



# Laccase-Mediated Synthesis of Bio-material Using Agro-residues

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## Abstract

Laccase has the ability to act on a broad range of substrates, as a result of which multiple compounds have been used for laccase assay and numerous applications have been discovered over time. Thus, considering the above points, in the present study, white and blue laccase from *Myrothecium verrucaria* ITCC-8447 and *Pleurotus ostreatus* were used for laccase assay using various substrates which includes 2,2' azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,6-dimethoxyphenol (2, 6 DMP), guaiacol (GCL), and syringaldazine (SYZ). The most effective substrate of the four substrates was ABTS for white and blue laccase. The white and blue laccase were further used for the synthesis of bio-material via fungal-assisted treatment of wheat bran (WB) and sugarcane bagasse (SB) followed by micro-wave pre-treatment and the addition of starch and glycerol to the treated agro-residue in the ratio of 5:1:1. The thickness swelling and water absorption percentage was minimum for wheat bran synthesized bio-material using white laccase in comparison to the other synthesized bio-material.

## Keyword

ABTS · 2 · 6 DMP · GCL · SYZ · Agro-residue · Bio-material

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## 10.1 Introduction

Laccase is widely distributed in nature and is found in plants, fungi, insects [1], and bacteria [2]. Laccase, due to its substrate specificity, can act on a broad range of substrates, as a result of which various substrates have been used for laccase assay, and the most widely used substrates include ABTS, 2,6 DMP, GCL, and SYZ. The other aspect of the enzyme involves the reuse of agro-residues which are generated in huge amounts throughout the globe. Only a small fraction of these residues is used, and the rest are either dumped in wasteland or are burned, thereby contributing to environmental pollution. They have immense applications, but the lignin content in these agro-residues acts as a barrier for its effective utilization [3]. However, laccase has been used for the delignification of agro-residues [4] which can further help in the utilization of these agro-residues in lignocellulosic biorefinery and synthesis of bio-material. Thus, laccase substrate affinity and its application in various sectors have attracted considerable attention from environmental, industrial, and biotechnological prospect [5].

In the present study, the focus was on two aspects: first is on laccase assay using various substrates, which include 2,2' azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,6-dimethoxyphenol (2,6 DMP), guaiacol (GCL), and syringaldazine (SYZ) from newly isolated white laccase-producing strain *Myrothecium verrucaria* ITCC-8447 and blue laccase-producing strain *Pleurotus ostreatus* as the reference strain. This was followed by fungal-assisted synthesis of bio-material using wheat bran (WB) and sugarcane bagasse (SB) with white and blue laccase-producing strain *Myrothecium verrucaria* ITCC-8447 and *Pleurotus ostreatus*. The fungal pre-treated substrate was subjected to optimized microwave treatment in our laboratory. The bio-material was synthesized using starch and glycerol. The volume, thickness swelling, and water absorption percentage of the synthesized bio-material were determined. These synthesized bio-materials can help in effectively reutilizing agro-residue which would otherwise be rendered useless, thereby contributing toward management and recycling of agricultural waste.

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## 10.2 Materials and Methods

### 10.2.1 Materials

The chemicals used were of analytical grades and purchased from Sigma, Hi-Media, and Merck, India.

### 10.2.2 Culture Condition and Inoculum Preparation

The fungal strain *Myrothecium verrucaria* ITCC-8447 and *Pleurotus ostreatus* were used in this study. The newly isolated strain *Myrothecium verrucaria* ITCC-8447 was isolated from Rajasthan, India, and maintained on malt extract agar plates,

whereas *Pleurotus ostreatus* was a gift culture from the Institute of Forstbotanik, Göttingen, Germany, used as the reference culture and maintained on potato dextrose agar plates. The medium composition used for laccase assay was as follows for *Myrothecium verrucaria* ITCC-8447 (g/L): glucose 5, peptone 5, yeast extract 2,  $\text{KH}_2\text{PO}_4$  1,  $\text{MgSO}_4$  0.5,  $\text{ZnSO}_4$  0.01,  $\text{MnSO}_4$  0.001,  $\text{CaCl}_2$  0.01,  $\text{FeSO}_4$  0.01, and pH  $7.0 \pm 0.5$  [6] with slight modifications, and for *Pleurotus ostreatus*, Bushnell Haas Media (BHM) supplemented with (g/L)  $\text{C}_6\text{H}_{12}\text{O}_6$  pH  $5 \pm 0.5$  [7] was used for laccase assay. For the inoculum preparation, 7-day-old culture was used, and two 6 mm cubes were inoculated in 50 mL of media using 250 mL Erlenmeyer flask and incubated at  $28 \pm 2$  °C under static condition. The crude enzyme was withdrawn every 48 h for laccase assay, and the experiment was carried out for a period of 12 days. The crude extract from the 10th day was centrifuged at 10,000 rpm for 5 min used for further study.

