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Synthetic dye decolourization, textile dye and paper industrial effluent treatment using white rot fungi *Lentines edodes*

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ABSTRACT

The laccase producing fungi *Lentines edodes* was screened for laccase production using various indicator compounds like guaiacol, tannic acid and the polymeric dyes such as Remazol brilliant blue R and Poly R-478. The organism *Lentines edodes* that produce laccase was cultivated on basal medium. Potato dextrose agar medium (PDA) and malt extract agar medium (MEA) were used for the first subculture of chosen isolate and the plates were examined by light microscopy to check the absence of bacteria and the unique fungi isolation. For testing the crude enzyme laccase activity an agar plug (1 cm^2) from a 7-day old *Lentines edodes* agar plate was transferred to 50 ml of potato dextrose broth in Erlenmeyer flasks. The cultures were maintained at 25°C for 7 days. Screening was performed in Petri dishes (60 mm diameter) with 15 ml of malt extract agar (MEA) medium and potato dextrose agar (PDA) medium from Hi media, Mumbai, India, containing indicator compounds such as 0.04% (w/v) of Remazol Brilliant Blue-R (RBBR) and Poly R-478, 0.01% (v/v) of guaiacol and tannic acid 0.05% (w/v). Guaiacol (Sigma) RBBR (Sigma) and Poly R-478 (Sigma) were added to the media after autoclaving as sterile-filtered solutions. Tannic acid (Merck Chemicals Ltd., UK) was autoclaved separately before addition to the media. Guaiacol is a sensitive substrate that allows a rapid screening of fungal strains producing extracellular guaiacol oxidizing enzymes by means of a colour reaction. The white-rot fungus *Trametes hirsuta* that produces laccase, manganese peroxidase and lignin peroxidase was used as a positive control. The identity of laccase producing fungal species was confirmed by brown color development surrounding the fungal growth. Any colony produce yellow or which causes decolourization of Poly R-478 was considered as ligninolytic positive and isolated. Decolourization of Poly R 478, RBBR, guaiacol and tannic acid oxidizing strains were also studied on liquid cultures for lignin peroxidase, manganese peroxidase and laccase activities. For laccase activity, the isolated fungi was grown at 25°C for 12 days with rotary shaking (150 rpm) in 500-ml baffled Erlenmeyer flasks containing 50 ml of potato dextrose broth. The crude extract of *Lentines edodes* revealed promising results on decolourization of various dye stuffs, paper and textile effluents. About 74.1% of reactive yellow, 77.5% reactive blue and 75% RBBR dye stuffs were effectively decolorized on the third day. Similarly, more than 90% of textile and paper effluents were decolorized on first day itself.

Keywords: Laccase; RBBR; Decolourization; Effluents

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1. Introduction

The textile industry and paper industry account for two-thirds of the total dyestuff market [1] and consume large volumes of water and chemicals for wet processing of textiles and paper processing. The chemical reagents used are very diverse in chemical composition, ranging from inorganic compounds to polymers and organic products [2,3]. There are more than 100,000 commercially available dyes with over 7×10^5 ton of dyestuff produced annually [4]. Due to their chemical structure, dyes are resistant to fading on exposure to light, water and different chemicals [5] and most of them are difficult to decolorize due to their synthetic origin. The use of laccase in the textile and paper industry is growing very fast, since besides to decolorize textile effluents as commented above, laccase is used to bleach textiles and even to synthetic dyes [6]. The industrial preparation of paper requires separation and degradation of lignin in wood pulp. Environmental concerns urge to replace conventional and polluting chlorine-based delignification/bleaching procedures. The capability of laccases to form reactive radicals in lignin can also be used in targeted modification of wood fibers. Laccases can be used in the enzymatic adhesion of fibers in the manufacturing of lignocellulose-based composite materials such as fiberboards. Laccases have been proposed to activate the fiber bound lignin during manufacturing of the composites, thus, resulting in boards with good mechanical properties without toxic synthetic adhesives [7,8]. Another possibility is to functionalize lignocelluloses fibers by laccases in order to improve the chemical or physical properties of the fiber products. Preliminary results have shown that laccases are able to graft various phenolics acid derivatives onto kraft pulp fibers [9,10]. Shanmugam et al. [19] has reported that the crude extract of laccase from *Pleurotus florida* possessed an effective dye, textile and paper effluents decolourization. In the light of the aforementioned studies, this study was designed to investigate the effect of *Lentines edodes* obtained from Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India was used for decolourization of textile dye-containing wastewater and paper industry effluent.

