CELLULASE PRODUCTION FROM MONO-CULTURE AND CO-CULTURE OF INDIGENOUS FUNGI DURING SOLID STATE FERMENTATION OF GROUNDNUT SHELL BIOMASS.

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ABSTRACT

The use of cellulolytic enzymes has over the years attracted considerable interest as a means for the production of value-added products from cellulose containing materials in the environment. Fungi were isolated from decaying groundnut shell and subjected to cellulolytic screening on modified Czapek-Dox agar supplemented with 1% carboxymethyl cellulose. Four out of the five isolates screened gave highest zones of clearance and were identified as *Fusarium oxysporum*(B_1), *Fusarium* sp (B_2), *Aspergillus flavus*(D_2), *Trichoderma harzianum* (D_3). These four fungi were selected on the basis of performance as screened. Assaying for fungal cellulase enzymes among the single isolates (mono-culture), *Aspergillus flavus* had the highest cellulase (FPase) activity of 2.424 FPU/ml after the 4th day of solid state fermentation (SSF) of the pretreated groundnut shell while *Trichoderma harzianum* gave an enzyme activity optimum of 2.312 FPU/ml after the 5th day of SSF. In the mixed fungal cultures, the co-cultures B_1D_3 gave the highest enzyme activity of 7.206 FPU/ml after the 3rd day of fermentation while B_2D_2 had an activity optimum of 5.402 FPU/ml after the 4thday of fermentation. For mixed culture B_2D_3, an optimum activity of 5.012 FPU/ml was obtained after the 3rd day of SSF, while co-culture D_2D_3 had enzyme activity optimum of 4.450 FPU/ml after the 4th day of fermentation. However, a consortium of all the isolates applied in the fermentation gave a maximum cellulase activity yield of 6.982 FPU/ml after the 3rd of SSF respectively.

Keywords: **Cellulase, Groundnut shell, indigenous fungi, Fermentation.**

INTRODUCTION.

Cellulose is a biopolymer of glucose linked by β-1, 4 glycosidic linkages (Stryer 1996).

It is an abundant component of plant biomass. It is found in nature almost exclusively in plant cell walls (Lynd *et al.*, 2002). It accounts for 50% of the dry weight of plant biomass and approximately 50% of the dry weight of secondary sources of biomass such as agricultural wastes. The degradation of cellulosic biomass represents an important part of the carbon cycle within the biosphere and this underlies its potential as a source of energy (Coral *et al.*, 2002).An important feature of cellulose, relatively unusual in the polysaccharide world, is its crystalline structure. (Zhou and Ingram, 2000).

Cellulose is degraded by an enzyme called cellulase which belongs to a class of inducible enzymes that are synthesized by large number of microorganisms, including fungi, bacteria and protozoans, either cell-bound or extracellular during their growth on cellulosic materials (Lee and Koo, 2001; Immanuel *et al*., 2006). Although a large number of microorganisms can degrade cellulose, only a few of them produce significant quantities of free enzyme capable of completely hydrolyzing crystalline cellulose (Koomnok, 2005). The three main types of cellulases have been established in the cellulolytic enzyme system are: endo-β-1, 4-glucanase, exo-β-1, 4-glucanase and β-glucosidase. The endoglucanases act internally on the chain of cellulose cleaving β-linked bonds liberating non-reducing ends, and exoglucanases remove cellobiose from this non-reducing end of cellulose chain. Finally, β-glucosidase completes the saccharification by splitting cellobiose and small cello-oligosaccharides to glucose molecules (da Silva *et al*., 2005).

Groundnut shell, a lignocellulosic material, is a derived post-harvest product of the groundnut plant – *Arachis hypogea* L. The shell is an agricultural waste obtained from milling of groundnut. It contains 65% cellulose (Masenda*et al*.,2004) and can be used for the production of extracellular enzymes (Tewari*et al*., 1988).

Groundnut is widely grown in the northern part of Nigeria and a large amount of its shells are generated as wastes during its processing.This agro-waste is known to resist degradation for the periods of between 1-3 years, thus predisposing the environment to deleterious impacts that could have been averted and also creating a pollution problem. The shell most often burned in the environment releases carbon dioxide gas into the atmosphere which affects the ozone layer contributing to global warming.

Considering therefore the deleterious impacts and the pollution problems associated with groundnut shell due to its persistence in the environment, appropriate knowledge of the shell being used as substrate for the production ofcellulase enzymesnecessitated this research.

