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Optimization of Fungi Lignocellulose degrading enzymes, isolated from Fadama Soil at Dutse Local Government Area of Jigawa State.

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Abstract
This research work was aimed at isolating and identifying fungal species with the potential to produce lignocellulose degrading enzymes and optimizing their culture producing condition. Soil samples were obtained from Madaki, Dadin Duniya and Federal University Dutse from Dutse local government area of Jigawa State. The fungal species isolated from the soil were *Aspergillus flavus, Aspergillus fumigatus, Alteneria* spp. *Mucor* spp and *Rhizopus* spp. The five fungal species were screened for their ability to grow on wood containing agar medium and decolorization of synthetic dye, Remazol Brilliant Blue R (RBBR) and methyl green as an inidicator for the production of lignocellulose degrading enzymes. *A. fumigatus* was found to have the highest lignocellulose degrading enzymes ability and hence selected for optimization process. The optimum temperature for cellulase activity using wheat bran as substrate was obtained at 45ºC with reducing sugar concentration of 0.47mg/ml with pH of 6 with concentration of 0.50mg/ml at 20 days of incubation with concentration of 0.51mg/ml, 1% substrate concentration of 0.53mg/ml and 10^{-3} spore suspension of inoculum size with reducing sugar concentration of 0.49mg/ml. For xylanase, optimization was observed at temperature of 35ºC with reducing sugar concentration of 0.62mg/ml at pH of 6 with concentration of 0.57mg/ml at 20days of incubation using 1% substrate concentration and 10^{-3} spore suspension with concentration of 0.58mg/ml and 0.56mg/ml respectively. Finally, the production of lignin degrading enzymes was negligible.

Keywords: optimization, lignocellulose, fadama soil, fungal species.

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Introduction

Lignocellulose describes the three major constituents in plants, namely cellulose, hemicelluloses and lignin. The composition of lignocelluloses depends not only on the species but also on the growth conditions, the different parts of the plant and their age (Jørgensen, 2003). Lignocellulose is a network of lignin, cellulose and hemicellulose that is chemically bonded through non-covalent forces and covalent cross-linkages (Perez *et al*., 2002), and it is the major structural component of woody plants. Cellulose and hemicellulose are carbohydrate polymers, while lignin is a complex aromatic polymer. In woods, lignin

physically surrounds and protects the carbohydrate polymers from enzymatic hydrolysis and is also the most recalcitrant component of plant cell wall (Chandel *et al*., 2013). Although, many microorganisms are capable of degrading and utilizing cellulose and hemicelluloses as carbon and energy sources, only a smaller group of filamentous fungi known as white-rot fungi possess the unique ability to efficiently degrade lignin (Sanchez, 2009). When wood-decomposing fungi attack woods, a range of degradative extracellular, enzymatic and non-enzymatic activities are carried out, and these alter the wood chemically and morphologically, resulting in three major types of rot: white, brown and soft rots (Blanchette, 1995; Sanchez, 2009). These enzymatic activities are performed by complex mixtures of cellulases, hemicellulases, and ligninases (Sanchez, 2009; Andlar *et al.,* 2018). Cellulase refers to a family of enzymes which act in concert to hydrolyze cellulose. Cellulase is used extensively in the textile and food industries, bioconversion of lignocellulosic wastes to alcohol, animal feed industry as additive, isolation of plant protoplasts, in plant virus studies, metabolic investigations and genetic modification experiments (Lo *et al.,* 2005). The potential applications of xylanases also include the bioconversion of lignocellulosic material and agro-wastes into fermentative products, the clarification of juices, the improvement of the consistency of beer and the digestibility of animal feedstocks (Wong *et al.,* 1988). One of the most important biotechnological applications of xylanase is its use in pulp bleaching (Viikari *et al.,* 1994; Sabbadin *et al*., 2018). Lignin plays a significant role in the carbon cycle, sequestering atmospheric carbon into the living tissues of woody perennial vegetation. Lignin is one of the most slowly decomposing components of dead vegetation, contributing a major fraction of the material that becomes humus as it decomposes (Sjostrom *et al.,* 1993; Andlar *et al.,* 2018). The resulting soil humus, in general, increases the photosynthetic productivity of plant communities growing on a site as the site transitions from disturbed mineral soil through the stages of ecological succession, by providing increased cation exchange capacity in the soil and expanding the capacity of moisture retention between flood and drought conditions (Kuroda *et al*., 2001).

