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Effectiveness of application of lignolytic fungal strains, *Cladosporium uredinicola* GRDBF21 and *Bipolaris maydis* GRDBF23 in the treatment of tannery effluent

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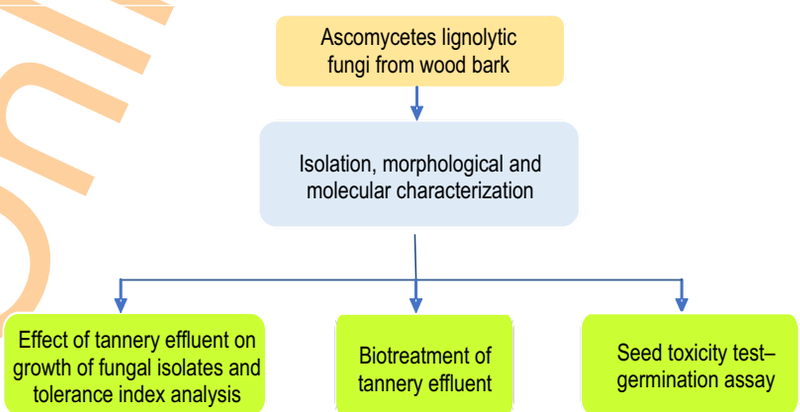
Abstract

Aim : This study aims to investigate the ability of laccase producing fungal strains *Cladosporium uredinicola* GRDBF21 and *Bipolaris maydis* GRDBF23 isolated from decaying wood bark in decolouration and detoxification of tannery effluent.

Methodology : Fungal strains from decaying wood bark samples were isolated by serial dilution technique followed by single spore isolation method. The selected fungal isolates were investigated for their laccase enzyme production. Their effect on physio-chemical properties of tannery effluent collected from final effluent drainage of a leather-tanning factory in Chrompet, Chennai, Tamil Nadu, India was analysed. Toxicity of treated and untreated tannery effluent was analysed by seed germination test.

Results : The lignolytic and constitutive producers of laccase enzyme, *C. uredinicola* GRDBF21 and *B. maydis* GRDBF23 exhibited a tolerance index of 1.2 and 1.5, respectively, at 60% effluent concentration. The isolates were able to increase pH and reduce colour, turbidity, total suspended solids and electrical conductivity of the effluent. Besides observing a decrease in the BOD and COD levels, there was also a reduction in the sodium and hexavalent chromium content. *C. uredinicola* GRDBF21 and *B. maydis* GRDBF23 treated effluent showed a seed germination percentage of 66.6% and 76.6%, respectively. The untreated effluent completely inhibited the seed germination.



Interpretation : The study confirms that the fungal species *C. uredinicola* GRDBF21 and *B. maydis* GRDBF23 could be effectively used in decolouration and detoxification of tannery effluent.

Key words: Ascomycetes fungi, Bioremediation, Laccase, Lignolytic enzymes, Tannery effluent

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Introduction

Tannery industries hold a major sector in the economy of many countries and are considered as an inevitable part of leather manufacturing (Hasegawa *et al.,* 2011). Tanning process converts the animal skin into useful leather material by altering the protein of rawhide into a flexible and stable material, which will not putrefy and is suitable for a wide variety of end applications. This complex process uses chemicals such as chromium, sulfides, ammonium, acids and different types of organic and inorganic ingredients. Majority of chemical compounds used for tanning, post tanning and dyeing process are toxic and recalcitrant compounds (Jnr and Spiff, 2005; Kongjao *et al.,* 2008; Abdallh *et al*., 2016; Chowdhury *et al*., 2015)).

Physico-chemical methods like recycling, coagulation, membrane filtration, reverse osmosis and adsorption are used for the treatment and detoxification of tannery effluents. These methods are not considered as a sustainable remedy for the effluent pollution problem, because of the transfer of contaminants takes place from one phase to another (Hasegawa *et al.,* 2011). Bioremediation of toxic industrial effluents using microorganisms is considered as an efficient method to replace the conventional treatment methods. Ascomycetes are efficient in degrading a large variety of dyes, phenolic and non-phenolic compounds, demonstrating a high potential for environmental bioremediation. Many fungi have the ability to biosorb and bioaccumulate metals and are involved in the reduction of hexavalent chromium to trivalent chromium (Arévalo-Rangel *et al.,* 2013). Fungal/microbial treatment methods for decolourisation and detoxification of industrial effluents have been reported earlier (Sharma and Malaviya, 2014; Choi *et al*., 2014; Hossain and Ismail, 2015; Hossain *et al*., 2016). Lignolytic fungi produce a special group of enzymes such as lignin peroxidase, manganese peroxidase and laccases, which are involved in the degradation and break down of complex aromatic polymers such as lignin and structurally complex toxic environmental pollutants (Bonugli-Santos *et al.,* 2012). Ascomycetes are known producers of laccase enzyme (Brijwani, 2010). Lignolytic fungi and their enzymatic system are involved in the degradation of xenobiotic compounds, dyes and treatment of various industrial effluents (Ravikumar *et al.,* 2011; Hasegawa *et al.,* 2011). Laccase enzyme is being widely used for the degradation and removal of various aromatic compounds and pollutants in industrial waste, contaminated water and soil (Madhavi and Lele, 2009). Several studies have reported the potential use of lignolytic fungi for bioremediation of various industrial effluents like tannery, textile, pulp and paper industry (Gómez-Bertel *et al.,* 2008; Anastasi *et al.,* 2010; Chopra and Singh, 2012; Kunjadia *et al.,* 2016). Against this background, the present study was undertaken to analyze the effect of laccase producing fungi isolated from tree barks for decolouration and detoxification of tannery effluent.

