

Screening, Production and Optimization of Extracellular Laccase Enzyme from *Pleurotus Ostreatus*

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Abstract

The enzyme laccase (p-diphenol: oxygenoxidoreductase; EC 1.10.3.2) is known to degrade many phenolic aromatic compounds. Many species of white rot fungi are well-known producers of the enzyme laccase. The first phase was the isolation of eight different strains of white rot fungi and screening them for laccase activity. Then highest laccase producing white rot fungal strains was identified. This strain *Pleurotus ostreatus* produced high amount of laccase compared to rest of the strains. In the second phase, the culture conditions for *Pleurotus ostreatus* were standardized to maximize the laccase production. The further steps involved the isolation, purification, and characterization of the enzyme laccase produced by *Pleurotus ostreatus* and in the final phase, the project deals with the textile industrial application of dyes and sludge treatment.

Keywords: Laccase; *Pleurotus ostreatus*

Introduction

The textile industry involves processing or converting raw material into finished cloth employing various operations. It consumes large quantities of water and produces polluting waste effluents. About 200 tonnes/day of textile sludge are generated in Thirupur (a textile industry in Coimbatore). Some of the sludge is disposed in an engineered landfill. Much of the sludge is openly dumped, which leads to soil, surface water and groundwater contamination. The inorganic salts and toxic metals in the sludge pose a threat to residents.

Synthetic dyes have a variety of uses and find applications in industries such as textile, paper printing, photography and petroleum. The largest group of synthetic colourants is the azo group of dyes which constitute almost a half of all known synthetic dyes. Azo dyes and their pigments are extremely versatile colourants and most of them are released into the environments during dyeing process. An estimated 10-15 % of dye is discharged or lost into the effluents during different dyeing processes [1,2]. They pose a serious threat to mankind due to their mutagenic, carcinogenic, and poisonous nature [3]. The dye industries effluents are more difficult to treat because of the high salinity and color and due to the presence of non-biodegradable organics [4]. The pollutants can be removed by treating the effluent with enzymes.

Laccase is one of the most actively investigated enzymes for the remediation of environmental pollutants. Laccase (p-diphenol: oxygen oxidoreductase, the most important number of lignolytic system, is multi copper enzyme belonging to the group of blue-copper proteins. Laccases are secreted by the family of fungi known as "white rots".

They are wood inhabiting fungi and are capable of efficiently degrading lignin [5]. White rot fungi such as *Bjerkandera adusta*, *Irpex lacteus*, *Plebia radiata*, *Pleurotus ostreatus*, *P. sajor-caju*, *Ganodema*

lucidum, *Pycnoporus cinnabarinus* and *Trametes versicolor* have been used for decomposition of several recalcitrant dyes [6-8].

The fungus, *Pleurotus ostreatus* produces both cellulolytic and lignolytic enzymes [9]. However, limited studies have been made on the physiology of lignocellulolytic enzymes and their application in bioremediation. *Pleurotus ostreatus* is a common edible mushroom. In the present study an attempt has been made to explore the potential decolourization of synthetic textile dyes by a white rot fungus *Pleurotus ostreatus* and attempts also have been made in remediation of dye and effluent.

Materials and Methods

Sample collection

Azo dyes: Six different textile azo dyes (Red F3B, T Blue G, Yellow F3R, Yellow mg 4G, Black B, Black GR) were collected from textile industry, Balu process group of units located at Thirupur.

Effluent: The textile industrial effluent was collected from Balu process group of units located at Thirupur and the pH and temperature of the effluent was estimated in the environment and also in lab and was found to be 5.7 and 45°C.

Organism and inoculum: The fungal culture *Pleurotus ostreatus* was obtained from Microbial collection centre, Thiagarajar college of Arts and science, Madurai, and was maintained in Potato Dextrose Agar medium (PDA) and used for the present investigation. A mycelial disc (6 mm) from 3-day-old culture of the fungus was grown on PDA plates and was used as inoculum for the experiments.

Screening of white rot fungus for laccase

Eight strains were selected for initial screening of laccase activity using plate assay such as ABTRI-1, CD-2, MDU-1, APK- 1, FLOWER, *Pleurotus ostreatus*, *Ganoderma lucidum*, *Pleurotus cystidiosus*.