Materials and Methods.

Sample collection

Soil samples were collected from different Areas at Dutse local government Area of Jigawa state from a depth of 1-5 inches from the top and sieved through a 2mm sieve constituted the soil sample. The samples were dispensed into clean polythene bag and immediately transported to Microbiology Research laboratory of Bayero University Kano.

Isolation and identification of fungi

The fungal isolates were isolated from soil by the serial dilution method of Gillman. One gram of the soil sample was suspended in test tube containing 9ml of sterile distilled water. An aliquot of 1ml was taken and serially diluted to six-fold dilution. After the serial dilution, 0.1ml from the 10^6 dilution was spread on potato dextrose agar plates. The plates were incubated at room temperature for 5 days. The colonies that developed were subcultured unto potatoe Dextrose Agar (PDA) and were identified using colonial appearance and microscopic examination, Refa'I (1979).

Screening of potential degraders

Screening test for the ability of organisms to grow on wood-containing agar plates was performed in wood-containing agar medium. The medium contained 2% w/v wood powder, 5% v/v stock salt solution, 0.02% v/v trace elements, 0.05% w/v glucose and 1.5 % w/v agar. The stock salt solution contained the following (per liter): $NaNO_3$ 6g, KCl 0.52g, $MgSO_4.7H_2O$ 0.52g and KH_2PO_4 0.82g. The trace elements solution contained the following (per liter) ZnSO4.7H2O 2.2g, H3BO3 1.1g, $MnCl_2.4H_2O$ 0.5 g, $FeSO_4.7H_2O$ 0.5g, $CoCl_2.6H_2O$ 0.16g, $CuSO_4.6H_2O$ 0.16g, $(NH4)_6Mo_7O_{24}.4H_2O$ 0.11g and Na_2EDTA 5g.

Dye decolorization test.

In dye decolorization tests, PDA medium containing 0.04% w/v Remazol Brilliant Blue R, (RBBR) and 0.04% w/v methyl green) were used. RBBR and methyl green were added as a sterilized filtered solution. Each fungal strain was inoculated onto the media plates and incubated at 30ºC and the growth was observed for a period of 2 weeks, (Kyi, 2011).

Assessment for lignocellulose degrading enzyme production.

Lignocellulose degrading enzymes production was assessed in wheat bran liquid media containing 2% (w/v) wheat bran, 0.05% (w/v) glucose, 5% (v/v) stock salt solution and 0.02% (v/v) trace element.

Enzyme extraction for cellulase and hemicellulase assay.

A cornical flasks filled with 150 ml of liquid media was autoclaved at 121ºC for 20 min. Mycelia from an agar plate were used for inoculation. The flasks were incubated at 30ºC while agitating and samples were collected after 5 days. The collected samples were centrifuged at 5100 rpm for 15 minutes and the supernatant was stored for further analyses.

Enzyme extraction for Laccase Assay

The enzyme extract was prepared by homogenizing 0.5ml of the liquid media in 2.0ml of an extraction medium containing tris HCl, sorbitol and NaCl. The homogenate was centrifuged at 5100rpm for 15 minutes and the supernatant was used for the assay.

Cellulase Assay

Cellulase was assayed by adding one millilitre of enzyme (fermented broth supernatant) in test tube containing 1ml of 0.1M citrate buffer pH 5 followed by the addition of 1ml of 1% carboxymethyl cellulose solution. The test tubes were incubated for 30 minutes at 50ºC. After incubation 3ml Dinitrosalicylic acid (DNS) reagent was added and then boiled for 15 minutes in boiling water bath followed by the addition of 1ml sodium potassium tartarate. After cooling to room temperature absorbance was measured at 540nm. Cellulase was defined as the amount of enzyme which released 1 unit of reducing sugar measured as glucose per minute under the assay condition.