MATERIALS AND METHODS

Sample collection.

Dry groundnut shells were collected from a local groundnut processing plant in Bambuka, Taraba State located on longitude: 11 22' 13.5"E; latitude 9 29' 30.2" Nand Altitude: 184. 32m. Decayed shells were also collected from a dump site near the processing plant for the purpose of fungal isolation.

Isolation of fungi.

Following the method of Lakshmi and Narasimha(2012), one gram of the decayed shell was transferred to 10 ml of distilled water in test tubes and shaken well. Serial dilutions were made up to 10^{-6} and 0.5 ml each from 10^{-5} and 10^{-6} dilutions were inoculated on sterile Potato Dextrose Agar (PDA) using streak plate method. Pure culture of the fungal isolates were maintained on Potato Dextrose Agar (PDA) slants and kept at 4°C in a refrigerator. Isolates were identified according to morphological characteristics as described by Domsch*et al*. (1980).

Screening for Cellulolytic Activity.

The isolates were screened for their ability to produce cellulase complex following the modification of the method of Teather and Wood (1982). Czapek-Dox medium (g/l) [sucrose 30, $NaNO_3$ 2, K_2HPO_4 1, $MgSO_4$ 0.05, KCl 0.5, $FeSO_4$ 0.01, carboxy methyl cellulose 1%, Agar agar 20] was used. Using a 6 mm size cork borer, wells were made in the solidified medium and inoculated with equal sizes of the agar plug containing the isolates from 5 day cultures. After the initial incubation at room temperature (28 ± 2 °C) for three days, the plates were further incubated for 18 h at 50 °C, the optimum temperature for cellulases activity (Lakshmi and Narasimha,2012). This is because the optimum temperature for enzyme activity varies between enzyme types. After incubation, 10 ml of 1% Congo - Red staining solution was added to the plates and allowed to stay for 15 min. The Congo - Red staining solution was then discarded. Each plate was flooded with 10 ml of 1 N NaOH for 15 minutes with a continuous shaking. The plates were observed for the formation of halo zones around the fungal plugs in the inoculated wells as indication of cellulase production.

Inoculum Preparation.

Sterile distilled water (5 ml) was added to each fungal agar slant and shaken vigorously for preparing uniform suspension which was used as inoculum for solid-state fermentation (Reddy *et al.*, 1998).

Pretreatment of Groundnut Shell for Fermentation.

The dried groundnut shell was milled using Hammer mill at the Department of Food Technology, University of Ibadan and was sieved using 180μm sieve. Dried powdered shell (100g) was soaked in 1NNaOH (500ml) for 24 h. The excess alkali used was decanted and the substrate washed repeatedly with distilled water till it reached a neutral pH after which it was dried over night at 60°C (Fan *et al.*,1987).

Substrate Preparation and Inoculation (Solid State Fermentation).

Pretreated groundnut shell (10g) was moistened with 50 ml modified Mandel and Reese medium (Reese and Mandel, 1957) [Peptone, 1.0; $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2.0; NH_2-CO-NH_2, 0.3; $MgSO_4.7H_2O$, 0.3; $CaCl_2$, 0.3; $FeSO_4.7H_2O$, 0.005; $MnSO_4.H_2O$, 0.0016; $ZnCl_2$, 0.0017; distilled water, 1000 ml; pH: 5.3] to initial moisture content of (50 %) and autoclaved in 250 ml Erlenmeyer flasks at 121° C for 1 h. The flasks containing the substrate were inoculated with 2 ml of the fungal spores upon cooling using both mono-cultures of the fungi [*Fusarium oxysporium*(B_1), *Fusarium* sp (B_2), *Aspergillus flavus*(D_2),and*Trichoderma harzianum*(D_3)] and the mixed cultures combined as follows (B_1+B_2; B_1+ D_2;B_1+ D_3; B_2 + D_2; B_2 +D_3; D_2+ D_3; and consortium $B_1B_2D_2D_3$). All tests were carried out in duplicates. The fermentation was carried out at 30°C under static conditions for a period of 8days. All samples were replicated to allow for single withdrawal. Control samples were also set up alongside the treatment.

Enzyme Assay.