Optimization of culture conditions for laccase production by *Pleurotus ostreatus*

The effect of pH, Carbon, Nitrogen, Concentration of copper sulphate on laccase production from *Pleurotus ostreatus* was studied.

Enzyme production

Enzyme was produced by Solid State Fermentation using wheat bran, rice bran, green gram husk as substrate. *Pleurotus ostreatus* was inoculated, filtered and centrifuged. The supernatant was collected and stored. This was used to estimate laccase.

Estimation of protein

The protein was determined according to the method of Bradford [10] for both the crude as well as partially purified enzyme.

Enzyme production in liquid state fermentation

The optimized media which showed higher laccase enzyme activity was taken and the various sources were amended like carbon source (Mannitol), Nitrogen source (Sodium nitrate), copper sulphate 0.012 g and the pH was adjusted to 6.0 and the media was incubated for 15 days. Then the enzyme activity was assayed and protein was estimated using BSA as standard.

Physiochemical properties of crude enzyme

pH optimum: For determination of pH stability of partially purified laccase enzyme from *Pleurotus ostreatus* using the substrates wheat bran, rice bran, green gram, the enzyme was incubated with different buffers of pH ranging from (pH 5.0; Sodium acetate buffer 200 mM); (pH 5.6 Sodium acetate buffer 200 mM); (pH 6.0, phosphate buffer 100 mM); (pH 6.5; Phosphate buffer 100 mM); (pH 7.0; Phosphate buffer 100 mM). After 30mins incubation the samples were assayed for enzyme activity under standard conditions.

Temperature optimum: To determine the temperature stability of partially purified laccase enzyme from *Pleurotus ostreatus* using the substrates wheat bran, rice bran, green gram, the enzyme was incubated with sodium acetate buffer of pH 5.4 was incubated with different temperature ranging from 45°C 50°C, 55°C, 60°C, 65°C, 70°C. After 15 mins incubation the samples were assayed for enzyme activity under standard condition.

Electrophoretic studies

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) SDS-Polyacrylamide gel electrophoresis was performed on slab gel with separating and stacking gels (10 and 5 % w/v) by the method of Laemmli [11].

Staining of separated proteins. At the end of electrophoresis, gel was removed and stained with silver staining method of Blum [12]. After staining, the gels were stored in 7 % (v/v) acetic acid.

Determination of molecular mass

The molecular mass of the purified laccase was determined on SDS-PAGE. After separation, the gels were stained with silver nitrate. Native polyacrylamide gel electrophoresis [13].

Polyacrylamide gel electrophoresis of the purified laccase was carried out to analyze the protein profile of the enzyme by using Bio-Rad Electrophoresis Apparatus, USA.

Application of laccase enzyme in textile industry

Dye decolorization on solid medium, liquid culture, effective pH and effective metal ions optimization in dye decolorizing process by enzyme immobilized technique was carried out.

The degree of decolorization was calculated using the formula [1]:

$$\frac{((\text{Initial absorbance} - \text{final absorbance}) / \text{initial absorbance}) \times 100}{[1]}$$

Results and Discussion

8 strains were selected for initial screening of laccase activity using plate assay such as ABTRI-1, CD-2, MDU-1, APK- 1, FLOWER, *Pleurotus ostreatus*, *Ganoderma lucidum*, *Pleurotus cystidiosus* 3 strains reported for maximal laccase activity from which *Pleurotus ostreatus* was selected with maximum 56mm zone using plate assay amended with Guaiacol.

Media optimization was carried out to ascertain the best carbon (sucrose, Mannitol, maltose, fructose, and xylose), nitrogen (sodium nitrate, ammonium chloride, ammonium sulphate, ammonium thiocyanate, di-ammonium tartrate), pH (5.0, 5.5, 6.0, 6.5, 7.0) and copper sulphate concentration (0.15 mM (0.003 g), 0.3 mM (0.006 g), 0.6 mM (0.012 g), 1.2 mM (0.024 g), 2.4 mM (0.048 g) sources. The studies revealed that Mannitol, sodium nitrate, pH 6, and 0.012 g were found to be the best carbon, nitrogen, pH and copper sulphate concentration sources, showing maximum Laccase activity of 0.017 U/mL (Figure 1, Table 1), 0.028 U/mL (Figure 2, Table 2), 0.027 U/mL (Figure 3, Table 3), and 0.019 U/mL (Figure 4, Table 4) respectively.

