



PRODUCTION OF LACCASE BY FUNGI ISOLATED FROM SOIL VIA SUBMERGED FERMENTATION USING CORN COB AS SUBSTRATE

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ABSTRACT

One of the limitations of large scale application of laccase (EC 1.10.3.2) is the inability to produce them in large quantity at an affordable cost. This study was carried out to screen indigenous fungi for their ability to produce laccase using the locally available substrate. Five soil samples were collected and diluted serially, 0.1 mL of the 10⁻⁵ and 10⁻⁶ dilutions were inoculated onto Potato dextrose agar (PDA) plates. The fungal isolates were identified based on their macroscopic and microscopic characteristics. The isolates were then screened for laccase production by growing them on PDA containing tannic acid as an indicator compound. The laccase producing isolates were further screened for their ability to utilize corn cob as a substrate for laccase production. Ten fungal species were isolated and identified as *Trichoderma viridae* (3), *Trichoderma harzianum* (3), *Aspergillus niger* (2), *Fusarium* sp. (1) and *Penicillium* sp. (1). Only two of the isolates namely *T. viridae* and *T. harzianum* were found to be laccase producers. Both laccase producing fungal species were able to utilize corn cob as substrate for laccase production. *T. viridae* had higher enzyme activity (2.228 U/mL) than *T. harzianum* (2.1583 U/mL) after 9 days of incubation. Laccase producing fungi were isolated in this study and they were able to use corn cob as substrate for laccase production.

Keywords: Laccase, fungi, corn cob, enzyme, activity.

INTRODUCTION

Lignocellulosic materials are the major component in plants and consist of cellulose, hemicellulose and lignin. Lignin is an important barrier that protects hemicellulose and cellulose from enzymatic attack (Wang *et al.*, 2019). Fungi are capable of breaking these barriers by the production of a different extra lignocellulosic enzyme such as laccases, lignin peroxidase and manganese (Emre and Ozfer, 2013).

Industries such as food agriculture, forestry, paper pulp and timber release large lignocellulosic waste. These waste cause serious environment pollution but rather than being burned they can be reused due to their rich sugar contents. The chemical composition of this lignocellulosic material/waste make them a crucial and cost-effective fermentation medium for biotechnological applications (Sahadevan *et al.*, 2013). Numerous microorganism especially fungi make use of cellulose and hemicellulose components of lignocellulosic waste while only limited number of the organism such as white rot fungi are capable of degrading lignin due to its high resistance to microbial attack (Kantharaj *et al.*, 2017).

In order to exploit the use of lignocellulosic wastes, several methods are employed for the separation of these materials, these processes are the physical, chemical and biological process. The physical process involves the use of microwave pretreatment, the chemical process involves the use of acid and alkali while biological process usually involves the use of an enzyme (biocatalyst) which improve the superiority of pretreatment processes (Brustarr M, Bakenhus, 2008). The use of biocatalyst is more preferred simply because they overcome the drawback of decrease in the quality of polymer and release of by-products that inhibit the fermentation of resulting sugars (Kantharaj *et al.*, 2017).

Laccase (EC 1.10.3.2) is one of the oldest known enzymes

and was first discovered in 1883 in the sap of Japanese lacquer tree *Rhus vernicifera*, leading to its polymerization and the product used commonly as Japanese lacquer varnish (Mendoza, 2011). They are copper-containing polyphenol oxidases which are capable of degrading both phenol and non-phenol substrate. They degrade lignin by oxidation of phenolic units of the lignin to phenolic radicals which cleave aryl-C while non-phenolic substrates are oxidized in the presence of an auxiliary substrate (Brijwani and Vadlani, 2011, Kantharaj *et al.*, 2017). Laccase is one of the three main ligninases but has peculiar characteristics of catalyzing the oxidation of lignin component using molecular oxygen as electron acceptor which reduced to water. Laccase uses electron mediator to degrade complex structures (Call and Muncke, 1997).