### 10.2.3 Enzyme Assay and Protein Concentration Determination

Laccase activity was determined quantitatively by measuring the absorption change of various substrates, which included ABTS at 420 nm,  $\epsilon_{420} = 36,000 \text{ M}^{-1} \text{ cm}^{-1}$  [8]; 2,6DMP at 469 nm,  $\epsilon_{469} = 14,800 \text{ M}^{-1} \text{ cm}^{-1}$  [9]; GCL at 465 nm,  $\epsilon_{465} = 12,100 \text{ M}^{-1} \text{ cm}^{-1}$  [10]; and SYZ at 525 nm,  $\epsilon_{525} = 65,000 \text{ M}^{-1} \text{ cm}^{-1}$  [11] for 5 min at 30 °C in UV-visible spectrophotometer (Make: Dynamica; Model: Halo DB-30). The concentration of the substrate was varied and was in the following range: ABTS – 0.5–1.5 mM; 2, 6 DMP, 1–5 mM; GCL, 20–30 mM; and SYZ, 10–30 mM, followed by the determination of the optimal concentration of substrates for laccase assay. The laccase activity was calculated as per Holme and Peck, 1996 [12]. One activity unit (U) was defined as the amount of enzyme required to oxidize 1 mmole ABTS per minute at 420 m, 2,6 DMP at 469 nm, GCL at 465 nm, and SYZ 525 nm. The protein estimation was done by Lowry's method, 1951 [13], with BSA as the standard.

### 10.2.4 Synthesis of Bio-material Using White and Blue Laccase

The fungal-assisted synthesis of bio-material was done by inoculating the agro-residue WB and SB with *Myrothecium verrucaria* ITCC-8447 and *Pleurotus ostreatus* under SSF for 15 days at  $28 \pm 2$  °C. The agro-residue was then soaked in 5 mM ammonium molybdate, followed by microwave-assisted pre-treatment using microwave reaction system SOLV, Multiwave Pro (Make: Anton Parr, Austria), at 150 °C, 6 lb. pressure for 45 min, as per Verma et al. [14], with slight modifications which were established in our laboratory. It was followed by the addition of starch and glycerol to the treated agro-residue in the ratio of 5:1:1. The bio-material was then synthesized at 140 °C for 30 min. The thickness swelling (%) and water absorption (%) were measured as per Jeefferie et al. [15]:

$$\text{Water absorption} = \left[ \frac{W_f - W_i}{W_i} \right] * 100$$

where  $W_i$  is the initial weight of bio-material before water absorption and  $W_f$  is the final weight of bio-material after water absorption.

$$\text{Thickness swelling} = \left[ \frac{T_f - T_i}{T_i} \right] * 100$$

where  $T_i$  is the initial thickness of bio-material before water absorption and  $T_f$  is the final thickness of bio-material after water absorption.