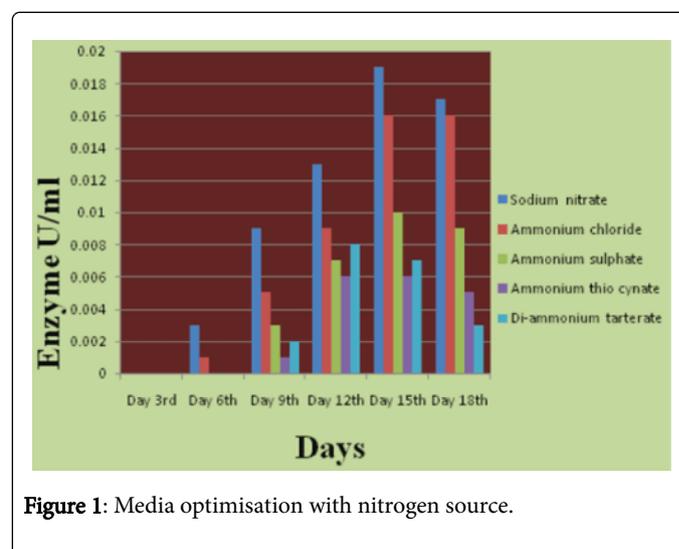


Figure 1: Media optimisation with nitrogen source.

Nitrogen Source	Day 3 (U/mL)	Day 6 (U/mL)	Day 9 (U/mL)	Day 12 (U/mL)	Day 15 (U/mL)	Day 18 (U/mL)
Sodium nitrate	Nil	0.003	0.009	0.013	0.019	0.017
Ammonium chloride	Nil	0.001	0.005	0.009	0.016	0.016
Ammonium sulphate	Nil	Nil	0.003	0.007	0.010	0.009
Ammonium thiocyanate	Nil	Nil	0.001	0.006	0.006	0.005
Di-ammonium tartrate	Nil	Nil	0.002	0.008	0.007	0.003

Table 1: Selection of suitable nitrogen source for maximum enzyme activity.

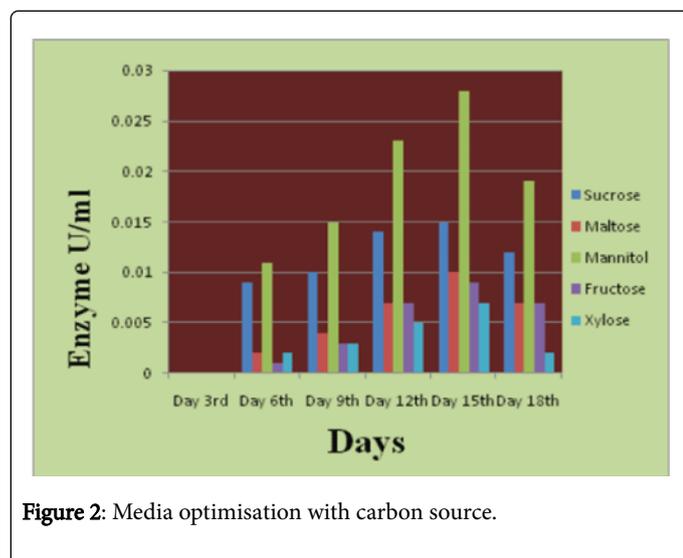


Figure 2: Media optimisation with carbon source.

Carbon Source	Day 3 (U/mL)	Day 6 (U/mL)	Day 9 (U/mL)	Day 12 (U/mL)	Day 15 (U/mL)	Day 18 (U/mL)
Sucrose	Nil	0.009	0.010	0.014	0.015	0.012
Maltose	Nil	0.002	0.004	0.007	0.010	0.007
Mannitol	Nil	0.011	0.015	0.023	0.028	0.019
Fructose	Nil	0.001	0.003	0.007	0.009	0.007
Xylose	Nil	0.002	0.003	0.005	0.007	0.002

Table 2: Selection of suitable carbon source for maximum enzyme activity.