2. Materials and methods

2.1. Microorganism

The fungi *Lentines edodes* obtained from Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India were maintained in potato-dextrose-agar slants (PDA) at 4°C. The fungus was transferred to PDA plates and incubated for 7–8 days at 27°C. Inoculum containing 10^6 spores ml^{-1} was prepared and was transferred to a sterilized 250-ml Erlenmeyer flask containing 10 g wheat bran (obtained from local market) moistened with distilled water (1:1, w/v). The flasks were incubated at 27°C for 15–20 days. Crude culture filtrate was obtained by adding 30-ml of distilled water to the flasks and filtering through a muslin cloth. The filtrate was again centrifuged at 10,000 X g for 10 min. The supernatant was used as the enzyme source.

Agaricus bisporus was obtained from Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, and India. The fungus was transferred to PDA plates and incubated for 7–8 days at 27°C. Inoculum containing 10^6 spores ml^{-1} was prepared and was transferred to a sterilized 250-ml Erlenmeyer flask containing 10 g wheat bran (obtained from local market) moistened with distilled water (1:1, w/v). The flasks were incubated at 27°C for 15–20 days. Crude culture filtrate was obtained by adding 30-ml of distilled water to the flasks and filtering through a muslin cloth. The filtrate was again centrifuged at 10,000 X g for 10 min. The supernatant was used as the enzyme source. A substrate specific for laccase, syringaldazine [*N, N*'-bis (3,5dimethoxyhydroxy benzylidenehydrazine)], was used. Spore suspensions were incubated with 50 mM syringaldazine and 10 mM $CuSO_4$ in 100 mM phosphate buffer. A purple color was developed which indicates laccase like activity.

2.2. Qualitative assay for laccase

Lentines edodes were screened for laccase production by growing them on basal medium containing 10 mM guaiacol. The basal medium contains (per liter of distilled water): 0.5 g of KH_2PO_4, 0.2 g of $MgSO_4.7H_2O$, 0.1 g of NH_4NO_3, 0.1 g of KCl, 0.02 g of $FeSO_4.7H_2O$, 0.05 g of $Ca(NO_3)_2.4H_2O$, 2.0 g of Bacto malt-extract (HiMedia chemicals) and 15 g of agar (Himedia Chemicals). Basal medium was autoclaved in flasks and cooled to 55°C, when the following were added aseptically: 5 ml of 1 M KOH; 0.4 ml of guaiacol; 30 mg of streptomycin sulphate; 30 mg of penicillin G (Na salt); 4 mg of benomyl (as benlate 50 WP) in 2 ml of 1:1 acetone-70% ethanol. The medium was stirred and poured into petridishes. 10 mm agar plug taken from fungal colony was used to inoculate 250-ml Erlenmeyer flask containing 100 ml potato dextrose medium. The culture was incubated at 30°C and shaken at 100 rpm. The production of intense brown colour around the fungal colony in the guaiacol-containing medium was considered as a positive reaction for presence of laccase activity.

2.3. Enzyme assay

Enzyme activity was assayed by using 50 μl of crude filtrate, 950 μl of 10 mM guaiacol in 0.1 M acetate buffer containing 10% (v/v) acetone, pH 5.0 [11]. The enzyme blank consists of 50 μl of crude filtrate and 950 μl of 0.1 M acetate buffer and the substrate blank consists of 50 μl 10 mM guaiacol in 0.1 M acetate buffer containing 10% (v/v) acetone and 950 μl of 0.1 M acetate buffer. The contents were mixed well and incubated at 30°C for 5 min

[12,13]. The brown coloureformed was spectrophotometrically read at 460 nm using Beckman DU-530 spectrophotometer. Enzyme activity was expressed in units ml^{-1}. One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 mmol guaiacol per 1 min.

In order to rule out the role of peroxidase oxidation and prove the oxidation only by laccase, the enzyme was pre-incubated with catalase (1000 units ml^{-1}) (E.C.1.11.1.6 from *Aspergillus niger*, Sigma C3515) for 30 min at 30°C prior to assay to remove any endogenous hydrogen peroxide [14]. Similarly, manganese independent peroxidase activity was measured by adding hydrogen peroxide (1 mM final concentration) to the laccase assay mixture and to subtract the activity due to laccase alone [14]. Also, lignin peroxidase activity was determined by measuring the production of veratraldehyde from veratryl alcohol at 310 nm in glycine-HCl buffer (pH 3.0) at 30°C, upon addition of hydrogen peroxide (1 mM concentration) [15].

