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Screening and Identification of Laccase Producing Fungi from Environmental Samples

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Abstract

Laccases are oxidases with broad substrate specificity and ability to oxidize various phenolic and non-phenolic compounds. This study was carried out to isolate and characterizes laccase producing fungi from environment samples. Soil and decaying wood samples were collected from different locations within Ahmadu Bello University, Zaria Main campus. Suspensions of the samples (1 g in 10 mL sterile distilled water) were serially diluted, inoculated onto Potato Dextrose Agar (PDA) containing 0.01% Chloramphenicol and incubated for 7 days at 30°C.The fungal isolates were characterized macroscopically and microscopically with the aid of an atlas. The identified fungal isolates were screened for laccase production by inoculating onto PDA containing 0.02% Guaiacol, 1mM ABTS (2 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) and 0.5% Tannic acid as indicator compounds and incubated at 25^{0}C for 7 days. The laccase producing isolates were confirmed molecularly by ITS rDNA sequence analysis using the FASTA algorithm with the Fungus database from the European Bioinformatics Institute (EBI).A total of 25 fungal species (11 from soil and 14 from decaying wood samples) were isolated. Two isolates from the soil origin identified as *Curvularia lunata* SSI7 (Accession No. QIE06317.1) and *Fusarium* clade VII SSI3 (Accession No. GQ505677) were found to produce laccase where *Curvularia lunata* SSI7 was able to oxidize all the indicator compounds used for the screening. Fusarium clade VII SSI3 was able to oxidize only 0.5% Tannic acid. Laccase producing *Curvularia lunata* and *Fusarium* clade VII were isolated from soil samples collected from ABU Zaria Main Campus.

Keywords: laccase, fungi, soil, decaying wood

INTRODUCTION

Laccases are multi-copper enzymes belonging to blue oxidase group which oxidize diphenol and allied substances using oxygen molecules as electron acceptor (Desai and Nityan and, 2011). Oxidation by laccase leads to the formation of free radicals which may undergo further oxidation or non-enzymatic reactions such as hydration, disproportionation and polymerization (Minari and Agho, 2018).

Laccases played vital roles in the degradation of lignocellulosic materials and other substrates like recalcitrants and phenolic compounds (Kumar and Chandra, 2020). The industrial and biotechnological applications of Laccasses are increasing by the day (Couto and Herrera, 2006; Mtui, 2012).

The importance of laccase lies in its ability to oxidize both phenolic and non-phenolic lignin-related compounds as well as highly recalcitrant environmental residues (Abdulredha, 2013). Unlike peroxidases, laccases do not require H_2O_2 for oxidation reaction as they oxidize wide range of chemical compounds such as aromatic amines, diaminesdiphenols, polyphenols, and benzenethiols (Forootanfar *et al.*, 2012).

Laccases are predominantly produced by fungi, bacteria and plants (Forootanfar *et al.*, 2012; Amutha and Abhijit, 2015). Laccases produced by the fungi are more advantageous over other laccase sources due to stability, broad substrate specificity and ability to oxidize various phenolic compounds (Wakil *et al.*, 2019). This study was carried out to screen and identify laccase producing fungi from soil and decaying wood samples.

MATERIALS AND METHODS

Collection of Environmental and Maize Cob Samples

Soil and decaying wood samples were collected from different locations within Ahmadu Bello University, Zaria Main campus. The samples were collected in sterile plastic bags, sealed and then taken to the Environmental Laboratory, Department of Microbiology, ABU, Zaria for further processing (Vantamuri and Kaliwal, 2015). Maize cobs were collected from Seed Processing Unit of Institute for Agricultural Research, Zaria. The maize cob samples were collected in clean plastic bags, labeled and taken to the Environmental Laboratory, Department of Microbiology, ABU, Zaria where the samples were crushed and grinded. The samples were then stored for further analysis.