Hemicellulase Assay

Hemicellulase was assayed by adding 1 millilitre of enzyme (fermented broth supernatant) in test tube, 1ml of citrate buffer pH 5 was added and finally 1ml xylan solution was added the mixture was incubated at 50ºC for 30 minutes on water bath. After incubation 3ml of DNS reagent was added and placed in boiling water bath for 15 minutes followed by the addition of sodium potassium tartarate. After cooling absorbance was measured at 540nm. Xylanase activity was defined as the amount of enzyme which released 1 unit of reducing sugar measured as xylan per minute under the assay condition.

Lignin Assay

Phosphate buffer (2.5ml) and 0.3ml of catechol solution was added in the cuvette and the spectrophotometer was set at 495nm.The enzyme extract (0.2ml) was added and change in absorbance was recorded for every 60 seconds up to 5 minutes in a spectrophotometer. One unit of laccase is defined as the amount of enzyme that transform 1µ mole of quinone per minute

Optimization of enzyme production

Effect of Temperature.

To evaluate the effect of temperature on lignocellulose activity, the selected fungal isolate was grown at 30ºC in wheat bran. After incubation at 25, 30, 35, 40 and 45ºC, culture broths were centrifuged at 140 rpm for 20 minutes to obtain supernatants which were later used for the assay.

Effect of pH.

The effect of media pH on lignocellulose activity was conducted by adjusting the pH to 3, 4, 5, 6, and 7 with NaOH and HCl before fungal inoculation. After 5days of incubation at 30ºC, culture broths were then centrifuged at 140 rpm for 20 minutes to obtain supernatants which were later used for assay.

Effect of incubation period.

In order to determine the effect of incubation period on lignocellulose activity, the selected fungal isolate was grown in wheat bran and then incubated for 5, 10, 15, 20 and 25 days. Culture broths were then centrifuged at 140

rpm for 20 minutes to obtain supernatants which were later used for assay.

Effect of substrate concentration.

To evaluate the effect of substrate concentration on lignocellulose activity, the selected fungal isolate was grown at 30ºC in 1, 2, 3, 4 and 5% of wheat bran and incubated for 5 days, culture broths were centrifuged at 140 rpm for 20 minutes to obtain supernatants which were later used for the assay.

Effect of inoculum size.

To evaluate the effect of inoculum size on lignocellulose activity, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} using the spore suspension of the selected fungal isolate was grown at 30ºC in 2% of wheat bran and then incubated for 5days, culture broths were centrifuged at 140 rpm for 20 minutes to obtain supernatants which were later used for the assay.

Results and Discussion

A total of five (5) fungal species were isolated from the soil samples, they are *Aspergillus flavus, A. fumigatus, Rhizopus* spp, *Mucor* spp and *Alternaria* spp. *Aspergillus fumigatus*, being the most predominant species was selected to further investigate their lignocellulose degrading enzyme ability due to its capability to grow on wood and the ability to decolorize the synthetic dye.

Effect of Temperature on cellulase and xylanase activity

The effect of temperature on the enzyme activity is presented in Figure 1. The result shows that the cellulase and xylanase yield was maximum at 45ºC and 35ºC with reducing sugar concentration of 0.47mg/ml and 0.62mg/ml respectively and showed reduction in the activity as the temperature decreases. This coincides with the result obtained by Padmavathi *et al.*, 2001, as *A. niger* produces maximum of xylanase at 45ºC. The enzyme activity gradually decreased probably due to enzyme denaturation, conformation change, as enzymes are proteins. Therefore, pre-treatment of lignocellulosic material enhances enzyme activity and maximum saccharification was achieved within the range 30-45ºC coinciding with the characteristics of mesophiles (Baig *et al*., 2004).