Samples were withdrawn every 24 hrs from each replicate for the period of 8 days and assayed for enzyme activity. The enzyme was extracted using phosphate buffer (pH 5). One gram of the sample was added to 10 ml of phosphate buffer and mixed by swirling to homogenize. The enzyme extract was obtained by filtering through Whatman filter paper no.1 and centrifuged at 9500 rpm for 20 minutes at 4°C. The clear supernatant obtained was used for enzyme assay (Vyas and Vyas, 2005).

Filter Paper Cellulase (FPase) Assay.

Filter Paper Activity (FPase): Filter paper activity of the crude enzyme was done according to the method of Ghose (1987). It is a combined assay for endo β-1, 4 glucanase and exo β-1, 4 glucanase. This analytical method measures the cellulase enzyme complex of a given organism. The standard reaction mixture containing 50 mg of Whatman No.1 filter paper strips (1×6cm) as a substrate was suspended in a mixture containing 1 ml of 0.05 M sodium citrate buffer (pH 4.8) and 0.5 ml of enzyme source in a test tube. The mixture was incubated for one hour at 50°C in water bath. The reducing sugar was estimated by dinitrosalicylic acid method (Miller, 1959). After incubation, 3 ml of DNS reagent was added to each test tube to stop the reaction. The mixture thereafter was boiled for 5 min in a boiling water bath. After boiling, the test tubes were transferred to a cold water bath to which 10 ml of distilled water was added. The content of the tubes were mixed completely by slanting the tubes several times so that the solution separates from the bottom of the tube at each slanting. The colour developed in the tubes was read at 540 nm using a spectrophotometer (Camspec M105). The enzyme control was also prepared simultaneously by adding distilled water instead of enzyme. The enzyme activity was expressed in filter paper units. Filter paper units were defined as the amount of enzyme releasing μ moles of reducing sugar from filter paper per minutes per ml.

FPase activity = Absorbance × standard factor (IU/ml/min)

Where

Standard factor = Conc. of glucose standard (mg/ml)

Absorbance at 540 nm

RESULTS

Identification of isolates:

Soil and decaying organic debris have long been implicated for association with different species of fungi and other microbes with varying genetic endowments. Cellulolytic fungi have been reportedly isolated from these sources. Using both morphological and microscospic examinations, the fungal isolates obtained were identified as *Fusarium oxysporium*, *Fusarium* sp, *flavus*, and

Trichoderma harzianum respectively.

The fungal cultures isolated from decaying groundnut shells were screened for their cellulolytic activity. All the fungal cultures produced zones of hydrolysis on 1% carboxymethyl cellulose supplemented modified Czapek-Dox agar plates within 3 days and results were presented in Table1.

Table 1. Screening of the isolates on modified CzapekDox agar supplemented with 1 % carboxylmethyl cellulose to determine their cellulolytic ability after 3 days.

Isolate	Organism	Colony Colour	Colony Diameter (mm)	Zone of Clearance (mm)
B_1	*F. oxysporum*	White with reflecting purple.	37	51
B_2	*Fusarium* sp	White.	34	51
D_2	*A. flavus*	Pale with yellow spores.	22	42
D_3	*Trichoderma harzianum*	Green.	17	44
D4	*R. oryzae*	Whitish brown	70	Undetermined

The cellulolytic activity of all the fungal isolates *F. oxysporum, Fusarium* sp, *A. flavus* and *T. harzianum* was confirmed by Congo Red Dye staining. Positive plates developed yellow zones of clearance around the colony of the fungi indicating the hydrolysis of the carbon source – CMC by the extracellular cellulase enzymes. The diameter of zone of clearing formed measures the cellulolytic endowment of the fungus. The best cellulase producing fungi upon screening were selected.

