Journal of Natural Sciences Research www.iiste.org
ISSN 2224-3186 (Paper) ISSN 2225-0921 (Online)
Vol.5, No.14, 2015

Qualitative Determination of Lignocellulolytic Enzymes in Eight Wood-Decomposing Fungi

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Abstract
Many fungal species are capable of degrading wood components by the secretion of specialized enzymes including the lignocellulotyic enzymes. A qualitative study was conducted to determine the production of lignocellulotyic enzymes in eight wood-decomposing fungi: *Armillaria mellea, Auricularia auricular, Chaetomium cupreum, Daedalea elegans, Fomes noxious, Ganoderma applanatum, Laetiporus sulphureus* and *Pleurotus pulmonarius.* The dye diffusion method was used in determining the cellulose and hemicellulose modifying enzymes, while brilliant blue agar clearance method of qualitative assay (using brilliant cresyl blue C.I.52010 dye) was used in determining the lignin modifying enzymes production by the fungi. The results showed that *Armillaria mellea, C. cupreum, F. noxious* and *L. sulphureus* tested positive for the production of cellulose and hemicellulose modifying enzymes, while *Auricularia auricular, D. elegans*, *G. applanatum* and *P. pulmonarius* tested positive for lignin modifying enzymes, negative for cellulose and hemicellulose modifying enzymes. *Armillaria mellea* and *Chaetomium cupreum* appeared to possess all the lignocellulose modifying enzymes tested. This study showed that all the 8 wood decomposing fungi have at least one lignocellulolytic enzyme that could be utilized for industrial purposes, especially in chemical, fuel and food industries.
Keywords: Cellulose, Enzymes, Fungi, Lignin, Lignocellulose

INTRODUCTION

Many fungal species are saprophytic and efficient degraders of major polymers such as lignin and cellulose (Sivaramanan, 2014). 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). These enzymatic activities are performed by complex mixtures of cellulases, hemicellulases, and ligninases (Bayer *et al.,* 1998; Ljungdahl, 2008; Sanchez, 2009). The extracellular ligninolytic enzymes of white rot fungi have the ability to degrade a wide range of recalcitrant organo-pollutants such as chlorinated phenols and various types of synthetic dyes (Heinfling *et al.,* 1997; Novotny *et al.,* 2001). Therefore, studying wood decay fungi and their abilities will not only help in the understanding of the economic threats they pose, but also in discovering their biotechnological abilities (Guillén *et al*., 2011).

It has been reported that qualitative assays are powerful tools used in screening fungi for lignocellulolytic enzyme production, especially when there is a large number of fungal isolates and definitive quantitative data are not required (Pointing (1999). Some of these assays are dye diffusion from a cellulose-dye complex and Brilliant Blue agar clearance methods. In this study, the dye diffusion method was used for qualitative determination of cellulose and hemicellulose modifying enzymes, while agar clearance method was used for qualitative determination of lignin modifying enzymes.

MATERIALS AND METHODS

Sample Collection and Identification

The eight fungal species used in this study were obtained from decaying woods in Akungba-Akoko, Ondo State, Nigeria. The collected samples were brought to the laboratory in clean plastic bags marked with number, date and location. The wood decomposing fungi were identified at the Environmental/Agricultural Unit (Mycology Section, Department of Microbiology, Adekunle Ajasin University, Akungba-Akoko, Nigeria. The samples collected were identified as *Daedalea elegans, Fomes noxious, Ganoderma applanatum, Auricularia auricular, Pleurotus pulmonarius, Armillaria mellea, Chaetomium cupreum,* and *Laetiporus sulphureus*. They were left in an open space for 24 hours for the production of spores.

Media Preparation
The medium used for this study was Malt Extract Agar (MEA), prepared according to the manufacturer's instructions.

Test for Cellulose and Hemicellulose Modifying Enzymes by Dye Diffusion
The method of Archibald (1992) was used for the determination of cellulose and hemicellulose modifying enzymes. This method yields clearer data than the filter paper degradation and cellulose agar clearance methods, since results are more visual. According to Pointing, 1999, the degradation of cellulose and hemicellulose by the modifying enzymes result in the release of a bound dye, while the vertical migration can be observed with naked eyes.