In industries like paper and pulp industry, certain chemical like chlorine and oxygen-based chemical oxidant are required for separation of lignin from lignocellulosic waste. Preparation of paper at an industrial level face certain problems such as cost recycling and toxicity which remain unresolved (Solomon, 1996). Laccase has great potential application in several areas of food and other industries, however, one of the limitation of large scale application of laccase is the lack of capacity to produce enzyme in large volume at an affordable cost (Shraddha *et al.*, 2011). The use of agricultural waste such as corn cob, yam peels, rice straw, rice husk and agro-waste as a substrate for fermentation can increase the yield of enzyme production at low cost and will reduce the use of chemicals for degradation of lignin-containing wastes, thereby reducing the risk of hazardous chemicals being released into the environment (Sadh *et al.*, 2018). Thus, the aim of this study was to isolate and characterize laccase producing fungi from the soil and screen for their ability to utilize corn cob as a substrate for the production of laccase enzymes that can be used for the

chemical degradation of lignin-containing waste.

MATERIALS AND METHODS

Collection of Samples

The soil samples used in this study were collected from 5 different locations of Botanical Garden, Department of Biological Sciences, Ahmadu Bello University, Zaria. The samples were collected in sterile polythene bags and brought to the laboratory for further study. Corn cobs were purchased from Samaru market, Zaria. The corn cobs were crushed and ground. The sample was then stored at room temperature in polythene bags.

Isolation and Identification of Fungi

One gram of the soil was weighed and dispensed into 10 mL of sterile distilled water. The suspension was mixed and serially diluted from 10^{-1} to 10^{-6} dilution. Then 0.1 mL of 10^{-5} to 10^{-6} dilutions were spread on the surface Potato Dextrose Agar (PDA) separately and incubated at 25 °C for 7 days. Distinct fungal colonies were sub-cultured until the pure culture was obtained. The cultures were maintained on PDA slant at room temperature (Adivappa and Basappa, 2015). The Fungi isolates were identified based on their macroscopic and microscopic characteristic using a colour Atlas of Mycology by Walsh *et al.* (2018).

Qualitative screening for laccase production

Mycelium from each fungal strain was inoculated on PDA plate containing 0.5 % tannic acid as an indicator compound for laccase production. The plates were incubated at 25 °C and observe daily for 12 days. Formation of reddish-brown halo around the fungal isolate on the plate indicated laccase production (Adivappa and Basappa, 2015).

Laccase production by submerged fermentation using corn cob as a substrate

Inoculum preparation

The slants of the laccase producing fungal isolates were used

The laccase activity in U/ml was calculated using the formula below (1):

$$E.A = A * V / t * e * v \dots\dots\dots 1.$$

Where E.A = Enzyme activity, A = Absorbance, V = Total mixture volume (mL), v = enzyme volume (mL), t = incubation time, e = extinction coefficient for guaiacol (0.6740 $\mu\text{M}/\text{cm}$).

Statistical analysis

ANOVA was performed to analyze the statistical significance of the differences observed in the laccase activities produced by *T. harzianum* and *T. viridae* using IBM SPSS Statistics. $P \leq 0.05$ was considered statistically significant.

RESULTS

Characterization of fungal isolate

Table 1 shows the macroscopic and microscopic appearances of the fungal isolates. Isolates DBS-1, DBS-6 and DBS-10 were identified as *T. harzianum* while DBS-2, DBS-5 and DBS-7 were identified as *T. viridae*. Two isolates (DBS-4 and DBS-8) were *A. niger* while DBS-3 and DBS-9 were identified as *Penicillium* sp. and *Fusarium* sp. respectively. Table 2 shows the distribution of the fungal species isolated from a soil sample. The percentage occurrences of *T. harzianum* and *T. viridae* were 30 % each while that of *A.*

to prepare spore suspension by the addition of 2 mL of sterile distilled water to 7 days old slant culture of the fungal isolates. With aid of a sterilized wire loop the spores were dislodged and counted with a haemocytometer to obtain a concentration of 1.0×10^6 spore/mL (Buddolla *et al.*, 2008).

