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Screening For Lignocellulolytic Enzymes-producing White Rot Fungi

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: The three major parts of lignocellulose are cellulose, hemicellulose and lignin which can be broken down by cellulase, xylanase and laccase, respectively, thereby making the reducing sugar in lignocellulose available for industrial processes.
Aims: This work aimed to screen for white-rot fungi with the potential of producing cellulase, xylanase and laccase which are vital in breaking lignocellulosic substrates.
Methodology: Some white rot fungi were screened for their abilities to produce cellulase, xylanase and laccase on potato dextrose agar supplemented separately with 1% carboxyl methyl cellulose (CMC), 1% of xylan, and 0.1% of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid), respectively and enzymes relative activity were determined.
Results: The highest average relative activity of cellulase (1.07 ± 0.073) was recorded with *Lentinus squarrosulus* while the same average relative xylanase activity (1.13) was produced by both *Lentinus squarrosulus* and *Pleurotus ostreatus*. *Pleurotus tuber-regium* did not produce cellulase and xylanase. The highest average relative laccase activity (1.43 ± 0.286) was produced by *Pleurotus tuber-regium* followed by *Pleurotus ostreatus* (1.24 ± 0.162) and the least was by *Lentinus squarrosulus* (1.12 ± 0.134).

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Conclusion: *Pleurotus ostreatus* and *Lentinus squarrosulus* produced cellulase, xylanase and laccase which are important in breaking down lignocellulose. *Pleurotus ostreatus* and *Lentinus squarrosulus* could be employed to break down lignocellulose.

Keywords: Pleurotus ostreatus; Lentinus squarrosulus; Pleurotus tuber-regium; white rot fungi; cellulose; xylanase; laccase; lignin-modifying enzymes.

1. INTRODUCTION

Lignocelluloses are wastes in the environment which consist of three main components such as cellulose, hemicellulose and lignin [1,2]. Cellulose is made up of hexoses while hemicellulose contains hexoses and pentoses. Reducing sugars in lignocellulolytic substrates that could be converted to value-added products are not readily available for bioconversion until lignocelluloses are pretreated. There are three main types of pretreatment: chemical, physical and biological [1]. Some advantages of the biological pretreatment method are low energy consumption, low cost and green technology [1,3]. Biological pretreatment is the use of organisms or their metabolites to break down lignocellulose. Organisms suitable for breaking down lignocellulose to simple sugar should be able to produce lignocellulolytic enzymes needed to break down the three main components of lignocellulose.

Cellulases are enzymes that break down the cellulolytic part of lignocellulosic substrates to simple sugars while xylanases are involved in the conversion of the hemicellulose portion of lignocellulose to sugar [4,5]. Lignases are used to degrade lignin that binds lignocellulose together. Production of three main lignocellulolytic enzymes by a single organism indicates that the organism could be used to break down lignocellulose yielding simple sugar that could be utilized for other value-added products.

There are different groups of fungi involved in the degradation of lignocellulosic substrates which are white-rot fungi, brown-rot fungi and soft-rot fungi [6]. White rot and brown rot fungi are Basidiomycetes while soft rot fungi are Ascomycetes [6,7]. White rot fungi is a group of fungi that breaks down lignocellulosic substrates leaving a white fibrous appearance on degraded substrates. This group of fungi produce lignocellulolytic enzymes (cellulase, xylanase and ligninolytic enzymes) which are important to many industries. Ligninolytic enzymes are also referred to as lignin-modifying enzymes, lignases or ligninases. Laccase, manganese peroxidase, lignin peroxidase and versatile peroxidase are examples of lignin-modifying enzymes. White rot fungi have been used for the degradation of lignocellulose by some researchers [1,2,8]. This work aimed at screening some white rot fungi for their ability to produce lignocellulolytic enzymes (cellulase, xylanase and lignin-modifying enzymes) on plate.

2. MATERIALS AND METHODS

2.1 Collection of White Rot Fungi

Three white rot fungi (*Pleurotus ostreatus*, *Pleurotus tuber-regium* and *Lentinus squarrosulus*) were collected from the Department of Botany, University of Ibadan, Ibadan. They were screened for their ability to produce cellulase, hemicellulase and lignase/laccase.