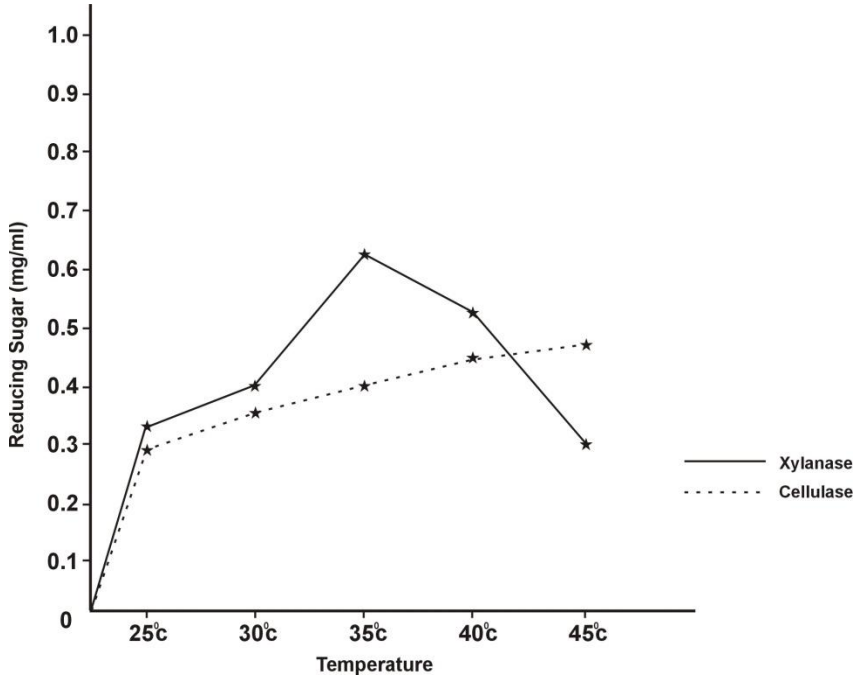

Figure 1: Effect of Temperature on cellulase and xylanase activity**.**

Effect of PH on cellulase and xylanase activity

The effect of PH on the cellulase and xylanase activity of *A. fumigatus* was examined at various pH ranging from 3 to 7 as shown in figure 2. The maximum activity was displayed at pH of 6 with reducing sugar concentration of 0.50mg/ml for cellulase and 0.57mg/ml for xylanase and least activity was displayed at pH of 3 with an activity of 0.30mg/ml and 0.33mg/ml of the respective enzymes. Enzymes have pH range within which they function best with their activity maximum at optimum pH and at higher or lower pH values, their activity decreases (Lehinger, 1993). The cellulase and xylanase produced showed that optimum pH was found to be near neutral (PH of 6). Endoxylanse I and II from *A. awamori* shows an optimum pH at 5.5 and 6 respectively (Kormelink *et al.*, 1993; Naitam *et al.*, 2022).

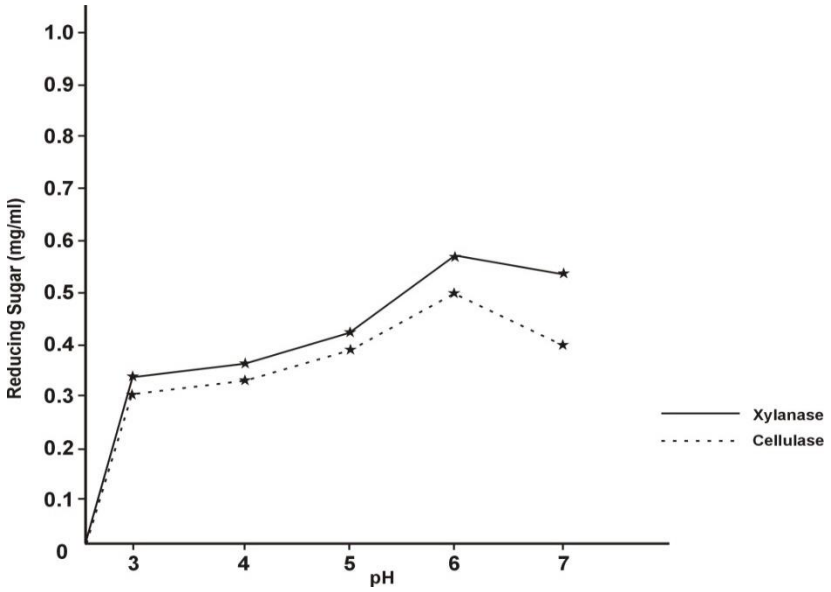

Figure 2: Effect of pH on Cellulose and Xylanase activity.