Isolation and characterization of fungi

One gram each of the soil and decaying wood samples were separately added to 9 mL of sterile distilled water and mixed. The suspension was serially diluted from 10^{-1} to 10^{-7}, subsequently, 0.1 mL of dilution 10^{-6} and 10^{-7} were spread on the prepared sterile Potato Dextrose Agar (PDA) medium plates containing 0.01% Chloramphenicol and incubated at 30^{o}C for 7 days. Distinct fungal colonies were repeatedly sub-cultured until pure isolates were obtained. The isolates were characterized using slide culture technique. Briefly, an agar block of desired dimensions was cut from PDA plate using sterilized scalpel blade. The agar block was placed on to the surface of a sterile microscope slide in a Petri dish containing moistened cotton wools. The four quadrants of the agar block were then inoculated with the fungal isolate and a sterile coverslip was placed onto the surface. The lid of the Petri dish was replaced and the plate was incubated at 30˚C for 5 days. After the incubation period, the cover slip was remove, placed on a microscope slide containing a drop of lactophenol cotton and observes microscopically for the characteristic shape and arrangement of spores. The identification was done both macroscopically and microscopically with reference to Larone Atlas of Mycology considering the colour of the mycelia and shape of the conidiophore, vesicle and conidia. The cultures were maintained on PDA slants at 25˚C for preservation (Daphne and Joel, 2013).

Qualitative screening

Screening for laccase production was carried out by the inoculation of mycelium from each strain onto PDA plates containing 0.02% Guaiacol, 1mM ABTS (2 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) and 0.5% Tannic acid as indicator compounds and the plates were incubated at 25^{0}C for 7 days. The formation of reddish-brown halo on media supplemented with Guaiacol and Tannic acid and/or dark-purple halo on media supplemented with ABTS indicates positive laccase secretion (Vantamuri and Kaliwal, 2015).

Molecular identification of Laccase producing fungi

Molecular confirmation of the laccase producing fungi was carried out at the Center for Agriculture and Bioscience International (CABI) UK, using ITS rDNA sequence analysis by FASTA algorithm with the database from EBI.

The procedures were in accordance with CABI's in-house methods (TPs 72-82 for filamentous fungi and yeasts). Briefly, molecular assays were carried out on each sample using nucleic acid as a template. A proprietary formulation [microLYSIS®-PLUS (MLP), Microzone, UK] was subjected to the rapid heating and cooling of a thermal cycler, to lyse cells and release deoxyribonucleic acid (DNA).

Following DNA extraction, Polymerase Chain Reaction (PCR) was employed to amplify copies of the Rdna *in vitro*. The quality of the PCR product was assessed by gel electrophoresis and PCR purification step was carried out to remove residual dNTPs, primers, polymerase and other PCR mixture compounds so as to obtain a highly purified DNA template for sequencing. This procedure also allows concentration of low yield amplicons.

Sequencing reactions were undertaken using BigDye® Terminator v3.1 kit from Applied Biosystems (Life Technologies, UK) which utilizes fluorescent labelling of the chain terminator ddNTPs, to permit sequencing. Removal of excess unincorporated dye terminators was carried out to ensure a problem-free electrophoresis of fluorescent labelled sequencing reaction products on the capillary array AB 3130 Genetic Analyzer (DS1) DyeEx™ 2.0 (Qiagen, UK).

Modules containing pre-hydrated gel-filtration resin were optimized for clean-up of sequencing reactions containing BigDye® terminators. Dye removal was followed by suspension of the purified products in highly deionized formamide Hi-Di™ (Life Technologies, UK) to prevent rapid sample evaporation and secondary structure formation.

Samples were loaded onto the AB 3130 Genetic Analyzer and sequencing undertaken to determine the order of the nucleotide bases, adenine, guanine, cytosine, and thymine in the DNA oligonucleotide.
Following sequencing, identifications of the fungi were done by comparing the sequence obtained with those available from the European Molecular Biology Laboratory (EMBL) database via the European Bioinformatics Institute (EBI).