2.2 Screening of White Rot Fungi for Enzymes Production

2.2.1 Screening of white rot fungi for cellulase production

Potato Dextrose Agar was prepared and supplemented with 1 % of Carboxyl Methyl Cellulose (CMC). It was sterilized at 121 °C and 1.05kg cm^{-2} for 15 minutes. It was dispensed into sterile Petri dishes and allowed to solidify. Each plate was inoculated with *Pleurotus ostreatus*, *Pleurotus tuber-regium* and *Lentinus squarrosulus* separately and incubated at 28 ±2 °C for five days. There were five sets for each white rot fungus. Each set was taken every 24 hours, flooded with 2% (w/v) aqueous congo red and left for 15 minutes. Excess stain was poured off after 15 minutes and washed with distilled water and the appearance of a clear area around colonies against a red colour for undegraded CMC indicates cellulase production [9]. Relative cellulase activities were determined by dividing the diameter of hydrolysed CMC by the diameter of the organism.

2.2.2 Screening of white rot fungi for xylanase production

Xylan is the main component of hemicellulose. One percent (1% w/v) xylan was used to supplement potato dextrose agar and was sterilized at 121 °C and 1.05kg cm^{-2} for 15 minutes. It was dispensed into sterile Petri dishes and allowed to gel. *Pleurotus ostreatus*, *Pleurotus tuber-regium* and *Lentinus squarrosulus* were inoculated into each plate separately and incubated at 28 ±2 °C for five days. There were five sets for each organism. Each set was taken every 24 hours and flooded with iodine stain (0.25% w/v aqueous I_2 and KI) and left for 5 minutes. The stain was poured off the plate after 5 minutes and was washed with distilled water. The appearance of a clear zone against a blue/reddish purple colour shows xylanase activities [9]. Relative xylanase activities were determined by dividing the diameter of hydrolysed xylan by the diameter of the organism.

2.2.3 Screening of white rot fungi for lignase production

Tannic acid agar was prepared by supplementing potato dextrose agar with one percent tannic acid. It was sterilized at 121 °C and 1.05kg cm^{-2} for 15 minutes. It was dispensed into sterile Petri dishes and allowed to solidify. Each plate was inoculated separately with *Pleurotus ostreatus*, *Pleurotus tuber-regium* and *Lentinus squarrosulus* and incubated at 28 ±2°C. Growth and colour were observed every 24 hours for five days. A brown oxidation zone around colonies indicates lignin degradation [9]. Relative lignase activities were determined by dividing the diameter of degraded lignin by the diameter of the organism.

2.2.4 Screening of white rot fungi for laccase production

Laccase is one of the enzymes involved in lignin degradation. Potato dextrose agar was supplemented with 0.1% (w/v) of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS). It was sterilized at 121 °C and 1.05kg cm^{-2} for 15 minutes, dispensed into sterile Petri dish, allowed to solidify and inoculated separately with *Pleurotus ostreatus*, *Pleurotus tuber-regium* and *Lentinus squarrosulus* and incubated at 28 ±2°C. The plates were observed every 24 hours for five days for the development of green or purple colouration around the colonies indicating the production of laccase [9]. Relative laccase activities were determined by dividing the diameter of oxidized ABTS by the diameter of the organism.

3. RESULTS

The three white rot fungi (*Lentinus squarrosulus*, *Pleurotus ostreatus* and *Pleurotus tuber-regium*) screened for cellulase, xylanase, lignase and laccase showed the ability to produce two or all four enzymes. The appearance of a clear area around fungus growth against a red colour of undegraded carboxymethyl cellulose (CMC) as shown by *Lentinus squarrosulus* indicated the ability of the organism to produce cellulase on the plate (Plate 1). A xylanase-producing *Lentinus squarrosulus* on potato dextrose agar supplemented with xylan is shown in Plate 2. The appearance of a clear area around the organism against a blue/reddish purple colour showed xylanase activities.

Plate 3 shows *Pleurotus ostreatus* with the ability to degrade lignin on tannic agar. The brown colouration around the growing fungus confirmed its ability to produce lignase. Lignase combines all the enzymes involved in lignin degradation which are known as lignin-modifying enzymes. Laccase-producing *Pleurotus tuber-regium* on potato dextrose agar supplemented with 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) is shown in Plate 4. The development of purple colouration indicated the ability of the fungus to produce laccase.