Effect of incubation period on cellulose and xylanase activity

Figure 3 shows a gradual increase in enzyme activity through 5, 10, 15 and maximum at 20days with reducing sugar concentration of 0.51mg/ml for cellulase and 0.60mg/ml for xylanase. The enzyme activity showed a gradual decrease on further extension of incubation period beyond 25days. The effect of incubation period on the enzymes production increased progressively and attained the peak activity at the 20th day of incubation and then declined on the 25th day. The current result differs from that of (Kyi, 2011) who reported peak cellulase and xylanase activity by *P. pinophilum* at the 17th day of incubation. It is also contrary to the findings of (Kaitam *et al*., 2022) where *A. nidulans* the optimum enzymatic activity was on the 7th day. The decline in enzymes activity on the 25th day may be explained by the fact that at this stage the isolates have entered their late stationary phase.

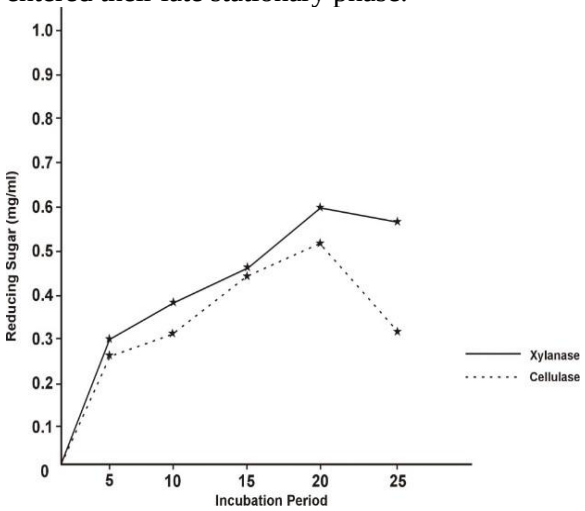

Figure 3: Effect of incubation period on Cellulose and Xylanase activity.

Effect of substrate concentration on cellulase and xylanase activity

The effect of substrate concentration (wheat bran) is shown in figure 4. From the figure, substrate concentration of 1% gave the highest activity of both enzymes with reducing sugar concentration of 0.53mg/ml for cellulase and 0.58mg/ml for xylanase while the substrate concentration of 5% gave the lowest activity of 0.32mg/ml and 0.35mg/ml respectively from *A. fumigatus*. Further increase in wheat bran beyond one percent (1%) did not result in proportionate increase in enzymes yield. Mandels and Reese (1959) also reported that maximum yield of cellulase were obtained on 1% substrate. This may be due to the fact that certain sugars are inhibitors of enzyme production while others stimulate enzyme production. Ibrahim *et al*, 2011 reported that *Adansonia digitata* fruit pulp inhibited the production of Amylase by *B. licherniformis.*

Effect of inoculum size on cellulase and xylanase activity

There was no increase in enzyme activity when the size of the inoculums was further increased (Figure 5). The maximum activity for cellulase and xylanase was 0.49mg/ml and 0.56mg/ml respectively when 10^{-3} of inoculums were used. Inoculum size of 10^{-3} dilution resulted in a higher cellulase and xylanase production compared to other inoculum sizes. Higher enzyme production of higher inoculum is related to the rapid growth of the fungus, which resulted higher degradation of the substrates and increase availability of the nutrients. Inoculum size beyond this level declined the enzymes production, inoculum size controls and shortens the lag phase, smaller inoculum size increased the moisture content which ultimately decreases the growth and enzymes production (Sharma and Behere, 1996).