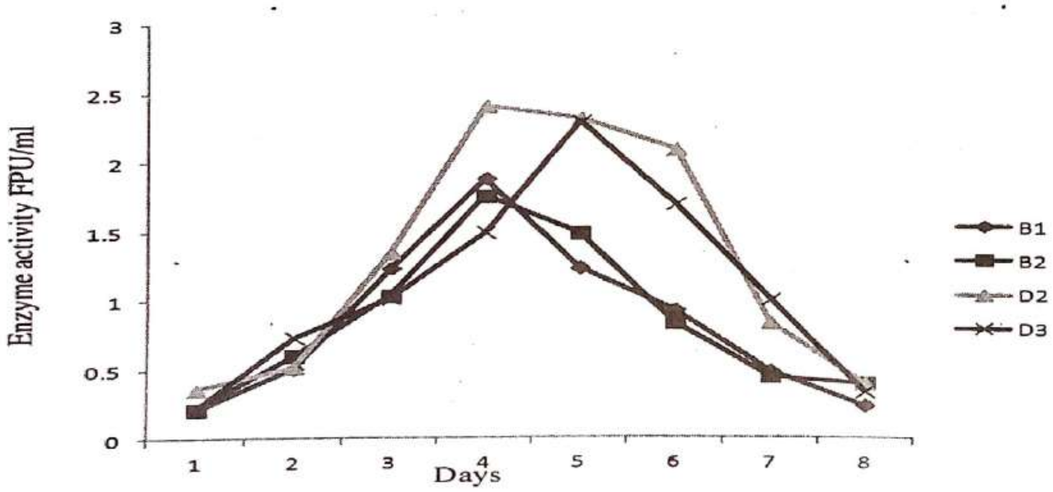

Figure 1:Cellulaseenzyme activity from the mono-cultures of the fungal isolates on groundnut shell substrate.

Keys:

B_1*F. oxysporum*
D_2*A.flavus*

B_2*Fusarium* sp.
D_3 *T. harzianum*

Figure 1, shows the result for cellulase enzyme assay under solid state fermentation.

The enzyme production condition was optimized based on the effect of incubation time on enzyme production. Here, mono or single-culture of the fungal isolates was used.

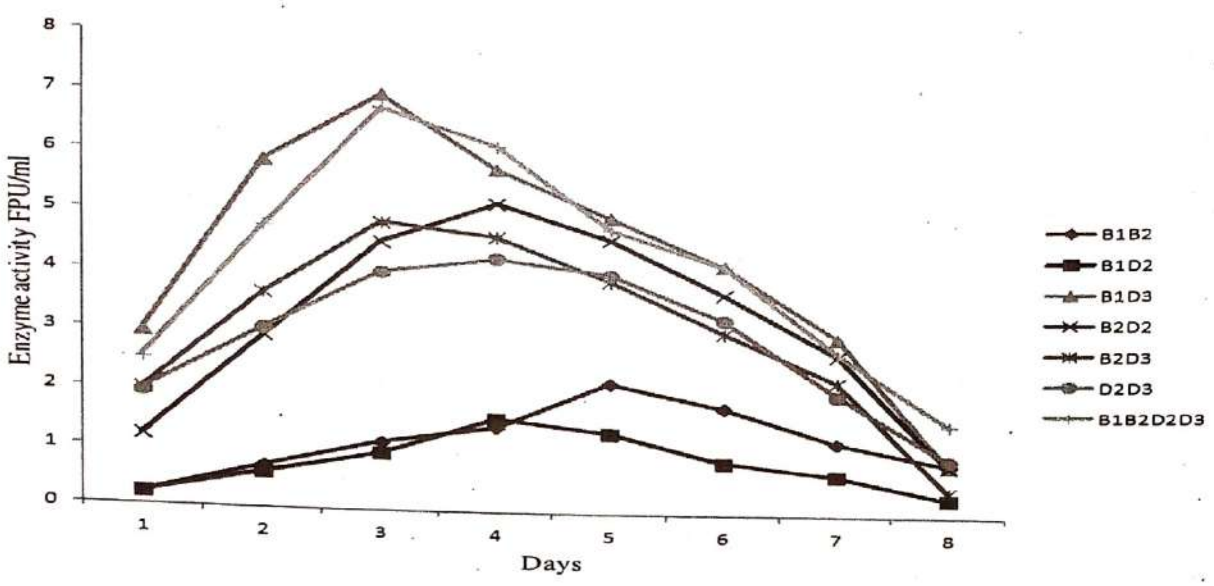

Figure 2: Cellulaseenzyme activity fromthe mixed-cultures of the fungal isolates on groundnut shell substrate.

B_1B_2 = *F. oxysporum* + *Fusarium* sp., B_1D_2 = *F. oxysporum* + *A.flavus*, B_1D_3 = *F. oxysporum* + *T. harzianum*, B_2D_2 = *Fusarium* sp. + *A. flavus*, B2D3 = *Fusarium* sp. + *T. harzianum*, D_2D_3 = *A. flavus* + *T. harzianum*, $B_1B_2D_2D_3$ = All isolates combined.