Procedure for dye diffusion from cellulose and hemicellulose-dye complex
Malt Extract Agar was prepared according to manufacturer's specifications. Ten millilitre of the culture medium was transferred to glass test tubes (12cm length and 1cm diameter), which were later autoclaved and allowed to solidify. The medium was prepared again and then supplemented with 1% w/v Brilliant Cresyl Blue dye (1g Brilliant Cresyl Blue in 100ml of distilled water). This was autoclaved and cooled until viscous, the mixture was gently and properly mixed and 0.1ml was transferred aseptically onto the surface of the solidified agar as an overlay. One test fungus each was inoculated into the test tubes, and were properly labeled for easy identification. Uninoculated tube served as control. The caps of the test tubes were loosely covered to allow adequate gas exchange, before they were incubated at 25^{o}C in darkness. They were examined daily for 10 days for diffusion of the dye through the clear bottom region.

Test for Lignin Modifying Enzymes:
Brilliant Blue agar clearance
A modified method of Archibald (1992) was used for the determination of lignin modifying enzymes. The brilliant blue dye was used instead of Azure-B dye. Machado *et al.* (2005) found a positive correlation between discolouration of brilliant blue dye and production of lignin modifying enzymes. This agar clearance test gives clear results when qualitative data on peroxidase-type lignin modifying enzymes are required (Pointing, 1999).

Procedure
The culture medium was prepared and supplemented with 0.01% w/v Brilliant Cresyl Blue (0.01g in every 100ml of sterile distilled water), after which it was autoclaved. Aqueous glucose solution containing 20g w/v of glucose was prepared to each 100ml of growth medium prepared, and 1ml of the aqueous glucose solution was aseptically added to the prepared Malt Extract Agar. The already prepared medium was then aseptically transferred to Petri dishes. One test fungus each was inoculated on each Petri dish and uninoculated plate served as control. They were later incubated at 25^{o}C in darkness and examined daily for 10 days.

RESULTS
Determination of Lignocellulolytic enzymes
The use of dyed cellulose yielded less ambiguous data since results were visual. This method also tested for simultaneous action of all cellulose and Hemicellulose enzymes. After 10 days, the extent of migration of the Brilliant Cresyl Blue to the clear lower zones was observed in each inoculated test tubes. The length of penetration in these test tubes was recorded in Figure 1. The deepest penetration was observed in the tube inoculated with *Fomes noxious* and *Laetiporus sulphureus,* both with 5.8cm penetration; while the least penetration was observed in the tube inoculated with *Pleurotus pulmonarius* with 0.2cm penetration. Plates 1 to 8 show the various levels of clearance of agar using the eight fungi compared with the control. The results of the lignocellulolytic abilities of test fungi were summarized in Table 1.

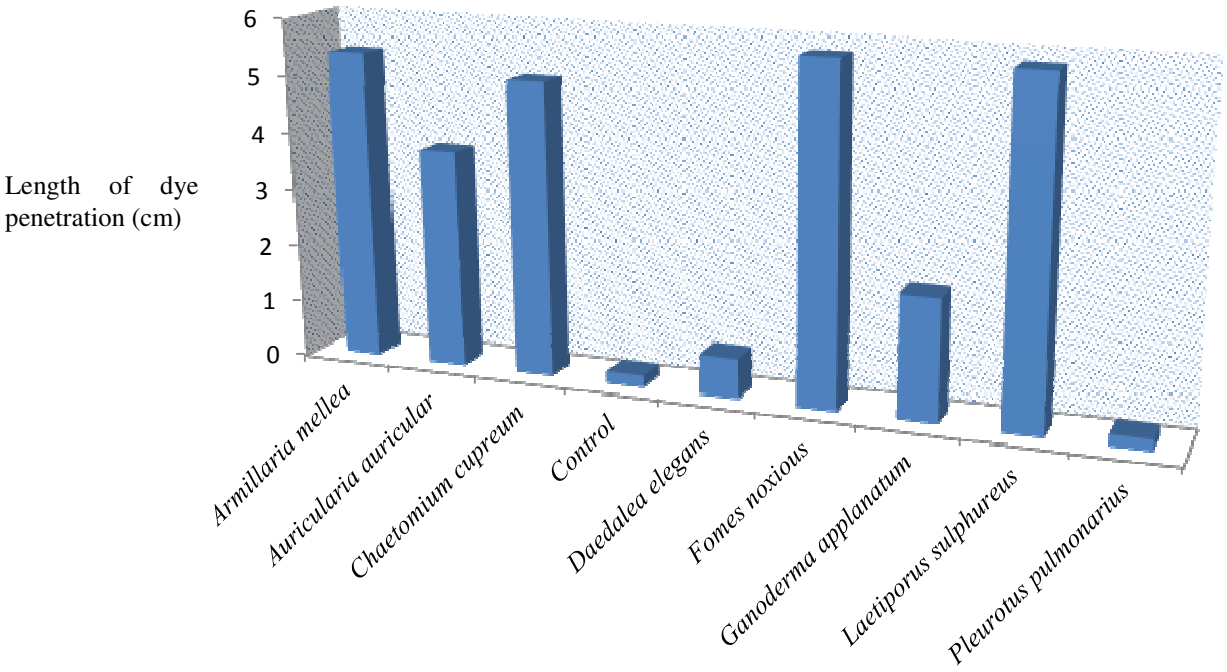

Figure 1. Level of penetration of brilliant cresyl blue dye in test tubes

Plate 1. Level of clearance of agar using *Daedalea elegans* compared with the control