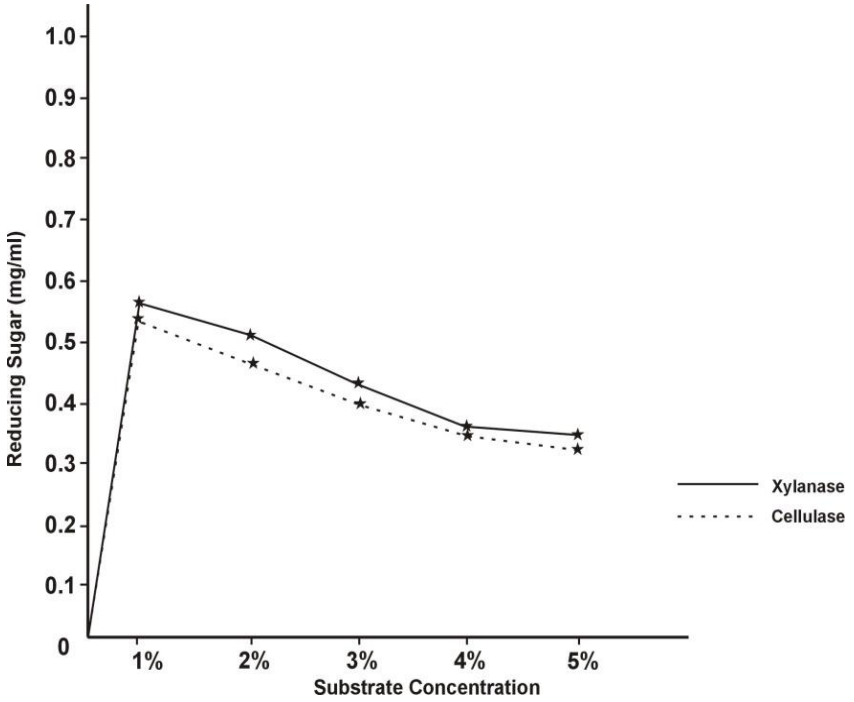

Figure 4: Effect of Substrate concentration on Cellulase and Xylanase activity.

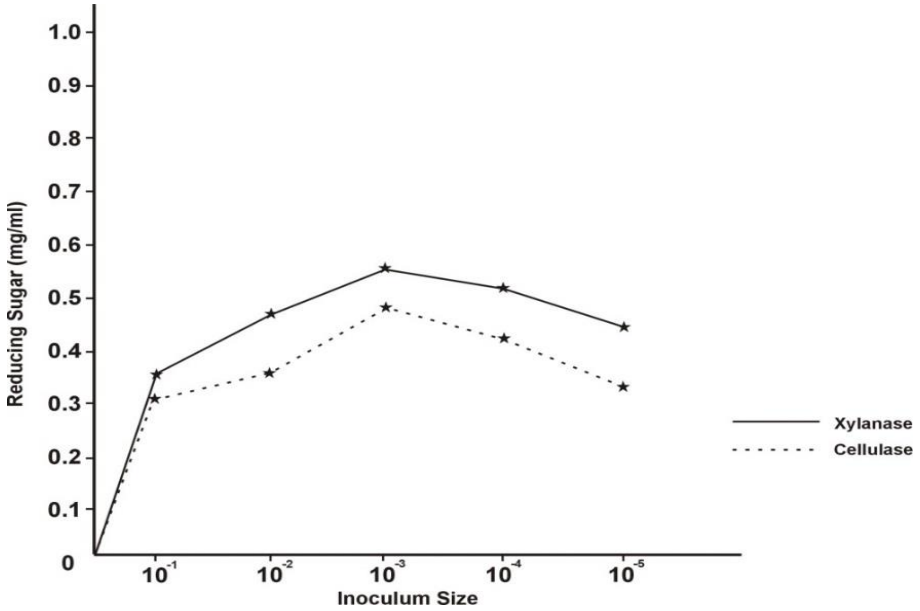

Figure 5: Effect of inoculum size on Cullulase and Xylanase activity.

Conclusion

In conclusion, the successful use of lignocellulosic material as carbon source is dependent on the development of economically feasible process for cellulase and xylanase production. The production of the enzymes by *A. fumigatus* on wheat bran was maximum at pH of 6, 20days of incubation using 1% substrate concentration with 10^{-3} of the inoculum.

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