The three white rot fungi (*Lentinus squarrosulus*, *Pleurotus ostreatus* and *Pleurotus tuber-regium*) were able to produce cellulase, xylanase, lignase and laccase except for *Pleurotus tuber-regium* that could not produce cellulase and xylanase as shown in Table 1. The relative lignase activity of *Lentinus squarrosulus* ranged from 1.15 (96 hours) to 1.43 (24 hours). An increase in relative lignase activity of *Pleurotus ostreatus* (1.10 – 1.72) and *Pleurotus tuber-regium* (1.10 – 2.40) was observed with increase in the period of incubation with the least and highest at 24 and 120 hours of incubation, respectively. Relative laccase activity of *Lentinus squarrosulus* and *Pleurotus ostreatus* decreased with an increase in days of incubation from 1.36 to 1.03 and 1.40 to 1.06, respectively whereas that of *Pleurotus tuber-regium* increased in the first 72 hours of incubation from 1.10 to 1.82 and thereafter decreased. A decrease in relative cellulase activity of both *Lentinus squarrosulus* and *Pleurotus ostreatus* with an increase in the day of incubation was recorded.

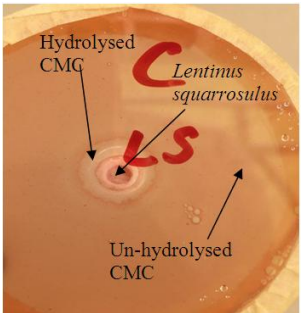

Plate 1. Cellulase-producing *Lentinus squarrosulus* on carboxymethyl cellulose agar

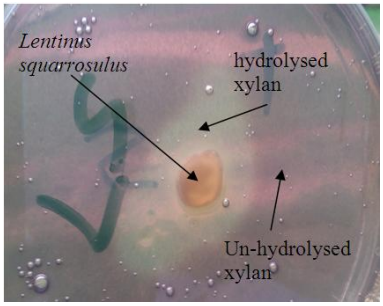

Plate 2. Xylanase-Producing *Lentinus squarrosulus on Potato Dextrose Agar Supplemented with Xylan*

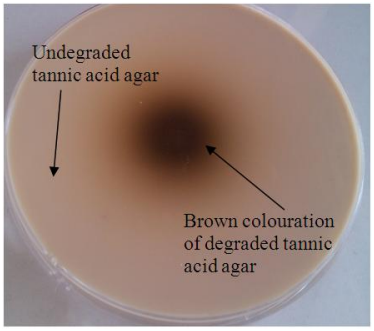

Plate 3. Lignase-producing *Pleurotus ostreatus* on tannic acid agar

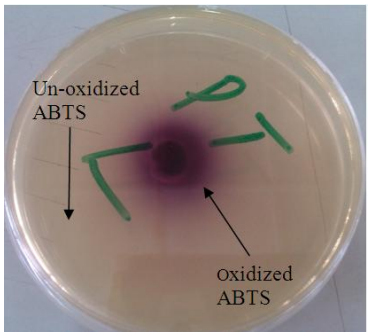

Plate 4. Lacasse-Producing *Pleurotus tuber-regium* on Potato Dextrose Agar Supplemented with 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS)

Table 1. Relative Activities of Lignocellulolytic Enzymes of Some White Rot Fungi on Plate

White Rot Fungi	Period (hours)	Lignocellulolytic Enzymes			
		Lignase	Laccase	Cellulase	Xylanase
Lentinus squarrosulus	24	1.43	1.36	1.19	1.40
	48	1.27	1.08	1.08	1.14
	72	1.27	1.10	1.03	1.06
	96	1.15	1.05	1.03	1.06
	120	1.22	1.03	1.01	1.01
	Average	1.27 ± 0.103	1.12 ± 0.134	1.07 ± 0.073	1.13 ± 0.156
Pleurotus ostreatus	24	1.10	1.40	1.09	1.40
	48	1.35	1.38	1.07	1.14
	72	1.61	1.28	1.03	1.06
	96	1.67	1.08	1.02	1.02
	120	1.72	1.06	1.02	1.02
	Average	1.49 ± 0.260	1.24 ± 0.162	1.05 ± 0.032	1.13 ± 0.159
Pleurotus tuber-regium	24	1.10	1.10	-	-
	48	1.40	1.20	-	-
	72	1.95	1.82	-	-
	96	2.15	1.50	-	-
	120	2.40	1.52	-	-
	Average	1.80 ± 0.537	1.43 ± 0.286	-	-