RESULTS

Eleven fungi species belonging six genera were isolated and characterized from the soil samples collected (Table 1). Fourteen fungi species belonging to five genera were isolated and characterized from decaying wood samples (Table 2). The macroscopic characteristics were the morphology of the mycelia and the colour of the reverse while the microscopic features were the morphology of the conidiophore, vesicle and conidia.
Table 3 shows the percentage occurrence of the fungi species isolated from soil and decaying wood samples. Eleven species of fungi were isolated from the soil samples, the isolates belong to six fungi genera. The six genera of fungi isolated were *Alternaria, Aspergillus, Curvularia, Fusarium, Penicillium* and *Trichoderma*. Out of the 11 fungi species isolated from the soil *Aspergillus niger* (27.27%) had the highest occurrence. Fourteen species of fungi were isolated from the decaying wood samples; the isolates belong to four fungi genera. The four genera of fungi isolated were *Aspergillus, Mucor, Penicillium* and *Trichoderma*. Out of the 14 fungi species isolated from the soil *Aspergillus flavus* (22.43%) had the highest occurrence.

Table 1: Macroscopic and Microscopic characteristics of fungi species isolated from soil samples

Isolate code	Macroscopic characteristic	Microscopic characteristic	Inference
SSI1	Black mycelium; uncoloured reverse	Brownish conidiophore, globose vesicle, globose conidia.	*Aspergillus niger*
SSI2	Orange floccose with exudates, yellow reverse	Long conidiophore, ampuliformphialides, subglose conidia	*Penicillium citrinum*
SSI3	Pink red colony Yellowish green colony, pale yellow reverse	Long branched conidiophore, cylindrical conidia	*Fusarium* sp.
SSI4	Black mycelium; uncoloured reverse	Branched conidiophore, cylindrical phialide, ellipsoidal conidia	*Trichoderma reesei*
SSI5	Black floccose mycelium	Brownish conidiophore, globose vesicle, globose conidia.	*Aspergillus niger*
SSI6	Brownish black colony, dark brown reverse	Elongated conidiophore, ovoid conidia	*Alternaria* sp.
SSI7	Black mycelium; pale yellow reverse	Brown branch and bent conidiophores, pyriform conidia	*Curvularia* sp.
SSI8	Dark green, dull yellow reverse	Brownish conidiophore, globose vesicle, globose conidia.	*Aspergillus niger*
SSI9	Dark green, amber reverse	Branching conidiophores, subglobose to obovoid conidia	*Trichoderma hariazum*
SSI10	Dark blue-green colonies	Long branching conidiophores, globose conidia	*Trichoderma viride*
SSI11		Long greenish conidiophore, dome shaped vesicle, globose conidia	*Aspergillus fumigatus*

Table 2: Macroscopic and Microscopic characteristics of fungi species isolated from decaying wood samples

Isolate code	Macroscopic characteristic	Microscopic characteristic	Inference
DWI1	Dark green, dull yellow reverse	Branching conidiophores, subglobose to obovoid conidia	*Trichoderma hariazum*
DWI2	Black mycelium; pale yellow reverse	Brownish conidiophore, globose vesicle, globose conidia.	*Aspergillus niger*
DWI3	Yellowish green colonies	Roughened conidiophore, subglobose vesicle, ellipsoidal conidia.	*Aspergillus flavus*
DWI4	White fluffy colony, white reverse	Non-septate sporangiophores	*Mucor* sp.
DWI5	Dark green colony, purple reverse	Long conidiophore, hemispherical vesicle, globose conidia	*Aspergillus nidulans*
DWI6	Yellowish green colonies	Roughened conidiophore, subglobose vesicle ellipsoidal conidia.	*Aspergillus flavus*
DWI7	Gray-green with yellow patches	Pale brown conidiophore, elliptical vesicle, globose conidia	*Aspergillus versicolor*
DWI8	Dark green, dull yellow reverse	Branching conidiophores, subglobose to obovoid conidia	*Trichoderma hariazum*
DWI9	Black mycelium; uncoloured reverse	Brownish conidiophore, globose vesicle, globose conidia.	*Aspergillus niger*
DWI10	Orange floccose with exudates, yellow reverse	Long conidiophore, ampuliformphialides, subglose conidia	*Penicillium citrinum*
DWI11	Dark green colony, purple reverse	Long conidiophore, hemispherical vesicle, globose conidia	*Aspergillus nidulans*
DWI12	Yellowish green colonies	Roughened conidiophore, subglobose vesicle ellipsoidal conidia.	*Aspergillus flavus*
DWI13	Gray-green with yellow patches	Pale brown conidiophore, elliptical vesicle, globose conidia.	*Aspergillus versicolor*
DWI14	Orange floccose with exudates, yellow reverse	Long conidiophore, ampuliformphialides, subglose conidia	*Penicillium citrinum*

