different substrates to determine the one with high cellulolytic effect of the yeast cultured, likewise see to the possible outcome on broiler chickens if incorporated to their conventional feeds via the hematological parameters. From this present study, the result showed that cellulolytic fungi can grow at optimized conditions of 120 h, pH of 4–5, temperature of 30-40 °C, substrate concentration of 1–5 %, and inoculum size of (10–13) $x10^5$ cfu/ml. Incorporation of the cellulase after purification in the conventional improved the weight gain, feed conversion and ration consumption, making it a better excipient in agricultural application. This study was carried out using a single strain of the yeast cultured from palm wine while the second yeast, though not abundant was not done. It is then recommended that further studies should be done on the effect of *Geotrichum candidum* on the agrowastes and compare the results with those obtained from this study.

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