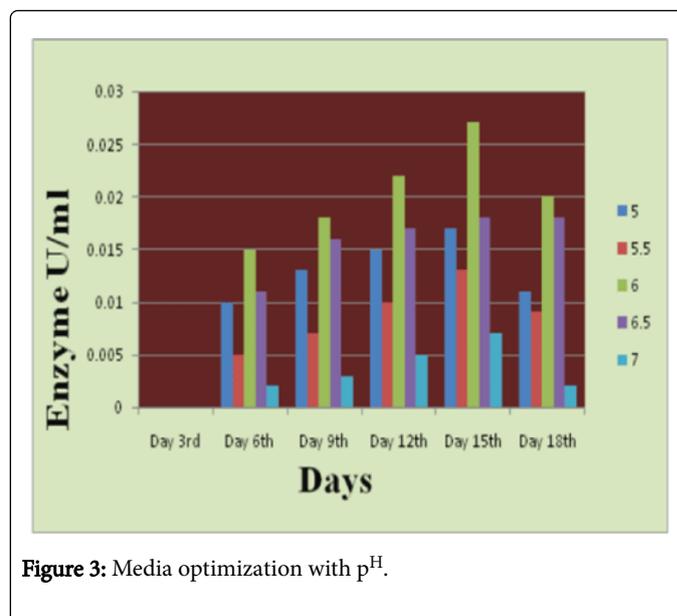


Figure 3: Media optimization with pH.

pH	Day 3 (U/mL)	Day 6 (U/mL)	Day 9 (U/mL)	Day 12 (U/mL)	Day 15 (U/mL)	Day 18 (U/mL)
5.0	Nil	0.010	0.012	0.015	0.017	0.011
5.5	Nil	0.005	0.007	0.010	0.013	0.009
6.0	Nil	0.015	0.018	0.022	0.027	0.020
6.5	Nil	0.005	0.006	0.008	0.009	0.007
7.0	Nil	0.002	0.003	0.005	0.007	0.002

Table 3: Selection of suitable pH for maximum enzyme activity.

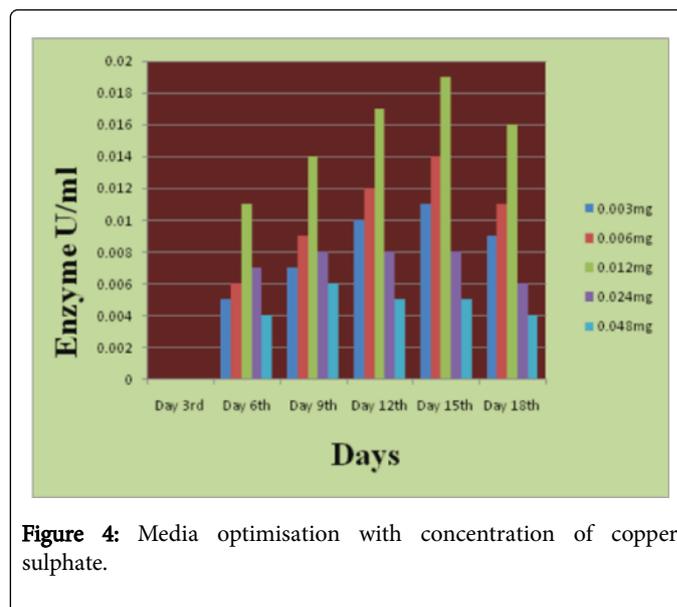


Figure 4: Media optimisation with concentration of copper sulphate.

Copper sulphate concentration (mg)	Day 3 (U/mL)	Day 6 (U/mL)	Day 9 (U/mL)	Day 12 (U/mL)	Day 15 (U/mL)	Day 18 (U/mL)
0.003	Nil	0.005	0.007	0.010	0.011	0.009
0.006	Nil	0.006	0.009	0.012	0.014	0.011
0.012	Nil	0.011	0.014	0.017	0.019	0.016
0.024	Nil	0.007	0.008	0.008	0.008	0.006
0.048	Nil	0.004	0.006	0.005	0.005	0.004

Table 4: Selection of suitable concentration of Copper sulphate for maximum enzyme activity.