Further, aryl alcohol oxidase activity was assayed under the same conditions without the addition of hydrogen peroxide and manganese dependent peroxidase activity was measured by the oxidation of phenol red at 431 nm in the presence of 100 mM $MnSO_4.5H_2O$ in glycine-HCl buffer (pH 3.0) at 30°C, upon addition of hydrogen peroxide (0.5 mM final concentration) [14].

2.4. Optimization of various parameters for maximum laccase activity

The pH optimum, temperature optimum, effect of optimized inhibitors and inducers of the crude laccase activity were investigated.

2.5. Treatment of synthetic dyes and effluent with crude laccase from Lentines edodes

A study was designed to see the efficiency of *Lentines edodes* to decolourize the various synthetic dyes and effluents. The dyes used for the study are Reactive blue, Reactive yellow, RBBR, Crystal violet, Brilliant green and Congo red. The synthetic and reactive are widely used in textile industries in and around Coimbatore, Tamilnadu, India. The dyes were monitored at their absorbance maxima at χ620 nm, χ401 nm, χ597 nm, χ589 nm, χ623 nm, and χ486 nm (all the dyes were from Sigma Chemicals, USA).

2.6. Treatment of effluent with laccase from Lentines edodes *and* Agaricus bisporus

Lentines edodes crude enzyme filtrate was utilized for investigating the decolourization efficiency of paper and textile effluents. The effluents were monitored at their absorbance maxima at 220 nm. The black liquor obtained from Seshhasayee paper mills, Erode, Tamilnadu, India was from bagasse and wood chip based newsprint manufacturing unit. As per the data provided by the paper mill, the effluent had COD of 416 mg l^{-1} and BOD of 190 mg l^{-1}.

3. Results and discussion

The test *Lentines edodes* isolate producing crude laccase activity was analyzed for optimum pH and temperature. It was found that the crude laccase possesses an optimum pH of 6.0 and 5.5 for the substrates guaiacol and o-dianisidine respectively. The optimum temperature was found to be 50°C for both the substrates. Also, the stability of crude laccase activity in the presence of several potential inhibitors like metal ions (Fe^{2+}, Zn^{2+}, Mg^{2+} and Mn^{2+}), azide, cyanide, hydrogen peroxide, sodium chloride, EDTA and arsenate and SDS were studied. The study was designed to see the effect of various metal ions which may be present in the effluent coming out from the industries and the concentration at which maximum inhibition of laccase activity occurs. Of the different inhibitors used, it was found that azide and cyanide, inhibit crude laccase activity completely, whereas metal ions like Fe^{2+}, Zn^{2+} and 1% SDS inhibit the enzyme activity partially. Further, the influence of three potential inducers (ethanol, xylidiene and veratryl alcohol) and the effect of two important substrates, guaiacol and o-dianisidine on laccase were investigated. From the study it was found that xylidiene is the best inducer to induce laccase activity among various compounds for *Lentines edodes*.

An ecofriendly, biological treatment method using laccase produced by *Lentines edodes* was tried out for the effective decolourization of wastewater containing dyestuffs and effluents. The treatment was carried out using crude laccase and a consortium of microorganisms that produces dye-degrading enzymes. A similar work reported using *Pleurotus florida* and *Agaricus bisporus* crude filtrates for decolourization of reactive dyes, revealed an efficient decolourization of Reactive Yellow of 89% followed by Reactive Green and reactive blue [19]. The efficiency of the crude extract to decolourize the dyes and effluents was monitored by change in their respective absorbance maxima every alternate day after the addition of dye/effluent for a period of 6 days. The final concentration of the dye on day 0 was considered to be 100%. The crude filtrate of *Lentines edodes* without any inducers showed activity of laccase (0.60 U/ml), Manganese-independent peroxidase (0.018 U/ml), Manganese dependent peroxidase (0.04 U/ml) and lignin peroxidase (0.10 U/ml). Aryl alcohol oxidase activity was found to be negligible in this species. However, in the presence of inducers the activity of crude laccase was still higher. Another fungus, *Agaricus bisporus* showed a greater extent of tyrosinase activity (1.6 U/ml), which plays an important role in the oxidation of phenolic compounds [16,17],

Table 1
Treatment of reactive dyes using *Lentines edodes* filtrate and using combinations of crude extracts of *Lentines edodes* and *Agaricus bisporus*