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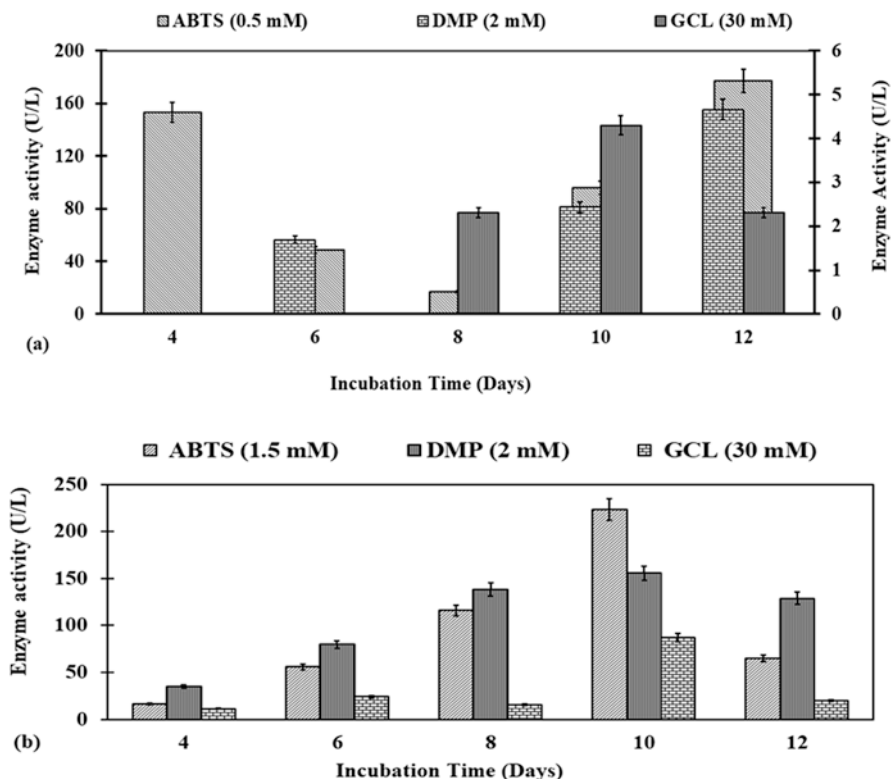
## 10.3 Results and Discussions

### 10.3.1 Laccase Activity Using Various Substrates

Laccase activity was evaluated with different substrates for a period of 12 days using *Myrothecium verrucaria* ITCC-8447 and *Pleurotus ostreatus* (Fig. 10.1a, b). The white laccase activity for *Myrothecium verrucaria* ITCC-8447 was detected 153.3 UL<sup>-1</sup> by ABTS, whereas no activity was observed for 2,6 DMP and GCL on the fourth day. However, on the tenth day, white laccase activity using 2,6 DMP and GCL was 2.4 UL<sup>-1</sup> and 4.3 UL<sup>-1</sup> which was negligible as compared to ABTS 95.8 UL<sup>-1</sup>. In the reference strain *Pleurotus ostreatus*; significant blue laccase activity was detected on the fourth day with 2,6 DMP and ABTS of 34.9 UL<sup>-1</sup> and 16.7 UL<sup>-1</sup>, respectively, and it was 11.4 UL<sup>-1</sup> for GCL, whereas on the tenth day, the laccase activity was 155.7 UL<sup>-1</sup>, 223.1 UL<sup>-1</sup>, and 87.4 UL<sup>-1</sup> for 2,6 DMP, ABTS, and GCL. The observed difference in laccase activity may be due to the specificity of the crude extract to different substrates.

### 10.3.2 Laccase Assay Using Various Substrates

The most effective substrate of the four substrates was ABTS for both white and blue laccase from *Myrothecium verrucaria* ITCC-8447 and *Pleurotus ostreatus* (Table 10.1). Multiple substrates were used for laccase assay in the work done by Eichlerová (2012) [7], and laccase activity using SYZ was only obtained with purified enzyme; similarly in the present study, both white and blue laccase did not exhibit oxidation with SYZ. The optimal concentration of various substrate was determined, and it was observed that, for white laccase from *Myrothecium verrucaria* ITCC-8447, it was ABTS (0.5 mM), 2,6 DMP (2 mM), and GCL (30 mM), and for blue laccase from *Pleurotus ostreatus*, it was ABTS (1.5 mM), 2,6 DMP (2 mM), and GCL (30 mM). Similarly, the protein concentration for white and blue laccase was 1.82 mg/mL and 0.29 mg/mL, respectively (Table 10.1).