DISCUSSION

From the result in Table 1, *F. oxysporum* and *Fusarium* sp. performed best in the production of extracellular cellulase enzymes. Both species gave a zone diameter of 51 mm (5.1cm) each while *T. harzianum* and *A. flavus* produced zone clearing diameters of 44 mm and 42 mm respectively on modified Czapek-Dox agar supplemented with 1% CMC. This finding is possible because *F. oxysporum* and some other *Fusarium* sp.have been reported as good producers of cellulase (Christakopoulos *et al.*, 1991). Meanwhile, Gautam*et al.* (2010) reported a zone diameter of 4.7cm (47mm) for *Trichoderma* sp.and 1.7 mm, 1.5 mm for both *A. flavus* and *A. nidulans* respectively screened on PDA amended with 5% CMC.

For the enzyme analysis in this study, (Figure. 1), *A. flavus* yielded maximum cellulase activity after 96hrs (4^{th} day) of fermentation with an activity of 2.424 FPU/ml, a result which contradicted that of Gajula*et al.* (2011) who reported maximum cellulase activity of 0.75 FPU/ml for *A. flavus* after the fourth day of SSF of NaOH treated wheat bran. Meanwhile, *T. harzianum* gave a maximum activity of 2.312 FPU/ml after the 5^{th} day (120hrs) of SSF of groundnut shell biomass. In a similar study for cellulase production by *T. viridae* during the SSF of an alkali treated wheat straw, maximum cellulase activity of 0.740 FPU/ml was obtained after the 5^{th} day of SSF (Mojsov, 2010). Meanwhile, *F. oxysporum* and *Fusarium* sp were optimal for cellulase enzymes after the 4^{th} day of SSF after which a gradual decline in activity was observed. An activity value of 1.896 FPU/ml and 1.769 FPU/ml were obtained for *F. oxysporum* and *Fusarium* sp respectively. A similar study by Ali *et al.* (2012) reported maximum cellulase activity after 4 days of aerobic growth of *F. oxysporum* strains.

The result in Figure 2 shows the activity in cellulase enzymes produced by co- culture of the four fungal isolates used in this study. The fungi were used in combination of two and at a point all the four isolates were combined into a consortium which was applied for the fermentation study too.

The co-culture of microbes in fermentation has been suggested to increase the quantity of desirable components of the cellulase complex, while SSF of the lingo cellulosic biomass is preferred because of some of the advantages over submerged fermentation. Co- culturing has been known to result in greater overall growth and higher specific yields based on both biomass and secreted protein. Other symbiotic associations, such as successive colonization and substrate penetration, co-metabolite production and metabolite inducers have all been reported (Kumar *et al,* 2008).

From Figure 2 above, the co-culture of *F. oxysporum* and *Fusarium* sp yielded 2.312 FPU/ml enzyme activity after 120 hrs (5 days), while the mixed culture of *F.oxysporum* and *T. harzianum* gave cellulase activity yield of 7.206 FPU/ml after 72hr (day 3). The yield in cellulase enzyme activity obtained for *Fusarium* sp and *A. flavus* mixed culture during the SSF of the lingo cellulosic material was 5.402 FPU/ml which was optimum after 96hrs (4th day) of fermentation. While the optimum cellulase activity of 5.012 FPU/ml for *Fusarium* sp and *T.harzanium* co-culture was obtained on the 3rd day of fermentation. This therefore means that the mixed cultures were capable of synthesizing cellulases faster and more in quantity compared to others. All the units of enzyme activity obtained from these mixed cultures of the fungi fell in a higher range compared to the 0.457 FPU/ml unit of enzyme activity obtained from the mixed culture of *A. terreus* and *T. viride* during the fermentation of alkali pre-treated groundnut shell as reported by Vyas and Vyas (2005). Meanwhile, the fungal consortium consisting of all the four fungal isolates, gave an activity of 6.982 FPU/ml after 72hr (3rd day) of fermentation. This could mean better colonization of the substrate by the consortium due to symbiotic association, with each species having its own niche for growth and substrate hydrolysis (Vyas and Vyas, 2005). The possible mechanism behind the enhanced cellulase production in mixed culture is the secretion of cellulase enzyme components acting synergistically on the cellulose yielding more production of the cellulases. One advantage of co-culture may probably be due to all the components of cellulase enzymes being produced.

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