No of days	*Lentines edodes* crude extract (% decolourization)					*Lentines edodes* crude extract + *Agaricus bisporus* (% decolourization)				
	0.1%	0.2%	0.3%	0.4%	0.5%	0.1%	0.2%	0.3%	0.4%	0.5%
Reactive yellow										
Day 1	75.7	70.5	70.4	67.6	60.38	74.3	69.8	67.8	66.6	65.3
Day 2	78.1	77.7	72.1	71.7	67.7	85.7	84.1	83.5	82.0	81.5
Day 3	86.5	84.4	81.3	78.1	74.1	90.0	88.5	87.3	86.2	84.0
Reactive blue										
Day 1	82.8	75.8	75.0	73.0	72.3	80.3	78.0	77.0	73.1	70.1
Day 2	84.0	80.0	76.0	79.5	76.3	82.0	81.0	80.0	76.1	77.0
Day 3	85.4	83.0	81.0	80.5	77.5	86.5	84.0	83.0	82.0	82.0
RBBR										
Day 1	83.0	76.0	74.0	72.0	70.0	85.0	84.4	78.0	72.5	71.5
Day 2	85.0	76.0	75.0	74.0	73.0	88.0	86.6	81.0	76.0	73.0
Day 3	87.0	81.0	78.0	78.0	75.0	90.0	87.2	86.0	85.2	82.0

The experiments were conducted in duplicate. Decoluorization of dyestuffs was carried out using 0.1–0.5% (w/v) concentrations. 0.2 ml of crude filtrate of *Lentines edodes* and *Agaricus bisporus* suspensions (1:1, v/v) was used.

was also selected for the study. All these enzymes have been implicated in dye decolourization as well as for effluent treatment [18,19]. In the study, the crude filtrate of *Lentines edodes* decolourized the reactive blue dye (77.5%) to a greater extent on the third day of incubation than the other dyes (Table 1). The experiment was conducted in duplicate. Decolourization of dyes was carried out using 0.1–0.5% (w/v) concentrations. About 0.2 ml of crude laccase of *Lentines edodes* and *Agaricus bisporus* extract was used (1:1 v/v).

The above mentioned enzymes have been widely adopted in the decolourization of various dyes as well as the textile and paper effluents [18,19]. The *Lentines edodes* filtrate when used individually or in combination with *Agaricus bisporus* suspension completely decolorized both paper and textile effluents within 1 day (Table 2).

Table 2
Treatment of paper and textile effluent using *Lentines edodes* filtrate and using combinations of crude extracts of *Lentines edodes* and *Agaricus bisporus* suspensions

No of days	*Lentines edodes* crude extract (% decolourization)	*Lentines edodes* crude extract + *Agaricus bisporus* suspensions (% decolourization)
	Paper and textile effluent	
Day 1	>90%	>90%

The experiment was conducted in duplicate. Decolourization of effluents was carried out using 0.2 ml of crude filtrate of *Lentines edodes* and *Agaricus bisporus* suspensions (1:1, v/v).

4. Conclusions

In this study, laccase producing fungi *Lentines edodes* was screened for laccase production using various indicator compounds like guaiacol, tannic acid and the polymeric dyes such as Remazol brilliant blue R and Poly R-478. The organism *Lentines edodes* that produce laccase was cultivated on basal medium. Potato dextrose agar medium (PDA) and malt extract agar medium (MEA) were used for the first subculture of chosen isolate, and the plates were examined by light microscopy to check the absence of bacteria and the unique fungi isolation. For testing the crude enzyme laccase activity an agar plug (1 cm^2) from a 7-day old *Lentines edodes* agar plate was transferred to 50 ml of potato dextrose broth in Erlenmeyer flasks. The cultures were maintained at 25ºC for 7 days. Screening was performed in Petri dishes (60 mm diameter) with 15 ml of malt extract agar (MEA) medium and potato dextrose agar (PDA) medium from Hi media, Mumbai, India, containing indicator compounds such as 0.04 % (w/v) of Remazol Brilliant Blue-R (RBBR) and PolyR-478, 0.01% (v/v) of guaiacol and tannic acid 0.05% (w/v). It was found that out of several indicators used, guaiacol is a sensitive substrate that allows a rapid screening of fungal strains producing extracellular guaiacol oxidizing enzymes by means of a colour reaction. The white-rot fungus *Trametes hirsuta* that produces

laccase, manganese peroxidase and lignin peroxidase were used as a positive control. The identity of laccase producing fungal species was confirmed by brown color development surrounding the fungal growth. Any colony produce yellow or which causes decolourization of Poly R-478 was considered as ligninolytic positive and isolated. Decolourization of Poly R 478, RBBR, guaiacol and tannic acid oxidizing strains were also studied on liquid cultures for lignin peroxidase, manganese peroxidase and laccase activities. The low price of *Lentines edodes* and *Agaricus bisporus* extracts containing various enzymes as a cocktail may prove to be an alternative source of decolourization and an efficient method of treating wastewater containing dye stuffs as well as in treating textile and paper effluents.

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