Table 3: Percentage occurrence of fungal species isolated from soil and decaying wood samples

Fungi species	No. (%) isolated from soil (n = 11)	No. (%) isolated from decaying woods (n = 14)	Total (%) (n = 25)
Alternaria sp.	1 (9.09)	0 (0.00)	1 (4.00)
Aspergillus flavus	0 (0.00)	3 (21.43)	3 (12.00)
Aspergillus fumigatus	1 (9.09)	0 (0.00)	1 (4.00)
Aspergillus nidulans	0 (0.00)	2 (14.29)	2 (8.00)
Aspergillus niger	3 (27.27)	2 (14.29)	1(20.00)
Aspergillus versicolor	0 (0.00)	2 (14.29)	2 (8.00)
Curvularia sp.	1 (9.09)	0 (0.00)	1 (4.00)
Fusarium sp.	1 (9.09)	0 (0.00)	1 (4.00)
Mucor sp.	0 (0.00)	1 (7.14)	1 (4.00)
Penicillium citrinum	1 (9.09)	2 (14.29)	3 (12.00)
Trichoderma hariazum	1 (9.09)	2 (14.29)	3 (12.00)
Trichoderma reesei	1 (9.09)	0 (0.00)	1 (4.00)
Trichoderma viride	1 (9.09)	0 (0.00)	1 (4.00)
Total (%)	11 (100.00)	14 100.00)	25 (100.00)

Out of the 25 fungal isolates screened (11 isolates from soil and 14 isolates from decaying wood), only two fungal isolates from soil origin namely *Curvularia* sp. (SSI7) and *Fusarium* sp. (SSI3) were found to be laccase producing (Table 4). However, none of the isolates obtained from the decaying wood samples showed evidence of laccase production. *Curvularia lunata* with isolate code SSI7 was found to be the isolate with best potential for high laccase production as it was able to oxidize all the indicator compounds (0.02% Guaiacol, 1mM ABTS and 0.5% Tannic acid). *Fusarium* sp. with isolate code SSI3 was able to oxidize only 0.5% Tannic acid based on the images showed in Plate I.

Table 4: Response of the fungi species to different indicators of laccase production

Isolate code	Isolate	0.5%Tannic acid	Indicators 0.02%Guaiacol	1mMABTS
SSI1	*Aspergillus niger*	-	-	-
SSI2	*Penicillium citrinum*	-	-	-
SSI3	*Fusarium* sp.	+	-	-
SSI4	*Trichoderma reesei*	-	-	-
SSI5	*Aspergillus niger*	-	-	-
SSI6	*Alternaria* sp.	-	-	-
SSI7	*Curvularia* sp.	+	+	+
SSI8	*Aspergillus niger*	-	-	-
SSI9	*Trichoderma hariazum*	-	-	-
SSI10	*Trichoderma viride*	-	-	-
SSI11	*Aspergillus fumigatus*	-	-	-
DWI1	*Trichoderma hariazum*	-	-	-
DWI2	*Aspergillus niger*	-	-	-
DWI3	*Aspergillus flavus*	-	-	-
DWI4	*Mucor* sp.	-	-	-
DWI5	*Aspergillus nidulans*	-	-	-
DWI6	*Aspergillus flavus*	-	-	-
DWI7	*Aspergillus versicolor*	-	-	-
DWI8	*Trichoderma hariazum*	-	-	-
DWI9	*Aspergillus niger*	-	-	-
DWI10	*Penicillium citrinum*	-	-	-
DWI11	*Aspergillus nidulans*	-	-	-
DWI12	*Aspergillus flavus*	-	-	-
DWI13	*Aspergillus versicolor*	-	-	-
DWI14	*Penicillium citrinum*	-	-	-