Medium preparation and fermentation

Determination of the ability of the laccase producing fungal isolates to use corn cob as a substrate for laccase production was carried out by growing the isolates using 250 mL Erlenmeyer flask containing 100 mL of mineral salt medium with the following composition (g/L): KCl, 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; $(\text{NH}_4)_2\text{HPO}_4$, 0.5; NaH_2PO_4 , 0.5; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002 and 10 g corn cob as was designed by Suprabha *et al.* (2008). The pH was adjusted to 5.0 and autoclaved at 121 °C for 15 mins.

The 2 mL spore suspension was transferred into the sterilized mineral salt medium and incubated at 25 °C in a rotatory shaker at 150 rpm for 9 days (Sanjeeviravar *et al.*, 2015). 2 mL of the fermentation medium were taken from each conical flask at an interval of 3 days. The laccase activity was determined using guaiacol assay method.

Determination of laccase activity using Guaiacol assay method

This assay is based on the oxidation of guaiacol by laccase enzyme which results in the development of reddish-brown colour which is used to measure laccase activity using spectrophotometer at 450 nm. The reaction mixture was prepared as follows: (a) 1 mL of 2 mM Guaiacol. (b) 3 mL of 10 mM Sodium acetate buffer. (c) 1 mL of fungal supernatant which serves as enzyme source. A blank which contains 1 mL of distilled water instead of the enzyme was also prepared. The mixture was incubated at 30 °C for 15 mins and the absorbance was read at 450 nm using UV spectrophotometer (Abd El Monssaf *et al.*, 2016).

niger was 20 %. The least occurring species were *Penicillium* sp. (10 %) and *Fusarium* sp. (10 %).

Qualitative screening for laccase production ability of the isolates

Table 3 shows the result of the screening for laccase producing ability of the fungal isolates. Isolate DSB-1 (*T. harzianum*) and DSB-2 (*T. viridae*) were positive for laccase production while the remaining isolates were negative for laccase production.

Laccase production by submerged fermentation using corn cob as a substrate

Figure 1 shows the laccase activities produced by the two positive isolates at 3 days interval for 9 days. At day 3, 6 and 9 days of incubation, *T. viridae* had higher laccase activity (1.1821, 1.3860 and 2.2280 U/mL respectively) compared to *T. harzianum* (0.7209, 1.0353 and 2.1583 U/mL). However, this difference was not statistically significant ($p = 0.6159$).