Various types of oxidative enzymes are produced by white rot fungi in order to make use of lignocellulosic substrates for nutrition. SSF has been considered as an efficient method for enzyme production due to its potential advantages and high yield. The high level of laccase production could be attributed to the presence of ferulic acid in wheat bran. The results reported that 6th day sample of green gram showed maximum laccase activity whereas other agro wastes showed lesser activity. After harvesting and dialysis, the expression of enzymes was higher. The maximum laccase activity was reported in 12th day sample of wheat bran whereas other samples showed declining activity. The results revealed that there must be interfering compound in the crude form of enzyme from wheat bran that could have been inhibiting whereas after salting out and dialysis due to the absence of interfering compounds wheat bran showed maximum activity. The laccase examined in this study had a temperature optimum 50°C and was stable at higher temperatures than the laccase purified from mesophyllic fungi. So this laccase enzyme is thermo stable. It had an optimum pH at 5.6.

Estimation of protein according to the method of Bradford [10] and enzyme activity according to the method of Wolfenden and Wilson [14] was done. The enzyme was collected using ammonium sulphate precipitation. The dialyzed protein samples of Laccase enzyme from *Pleurotus ostreatus* were analysed on SDS-PAGE and stained with Silver Nitrate. Multiple bands were seen indicating the dialyzed samples were electrophoretically not homogenous. The activity of purified laccase with crude positive control was determined on NATIVE PAGE using guaiacol as substrate. The oxidized region appeared as a clear dark brown band (Figure 5, Table 5).

Lane	Sample Name	Protein Volume
L1	Wheat Bran 6th day	50 µL
L2	Wheat Bran 12th day	50 µL
L3	Rice Bran 6th day	50 µL
L4	Rice Bran 12th day	50 µL
L5	Green Gram 6th day	50 µL
L6	Green Gram 12th day	50 µL

Table 5: SDS page.

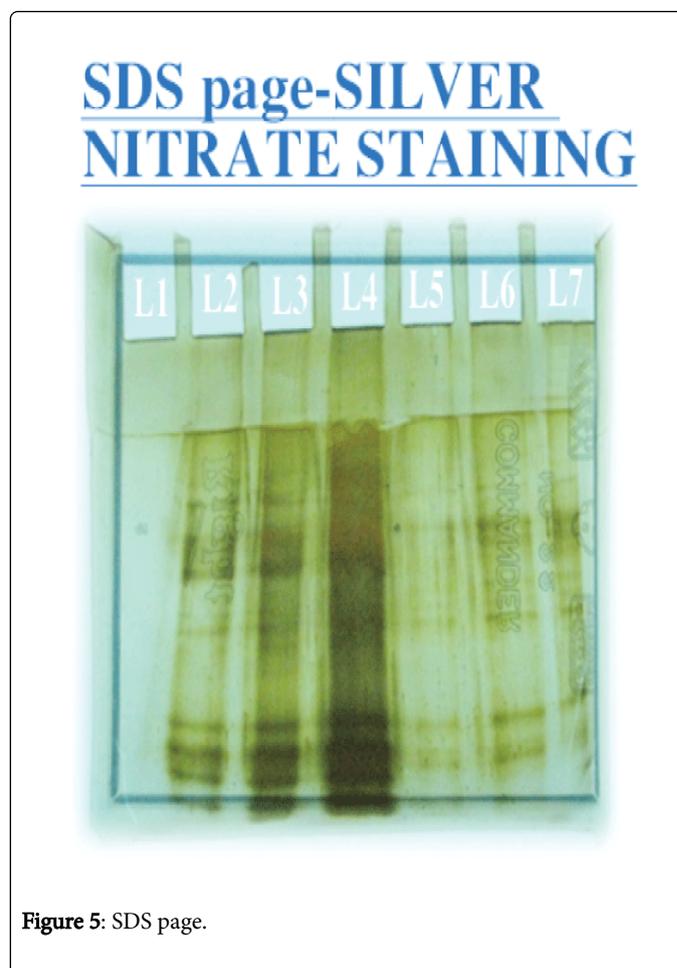


Figure 5: SDS page.

The release of dye based effluents into the water bodies is only a small proportion of water pollution, but as a result of their chromophoric groups, these dyes are visible even if they are present in small quantity. The decolorisation of these waste waters has acquired immense importance as they pose serious threat to mankind due to their mutagenic, carcinogenic and poisonous nature. There is no simple solution for this problem because the conventional physicochemical methods either are costly or are partially competent in treating these [15].