Key: - Enzyme not detected

Relative xylanase activity of *Lentinus squarrosulus* ranged from 1.01 to 1.40 while that of *Pleurotus ostreatus* ranged from 1.02 to 1.40. *Pleurotus tuber-regium* did not produce both cellulase and xylanase on the plate. Average relative activities of lignase and laccase by

Pleurotus tuber-regium (1.80 ± 0.537 and 1.43 ± 0.286) > *Pleurotus ostreatus* (1.49 ± 0.260 and 1.24 ± 0.162) > *Lentinus squarrosulus* (1.27 ± 0.103 and 1.12 ± 0.134), respectively. The highest average relative cellulase activity (1.07 ± 0.073) was obtained by *Lentinus squarrosulus* and the same average relative xylanase activity (1.13) was recorded by *Lentinus squarrosulus* and *Pleurotus ostreatus*.

4. DISCUSSION

The white rot fungi used in this work had the ability to produce hydrolytic (cellulase and xylanase) and oxidative (lacasse) enzymes. Production of hydrolytic enzymes by *Pleurotus ostreatus* and *Lentinus squarrosulus* was confirmed by their cellulose-degradation potential in the formation of clear halos around the fungi plugs against a pink Congo red-cellulose complex. An indication of their abilities to break down cellulose to simple sugars as reported by Olanbiwoninu and Fasiku, [10]. Production of cellulase by *Pleurotus ostreatus* and *Lentinus squarrosulus* as observed in this study has been reported by some researchers to be due to the ability of the organisms to excrete hydrolysing enzymes which effectively broke down the cellulolytic component of lignocellulose [11-19]. However, the inability of *Pleurotus tuber-regium* to produce cellulase in the course of this work could be an influence of environmental factors or the genetic makeup of the organism which might make the degradation of cellulose difficult.

In the hydrolysis of xylan, the clear zone against the blue-black colour observed is an indication of the fungal abilities to produce hemicellulase [5,20,21]. The fungi effectively converted xylan to hexoses and pentoses which resulted in clear zones around xylanase producing mushrooms on xylan agar. Xylanase production by both *Pleurotus ostreatus* and *Lentinus squarrosulus* could have been determined by the genetic makeup of the organisms because many researchers have reported using these organisms to produce xylanase. Akhmedova and Rashidova [18], Majumder [22] and, Han et al. [23] produced xylanase with *Pleurotus ostreatus* while Vichitraka et al. [19], Pukahuta et al. [24] and, Isikhuemhen et al. [25] used *Lentinus squarrosulus* to produce xylanase.

White-rot fungi are efficient in degrading lignin compounds because of their ability to produce lignin-modifying enzymes such as laccase and others. Production of lignase on tannic acid agar by *Pleurotus ostreatus*, *Pleurotus tuber-regium* and *Lentinus squarrosulus* in this work might be due to their abilities to utilise tannic acid which resulted in the brown coloration observed on the plates as earlier reported by Pointing [9]. Gramss et al. [26] explained that laccase-producing organisms oxidized 2, 2'-Azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) that was colourless to $ABTS^{2+}$ (purple) and purple colouration that appeared on medium supplemented with ABTS in this work indicated abilities of mushroom to produce laccase. The oxidative capability of lignin-modifying enzymes of *Pleurotus ostreatus* and *Lentinus squarrosulus* recorded in this research has been confirmed by many researchers [27-30].

Pleurotus ostreatus and *Lentinus squarrosulus* produced three enzymes responsible for breaking down lignocellulose in this work. Among other reported lignocellulolytic enzymes-producing mushrooms are *Phanerochaete chrysosporium* [31], *Stereum ostrea* [32], *Pleurotus florida* [33] and *Agaricus bisporus* [34].

5. CONCLUSION

Pleurotus ostreatus and *Lentinus squarrosulus* produced the three main lignocellulolytic enzymes (cellulase, xylanase and lignin-modifying enzymes) while *Pleurotus tuber-regium* produced cellulase and lignase. *Pleurotus ostreatus* and *Lentinus squarrosulus* with the potential of producing lignocellulolytic enzymes could be utilized to degrade lignocellulosic substrates releasing reducing sugars that are important for bioconversion.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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