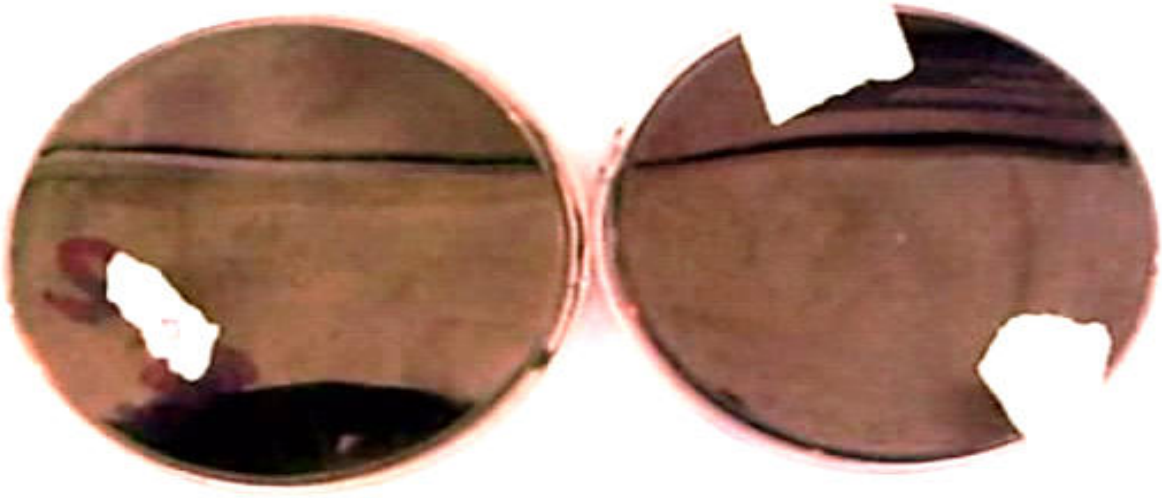

Plate 2. Level of clearance of agar using *Fomes noxious* compared with the control

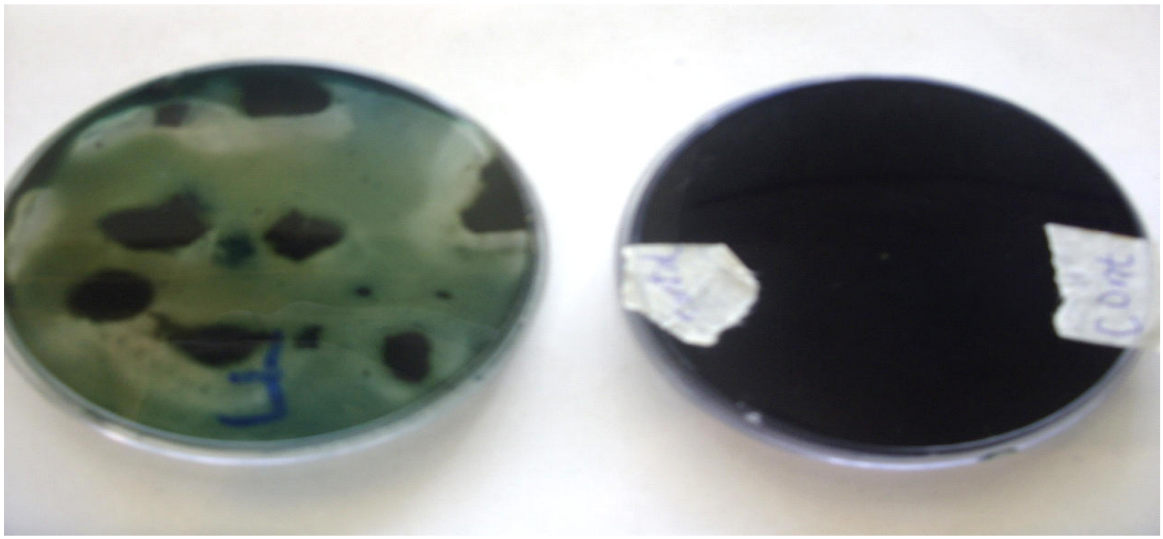

Plate 3. **Level of clearance of agar using *Ganoderma applanatum* compared with the control**