Table1: Macroscopic and microscopic characteristic of the fungal isolates

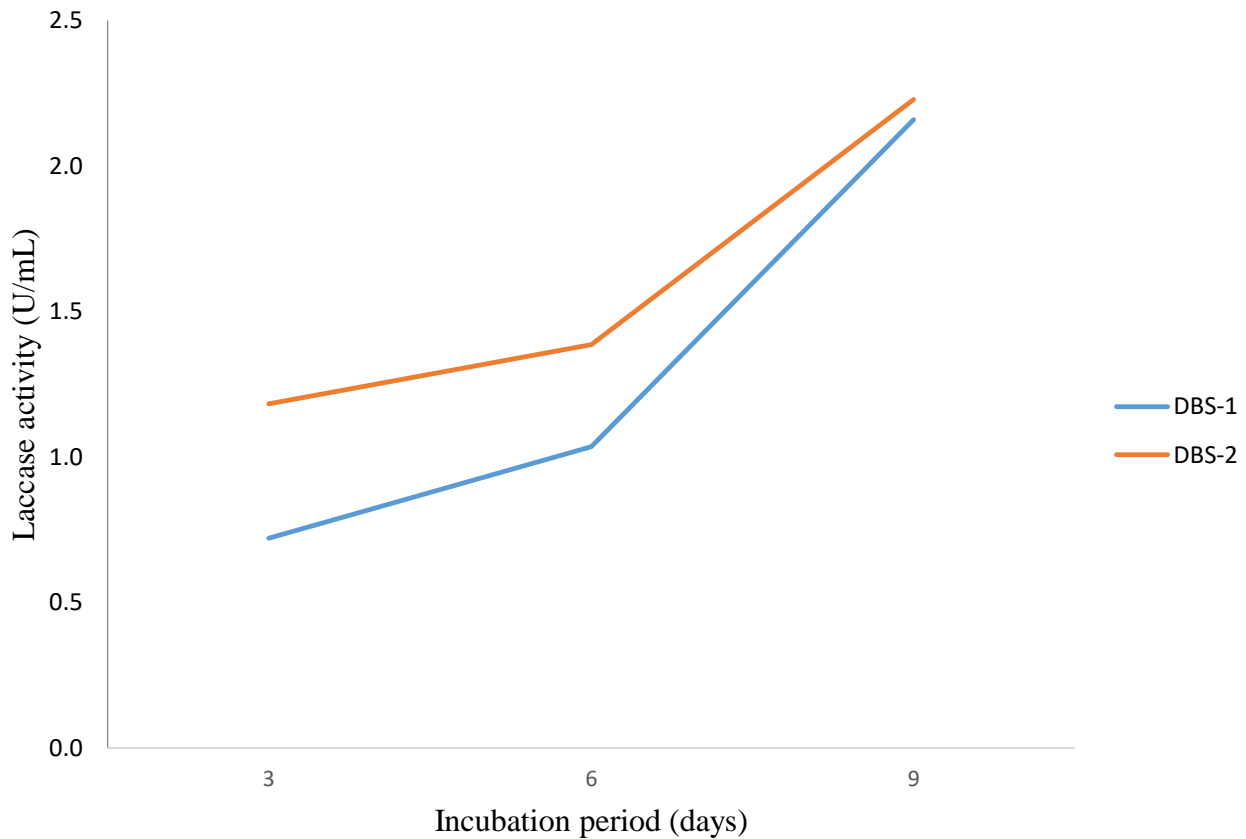
Isolate code	Macroscopic characteristic	Microscopic characteristics	Inference
DBS-1	Dark green, dull yellow reverse	Branching conidiophores, subglobose to obovoid conidia	<i>Trichoderma hariazum</i>
DBS-2	Dark green, amber reverse	Long branching conidiophores, globose conidia	<i>Trichoderma viride</i>
DBS-3	Orange floccose with exudates, yellow reverse	Long conidiophore, ampuliform phialides, subglose conidia	<i>Penicillium</i> sp.
DBS-4	Black mycelium; pale yellow reverse	Brownish conidiophore, globose vesicle, globose conidia	<i>Aspergillus niger</i>
DBS-5	Dark green, amber reverse	Long branching conidiophores, globose conidia	<i>Trichoderma viride</i>
DSB-6	Dark green, dull yellow reverse	Branching conidiophores, subglobose to obovoid conidia	<i>Trichoderma hariazum</i>
DSB-7	Dark green, amber reverse	Long branching conidiophores, globose conidia	<i>Trichoderma viride</i>
DSB-8	Black mycelium; pale yellow reverse	Brownish conidiophore, globose vesicle, globose conidia	<i>Aspergillus niger</i>
DSB-9	Pinkish red colony	Long branched conidiophore, cylindrical conidia	<i>Fusarium</i> sp.
DSB-10	Dark green, dull yellow reverse	Branching conidiophores, subglobose to obovoid conidia	<i>Trichoderma hariazum</i>

Table 2: Distribution of fungal species isolated from soil samples

Fungal species	Frequency	Occurrence (%)
<i>Trichoderma hariazum</i>	3	30
<i>Trichoderma viride</i>	3	30
<i>Penicillium</i> sp.	1	10
<i>Aspergillus niger</i>	2	20
<i>Fusarium</i> sp.	1	10
Total	10	100

Table 3: Qualitative screening for laccase production using tannic acid as indicator