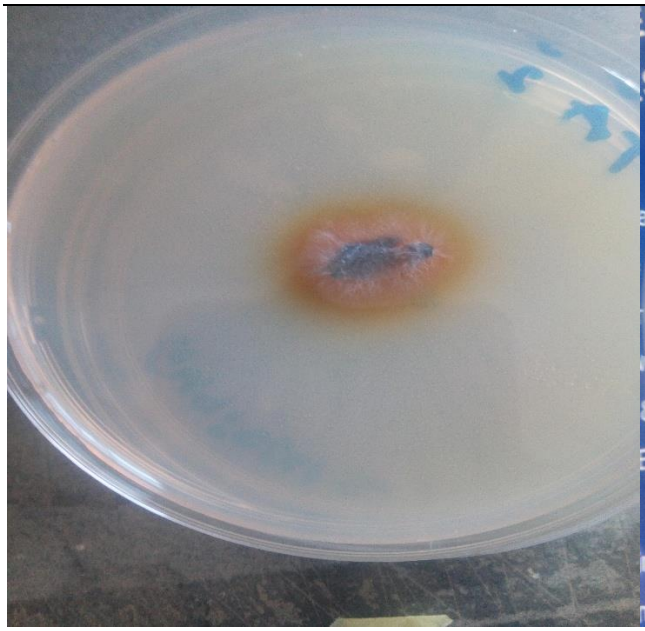

Plate Ia: Colonial morphology of *Fusarium* sp. with reddish brown halo PDA supplemented with 0.02% Tannic acid

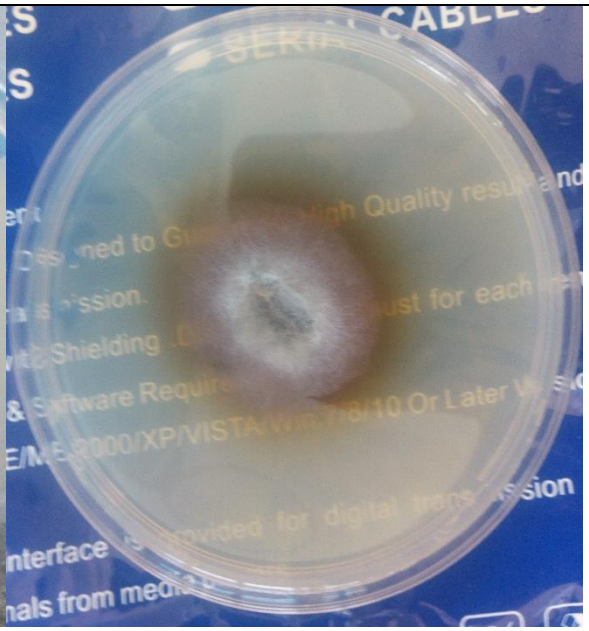

Plate Ib: Colonial morphology of *Curvularia* sp. with reddish brown halo PDA supplemented with 0.02% Tannic acid