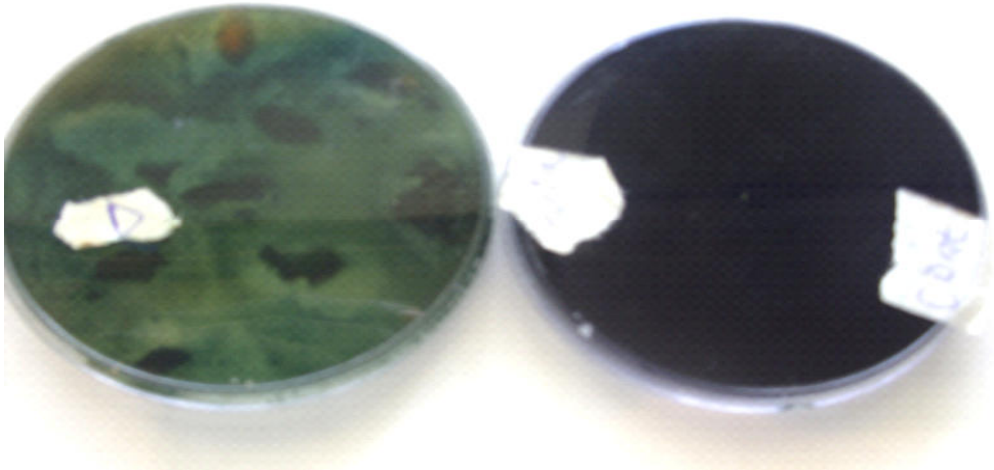

Plate 4. Level of clearance of agar using *Armillaria mellea* compared with the control.

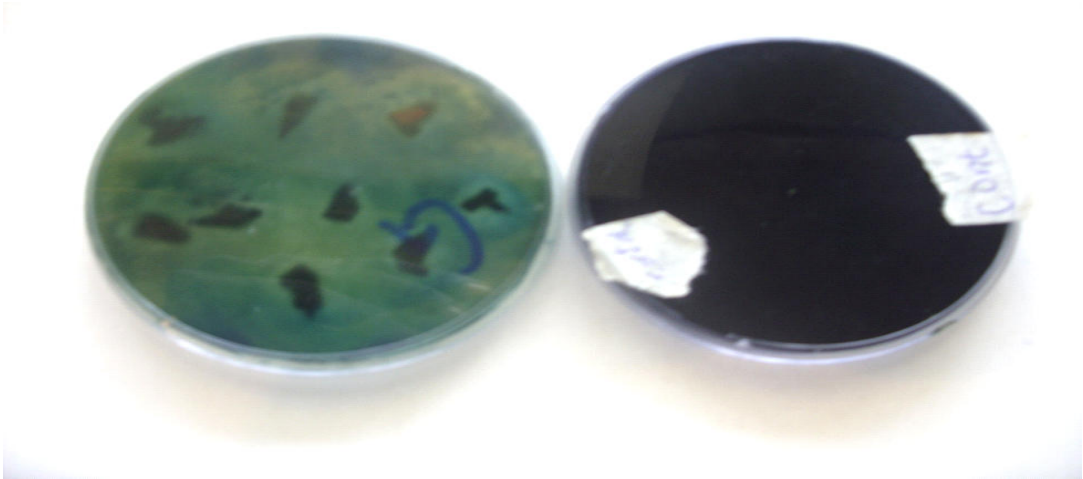

Plate 5. Level of clearance of agar using *Auricularia auricular* compared with the control

Plate 6. **Level of clearance of agar using *Pleurotus pulmonarius* compared with the control**

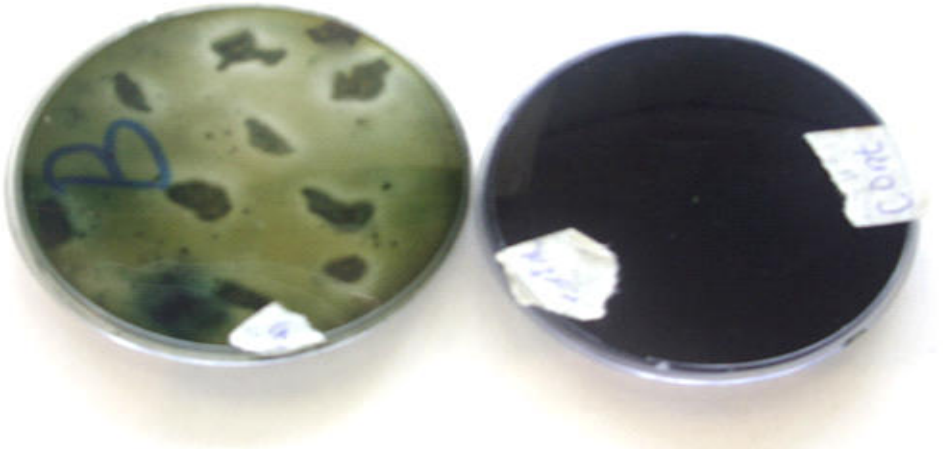

Plate 7. Level of clearance of agar using *Chaetomium cupreum* compared with the control