Isolate code	Isolate identity	Laccase production
DBS-1	<i>Trichoderma hariazum</i>	Positive
DBS-2	<i>Trichoderma viride</i>	Positive
DBS-3	<i>Penicillium</i> sp.	Negative
DBS-4	<i>Aspergillus niger</i>	Negative
DBS-5	<i>Trichoderma viride</i>	Negative
DSB-6	<i>Trichoderma hariazum</i>	Negative
DSB-7	<i>Trichoderma viride</i>	Negative
DSB-8	<i>Aspergillus niger</i>	Negative
DSB-9	<i>Fusarium</i> sp.	Negative
DSB-10	<i>Trichoderma hariazum</i>	Negative



F-value = 0.2949 p = 0.6159*

Figure 1: Laccase activity produced by *T. viridae* DBS-2 and *T. harzianum* DBS-1 at different incubation period.* The laccase activities produced by the two strains were not statistically significant (p>0.05).

DISCUSSION

Fungi have successfully inhabited the soil due to their ability to adopt to adverse conditions by adopting various forms (Sun *et al.*, 2005). They are able to degrade lignocellulosic wastes by producing a wide variety of extracellular enzymes (Žifčáková *et al.*, 2016). Fungi exist in the soil as spore and germinate upon inoculation on a mycological medium and incubation.

T. viridae, *T. harzianum*, *A. niger*, *Fusarium* sp. and *Penicillium* sp. were the fungal species isolated from the soil samples collected in this study. These fungal genera were among the most abundant in soil. The presence of these fungal isolates in the soil suggested they can produce a wide variety of extracellular enzymes, hence they can break down all kinds of organic matter and regulate the balance of carbon and nutrients (Frac *et al.*, 2018).

The laccase producers in this study were *T. viridae* and *T. harzianum*. These findings are in line with the result of Abd El Monssef *et al.* (2016) who reported that of all the isolates screened from biodeteriorated parchment, only *Trichoderma harzianum* was found to be laccase producer but disagree with the findings of Kumar *et al.* (2011) where *T. viridae* and *T. harzianum* isolates screened were not laccase producer. Only one out of the three isolates of *T. harzianum* was found to be laccase producer, this is likely due to strain variation among members of the same species.

Increase in laccase activity was observed with an increase in the incubation period for both *T. viridae* and *T. harzianum*. This implies that the isolates were able to use corn cob as a substrate for laccase production. Adivappa and Basappa (2015) also reported an increase in laccase production with an increase in the incubation period. However, the highest enzyme activity observed in this study (2.228 U/mL) was higher than 0.718 U/mL produced by *Marasmius* sp. as observed by Adivappa and Basappa (2015). This difference might be due to difference in enzyme production ability of different fungal species. Contrary to this finding, higher laccase activity (28.2 U/mL) was reported by Amanpreet *et al.* (2017). This wide difference was because Amanpreet *et al.* (2017) used optimized conditions and added enzyme inducers (2 mM CuSO₄ and 1 % MgSO₄) to the Czapek Dox medium used.

The higher activities exhibited by *T. viridae* as compared to *T. harzianum* in this study may be due to differences in enzyme production ability of different species of the same genus. This difference was however not statistically significant.

CONCLUSION

In conclusion, ten different fungal species were isolated from a soil sample and belong to the genus *Trichoderma*, *Penicillium*, *Fusarium* and *Aspergillus*. The fungal species with the highest frequency of occurrence were *T. viridae* (30 %) and *T. harziaum* (30 %) while the least occurring species were *Penicillium* sp. (10 %) and *Fusarium* sp. (10 %). *T. viridae* (DSB-2) and *T. harziaum* (DSB-1) were found to be laccase producing fungi while the remaining eight isolates were not. *T. viridae* (DSB-2) was a better laccase producer compared to *T. harziaum* (DSB-1). Both laccases producing isolate were able to utilize corn cob substrate for laccase production.

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