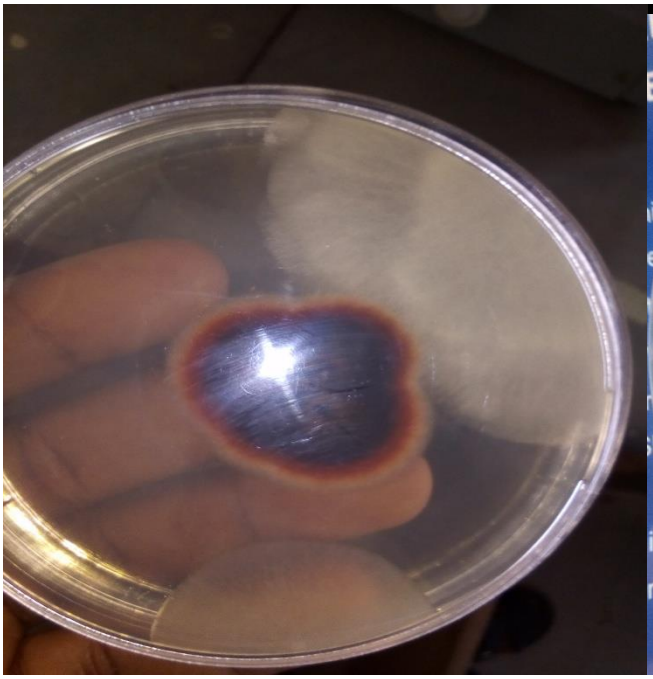

Plate Ic: Colonial morphology of *Curvularia* sp. with reddish brown halo on PDA supplemented with 0.5% Guaiacol

Plate Id: Colonial morphology of *Curvularia* sp. with dark-purple halo on PDA supplemented with 1mM ABTS

The two laccase producing fungi isolated were confirmed to be *Curvularia lunata* and *Fusarium* clade VII following the ITS rDNA sequence analysis using the FASTA algorithm with the Fungus database from EBI (Table 5).

Table 5: Confirmed identity and accession number of the laccase producing fungal isolates

Isolate code	Macroscopic & Microscopic identity	Molecular identity	Accession number	Comment
SSI7	*Curvularia* sp.	*Curvularia lunata*	QIE06317.1	100% similar to *Curvularia* sp. FIESC strain CBS 131009 [which belongs to the *Curvularia lunata* species complex
SSI3	*Fusarium* sp.	*Fusarium* clade VII	GQ505677	100% similar to *Fusarium* sp. strain NRRL 5537 [which belongs to the *Fusariumin carnatum-equiseti* species complex

DISCUSSION

In this study a total of 25 fungi species belonging to seven genera were identified as *Alternaria, Aspergillus, Curvularia, Fusarium, Mucor, Penicillium and Trichoderma*. The presence of these fungi in the samples is likely because fungi are very successful inhabitants of soil and decaying wood due to their high plasticity and their capacity to adopt various forms in response to adverse or unfavourable conditions (Naranjo-Ortiz and Gabaldon, 2019). Fungi have ability to produce a wide variety of extracellular enzymes as they are able to break down all kinds of organic matter thereby regulating the balance of carbon and nutrients (Frac *et al.*, 2018).

Out of the 25 fungal isolates screened for laccase production, two isolates namely *Curvularia* sp. and *Fusarium* sp. were found to be laccase producers. The fact that the laccase producing isolates were of soil origin implies that soil is a better source of laccase producers than decaying wood. These two fungal isolates were further identified molecularly by ITS rDNA sequence analysis using the FASTA algorithm with the Fungus database from EBI to be *Curvularia lunata* and *Fusarium* clade VII.

Curvularia lunata was able to oxidize all the indicators compounds used as against *Fusarium* clade VII that was only able to oxidize only one of the indicators (tannic acid). This difference could be due to difference in the type and nature of laccase produced by the fungal isolates. This is similar to the result of Vázqueza *et al*. (2019) where laccase activity was only exhibited by *Curvularia kusanoi L7* strain out of all the fungi strains screened. So also *Curvularia pallenscens* was identified by Sidhu *et al*. (2017) as one of the two fungal isolates that were positive for laccase production qualitatively.

CONCLUSIONS

In conclusion, 25 fungal species belonging to 7 genera namely *Alternaria, Aspergillus, Curvularia, Fusarium, Mucor, Penicillium and Trichoderma* were isolated in this study. Two fungal isolates namely *Curvularia* sp. and *Fusarium* sp. were found to be laccase producers with *Curvularia lunata* having the better laccase production potential. These two isolates were further identified molecularly to be *Curvularia lunata* and *Fusarium* clade VII respectively.

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