Plate 8. Level of clearance of agar using *Laetiporus sulphureus* compared with the control

Table 1: Lignocellulolytic ability of some wood decomposing fungi

S/N	Fungus	Cellulose-degrading ability	Hemicellulose-degrading ability	Lignin-degrading ability
1	*Armillaria mellea*	+	+	+
2	*Auricularia auricular*	-	-	+
3	*Chaetomium cupreum*	+	+	+
4	*Daedalea elegans*	-	-	+
5	*Fomes noxious*	+	+	-
6	*Ganoderma applanatum*	-	-	+
7	*Laetiporus sulphureus*	+	+	-
8	*Pleurotus pulmonarius*	-	-	+

Key: Positive +, Negative -

DISCUSSION

The correlation between decolorisation of dyes and ligninolytic abilities of fungi has been established by several authors (Babic and Pavko, 2007**;** Sarnthima *et al*., 2009**;** Singh *et al.,* 2010; Barrasa *et al.,* 2014). Four out of the eight fungal species studied (*Daedalea elegans*, *Ganoderma applanatum, Auricularia auricular* and *Pleurotus pulmonarius*) tested positive for lignin modifying enzymes, but tested negative for wood cellulose and hemicellulose modifying enzymes. This suggests that these fungi are capable of decomposing wood lignin leaving behind wood cellulose and hemicellulose. This selective delignification and simultaneous rot of wood has been reported to be carried out by white rot fungi (Martínez *et al*., 1994). The ligninolytic abilities of fungi in this study is in agreement with the reports of Dill and Kraepelin, 1986; Tychanowicz *et al.,* 2004; Adejoye and Fasidi, 2009; Liers *et al.,* 2010). The ability of these white rot fungi to modify lignin is due to their secretion of extracellular ligninolytic enzymes such as laccase (LAC), lignin peroxidase (LiP) and manganese peroxidase (MnP) (Baldrian, 2004; Schmidt *et al*., 2005), and the recently discovered dye-decolorizing peroxidase (DyP) obtained from *Auricularia auricula-judae* (Liers *et al.,* 2010). In the qualitative determination of cellulose and hemicellulose modifying enzymes, *Fomes noxious, Laetiporus sulphureus, Armillaria mellea,* and *Chaetomium cupreum* tested positive for the production of the enzymes. *Armillaria mellea* and *Chaetomium cupreum* were able to modify all the components of lignocellulose (Table 1).

The production of cellulose and hemicellulose modifying enzymes by *Fomes noxious* corresponds to the findings of Corner (1932) who first described *Phellinus noxius* as *Fomes noxious,* and classified it as a brown rot fungus capable of decomposing dead wood cellulose and hemicellulose. *Laetiporus sulphureus* has shown ability of decolorisation of dyes and production of lignocellulolytic enzymes in previous reports (Ferraz *et al*., 2001; Mtui and Masalu, 2008). Furthermore, Worrell *et al.* (1986) showed that *Armillaria mellea* produces laccase enzyme, which is important in the modification of lignocellulose. This explains why the fungus was able to modify lignocellulose in this study. The positive test showed by *Chaetomium cupreum* on cellulose and hemicellulose modifying enzymes in this study, corroborates with the study of Suphalucksana and Soytong (2006), who reported that sterilized rice straw and para-grass were rapidly degraded when specific strains of *C. cupreum* were inoculated. The Brilliant Cresyl Blue dye used in this study proved to be an efficient dye in the test for lignocellulolytic enzymes production by fungi, hence it could be employed in related studies in the future.

CONCLUSION

The study of lignocellulolytic abilities of fungi is important for biotechnological purposes such as pretreatments of biofuels, production of enzymes and animal feeds, and pulp generation in the paper industry. This study has shown that fungi that are readily isolated from decaying wood have the potential to digest lignocellulose, and if their abilities are properly harnessed they can be used in biopulping, waste management and manufacture of valuable products. However, further investigations aimed at determining the quantity of laccase, lignin peroxidase, manganese peroxidase, cellulose and hemicellulase are necessary to explain the differences in the lignocellulolytic efficacies of the fungal species.

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