EFFECT OF ENZYME SUPPLEMENTATION ON THE HEMATOLOGICAL INDICES OF BROILER CHICKENS

BY

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Abstract

A total of one hund red and fifty day old chicks was used for this experiment, enzymes was produced from solid state fermentation and was used to feed the chicks by adding it to their feed for six (6) week, it was added in 100, 250, 500 and 1000 IU/bird/day, at tye end of the experiment, it was found that the enzyme has no effect on white blood cell ($P>0.05$) but there was a significant different on ($P<0.05$) PCV, Hb, RBC, MCV, MCH and MCHC, also significant difference ($P<0.05$) was noticed feed intake, weight gain and feed to weight gain, it was found that the more the enzyme added the more the feed consumed and better corresponding weight gain, however the best feed conversion ratio was obtain ed from birds that consumed the 100IU/bird/day

Introduction

The world's population has been increasing in an unprecedented rate with a current population of about 7.95 billion (UNFPA, 2022), and projected to reach 9.7 billion by 2050 (UN, 2022). To cater for this increasing population, agricultural productivity has also increased globally attaining about 23.7 million tons of foods on a daily basis (Duque-Acevedo et al., 2020). The increased agricultural productivity is accompanied with the generation of huge amounts of agricultural wastes, whose disposal creates greater pressure on the environment causing negative impacts on soil, air and water resources; thus putting sustainability of the ecosystem at risk. These impacts may become more intense following projection that the world agricultural production must increase by 70% so as to meet the demands for food by 2050 (FAO, 2009). 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 Discussions

The effect of enzyme supplementation of basal diets on the hematology of broiler chickens are presented on Table 1. The results show that White blood cell (WBC) has no significant (P>0.05) difference among the groups meaning that, no amount of enzyme supplemented that will have effect on the white blood cell, whereas packed cell volume

(PCV), hemoglobin concentration (Hb) and erythrocyte concentration (RBC), indicated a significant difference effects ($P<0.05$) among the groups but other hematological indices that were significantly ($P<0.05$) affected by treatments were the Mean cell volume (MCV), Mean cell hemoglobin (MCH) and Mean cell hemoglobin concentration (MCHC), meaning addition of enzyme to basal feed had effect on the parameters

Table 1: Effect of enzyme supplementation on the hematological indices of broiler chickens

PARAMETER	T_0	T_1	T_2	T_3	T_4
Hb (g/dl)	6.31 ± 0.32^a	11.50 ± 0.32^b	18.58 ± 0.32^c	21.67 ± 0.32^d	29.71 ± 0.32^e
RBCx10^3/M	3.43 ± 0.27^a	5.48 ± 0.27^b	8.51 ± 0.27^c	10.59 ± 0.27^d	13.64 ± 0.27^e
PCV(%)	26.41 ± 0.34^a	34.73 ± 0.34^b	37.83 ± 0.34^c	42.87 ± 0.34^d	46.96 ± 0.34^e
WBCx10^3/M	11.21 ± 0.41	11.23 ± 0.41	11.23 ± 0.41	11.25 ± 0.41	11.27 ± 0.41
MCV (FL)	54.34 ± 2.17^a	74.47 ± 2.17^b	95.18 ± 2.17^c	106.38 ± 2.17^d	113.72 ± 2.17^e
MCH (PG)	23.24 ± 1.35^a	29.39 ± 1.35^b	36.25 ± 1.35^c	41.72 ± 1.35^d	53.13 ± 1.35^e
MCHC (G/DL)	21.04 ± 1.05^a	28.17 ± 1.05^b	38.03 ± 1.05^c	44.37 ± 1.05^d	51.31 ± 1.05^e

T_0:broiler chickens fed Basal diet, T_1: broiler chickens fed Basal diet + cellulase 100 IU/day, T_2:broiler chickens fed Basal diet + cellulase 250 IU/day, T_3:broiler chickens fed Basal diet + cellulase 500 IU/day, T_4:broiler chickens fed Basal diet + cellulase 1000 IU/dayEach 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.

Agricultural Application

The table 2 below shows the ration consumption, weight gain, and feed conversion in the broilers chickens fed cellulase-supplemented rations. There was influence of cellulase enzyme supplementation on the ration consumption, weight gain and feed conversion parameters. The addition of cellulase at different concentration significantly improved feed intake during this period compared to the control that was feed basal diet only. There was a significant difference ($p<0.05$) in the weight gain and feed conversion between the groups. The significant difference occurred at all levels of inclusion, the more the concentration of the enzyme added to their feed, the more the feed consumed and better corresponding weight gain, however the chicks that consumed the 100IU/bird had the best feed conversion ration.

Table 2 Ration consumption, weight gain, and feed conversion in chickens fed cellulase-supplemented rations.

Treatments	Ration Consumption (g)	Weight Gain (g)	Feed Conversion
Basal diet	752.71[a]	465.48[a]	1.62[a]
Basal diet + cellulase 100 IU/day	743.27[b]	457.37[b]	1.58[b]
Basal diet + cellulose 250 IU/day	846.68[c]	475.82[c]	1.67[c]
Basal diet + cellulose 500 IU/day	873.74[d]	504.63[d]	1.74[d]
Basal diet + cellulose 1000 IU/day	896.91[e]	512.51[e]	1.86[e]
SEM	0.065	0.054	0.072

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

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 compares 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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