The treatment system based on fungi, especially the white rot, have not been applied extensively due to many factors such as high costs, longer fungal growth periods and an early inactivation of enzymes [16]. Further, the use of alternate biological means e.g. dead or living mycelia for dye absorption, itself face disposal problem [17]. The aim of this study is to ascertain the role of relatively little explored ligninolytic white rot fungi and their cell free enzyme extracts in bio cleaning of industrial dyes. The results obtained show the differential susceptibility of dyes to fungal decolourisation.

As a part of applications, the laccase system was applied for dye decolourisation studies. 6 industrial dyes (Red F3B, T Blue G, Yellow F3R, Yellow mg 4G, Black B, Black GR) were selected and studied. Out of these 6 dyes, Red F3B showed maximum decolourisation activity based on agar plate assay amended with dyes. Liquid broth media amended with dyes (100 ppm) were inoculated with mycelia disc and used to study the decolourisation pattern of 6 industrial dyes. The Red F3B showed maximum decolourisation activity which is reflected from the spectrophotometric studies.

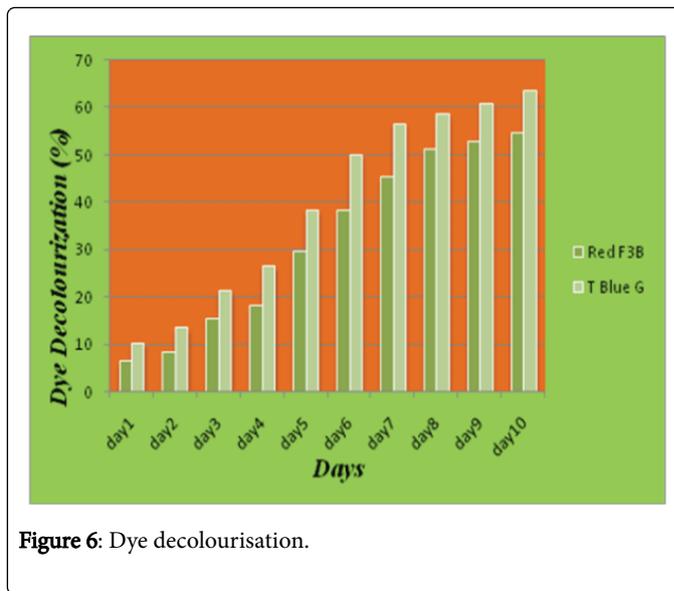


Figure 6: Dye decolourisation.

S.no	Name of the dye	Percentage of Dye Decolourisation for 10 days									
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
1	Red F3B	6.37	8.21	15.31	17.98	30	38.01	45.08	51.09	52.56	44.78
2	T Blue G	10.19	13.34	21.27	26.45	38	49.76	56.23	58.34	60.67	63.34

Table 6: Dye decolourisation.

2 dyes Red F3B, T Blue G were used to study the immobilization of laccase (Figure 6, Table 6), 2 parameters such as Calcium, Zinc metal ions and 3 different pH 4.5, 5.5 and 6.5 were chosen for the optimal metal ion and pH optimization studies. The optimal metal ion and pH for dye decolourisation using immobilized system was found to be calcium metal ion and pH 5.5. The calcium alginate at pH 5.5 showed maximum activity and stability during the course of experiment. The above 2 dyes were used to study the pH optimization in liquid broth medium with 3 different pH 4.5, 5.5 and 6.5. The results revealed that pH 5.5 is optimum in dye decolourisation. The effluent treated by crab shell chitin spectrophotometrically showed that the effluent was degraded.

The growth of plant on the treated sludge suggests that sludge after treatment could be used for agricultural purposes. The use of sludge in this application could serve as an alternative solution to disposal.

Conclusion

The utilization of bioremediation techniques in the degradation of industrial pollutants is a research field of high focus. Particularly, laccase enzymes play a vital role in biodegradation of toxic substances. Eight fungal strains were isolated. Isolated fungal strains were screened for its degradation activity of dyes. All the 8 fungal isolates were subjected to dye degradation study with Red F3B and T Blue G. Among the 8 fungal isolates, *Pleurotus ostreatus* showed high degradation on Red F3B and T Blue G. To conclude, the selected strains appear to be an attractive option for the treatment of industrial effluents contaminated with dye. *Pleurotus ostreatus* can be used for

the treatment of effluents and can be performed in low cost at the industrial site as compared to the anaerobic treatment which requires large input.

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