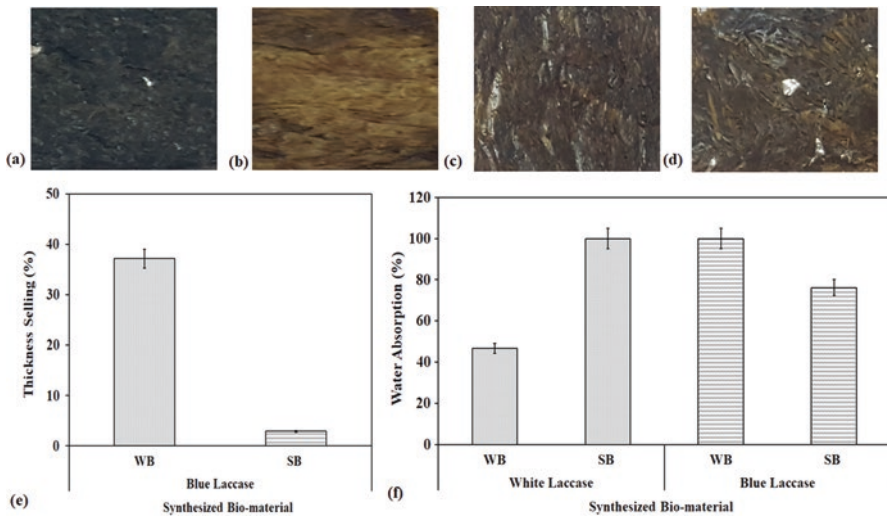
**Fig. 10.1** (a) *Myrothecium verrucaria* ITCC-8447: detection of white laccase activity by ABTS, 2,6 DMP, and GCL for 12 days. (b) *Pleurotus ostreatus*: detection of blue laccase activity by ABTS, 2,6 DMP, and GCL for 12 days

**Table 10.1** White and blue laccase activity from *Myrothecium verrucaria* ITCC-8447 and *Pleurotus ostreatus* with different substrates (ABTS, DMP, and GCL), protein concentration, and enzyme activity on the tenth day

Substrate concentration (mM)	Protein concentration (mg/mL)	Enzyme activity (UL <sup>-1</sup> )
<b><i>Myrothecium verrucaria</i> ITCC-8447</b>		
ABTS (0.5)	1.82	95.8
DMP (2.0)	1.82	2.4
GCL (30.0)	1.82	4.3
<b><i>Pleurotus ostreatus</i></b>		
ABTS (1.5)	0.29	223.1
DMP (2.0)	0.29	155.7
GCL (30.0)	0.29	87.4

### 10.3.3 Synthesis of Bio-material Using White and Blue Laccase

The bio-material synthesized from WB and SB using white and blue laccase from *Myrothecium verrucaria* ITCC-8447 and *Pleurotus ostreatus* had a volume of 15.3 mm<sup>3</sup> and 10.5 mm<sup>3</sup> (Fig. 10.2a, b) and 25.7 mm<sup>3</sup> and 54.9 mm<sup>3</sup> (Fig. 10.2c, d),



**Fig. 10.2** Synthesis of bio-material via fungal-assisted treatment of wheat bran (WB) and sugarcane bagasse (SB) using white laccase (a–b) and blue laccase (c–d) followed by microwave pre-treatment and addition of starch and glycerol. (e) Thickness swelling (%) of blue laccase-synthesized bio-material (f) water absorption (%) of the white and blue laccase-synthesized bio-material

respectively. The percentage of thickness swelling observed in case of both WB and SB was negligible when synthesized using white laccase; however, in blue laccase-synthesized bio-material, it was 37.2 and 3.0 (Fig. 10.2e). These results are in accordance with Jeefferie et al. [15] which states that the thickness swelling is directly proportional to the size of fiber, i.e., smaller the size of the fiber less is the thickness swelling and vice versa [15]. The percentage of water absorption for white laccase-synthesized bio-material was 46.7 and 100 for WB and SB, respectively, whereas for blue laccase-synthesized bio-material, the percentage of water absorption was 100 and 76 (Fig. 10.2f). The water absorption of the bio-material significantly affects the structural integrity of bio-materials and results due to the saturation of the fibers in the presence of water; it also gives an insight into the water diffusivity of the bio-material [15].

## 10.4 Conclusion

The present study states that the most effective substrate was ABTS for white and blue laccase-producing strain *Myrothecium verrucaria* ITCC-8447 and *Pleurotus ostreatus*. The fungal-assisted treatment using white and blue laccase followed by pre-treatment was employed for the synthesis of bio-material. The WB-synthesized bio-material via white laccase exhibited minimum water absorption percentage in comparison to the other synthesized bio-material. Thus, the delignification capability of the agro-residues and its role in bio-material synthesis using pre-treatment

and binding agents can be effectively utilized for the reutilization of waste agro-residues in synthesis of bio-material, thereby fitting in the recent concept of “waste recycling.”

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