Materials and Methods

Sampling and isolation of fungi : Eighteen bark samples from trees and decaying woods of Painavu forest (9.7824° N, 76.9643° E) in Kerala were collected. One cm cubic sections of these barks were collected in sterile polythene and were transferred to laboratory. The collected bark samples were incubated in 100 ml sterile Sabouraud dextrose broth at 25°C for 7 days. The experiments were carried out in duplicates. Subsequently, the broth was serially diluted and plated on Sabouraud dextrose agar and incubated at 25°C for 5-7 days. Further, purification of isolates was done by single spore culture method (Ko *et al.,* 2001). The fungal isolates were stored as a small agar block from the pure culture in sterile 0.85% saline solution at 27°C. The axenic cultures were grown in malt extract agar plates until profuse sporulation was obtained and mycelial discs from these plates were used as inoculum for further studies.

Laccase screening : The fungal isolates were grown on laccase detection medium containing 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) at 28°C for 3-5 days, and the plates were observed for dark green colouration around the colony which suggested laccase enzyme production (Jin *et al.,* 2012). For the assay of laccase enzyme, fungal agar disc of 5 cm was placed in modified Tien and Kirk's broth medium (Mabrouk *et al.,* 2010). After 15 days of incubation at 28°C, 5 ml of broth was centrifuged (Plastocrafts, Model superspin R-V/FM) at 4°C for 10 min at 10,000 rpm. The supernatant was collected and used as enzyme source for the assay procedures (Bourbonnais *et al.,* 1995). One unit of laccase activity was defined as activity of an enzyme that catalyzes the conversion of one mole of ABTS (E_{420} = 36,000 M^{-1} cm^{-1}) min^{-1}.

Identification of fungal isolates : The test mycelial discs (1cm) were inoculated at the center of SDA and incubated at 28°C for 5–7 days. After incubation, the appearance, texture and colour of the colonies were observed. The microscopic observation of fungal isolates was done after lacto-phenol cotton blue staining.

The DNA extraction from fungal cultures were carried out using NucleoSpin® Plant II genomic kit following manufacturer's instructions and were assessed for their purity on an agarose gel before they were subjected to amplification by polymerase chain reaction. The 28S ribosomal RNA gene (partial sequence) incorporating D1/D2/D3 regions of the fungal DNA samples were amplified using the following LSU primer sets: Forward primer: UL18F: 5' - TGTACACACCGCCCGTC- 3'; Reverse primer: UL28R: 5'- ATCGCCAGTTCTGCTTAC -3'. Sequencing of LSU 28S rRNA gene was performed commercially at ProGen Life Science Solutions (Salem, Tamil Nadu, India). For the analysis of sequenograms, the following software was used: Chromas Lite (Technelysium Pty. Ltd.), BioEdit, ClustalX 2.0.11 and the findings were critically evaluated (Hall, 1999; Higgins *et al.,* 1996). DNA sequences representing LSU 28S ribosomal RNA gene was compared with similar sequences available in the National Centre for Biotechnology Information (NCBI) and was submitted to GenBank.

Characterization of tannery effluent : Tannery effluent samples were collected in sterile plastic containers from the final effluent drainage of a leather tanning industry located at Chrompet,

Chennai, Tamil Nadu, India. The samples were transferred to laboratory within 24 hours for further processing. The effluent samples were stored at 4°C until further use. For broth studies, 250 ml of sterile tannery effluent (100%) amended with 2% (w/v) malt extract was inoculated with 5 cm mycelial disc of test fungal isolates and incubated at 28°C for 10 days on a rotary orbital shaker (REMI, Model RIS-24). The analysis of physico-chemical characteristics, such as pH, colour, chemical oxygen demand (COD), biochemical oxygen demand (BOD), total suspended solids (TSS), electrical conductivity (EC), levels of hexavalent chromium and sodium of biologically treated and untreated tannery effluent were performed using the standard methods of APHA (2012). The pH was determined using digital pH meter (EUTECH pH 510). Colour was checked using spectrophotometer (Merck, Spectroquant Pharo 100). Turbidity was measured in a turbidimeter (Merck, Model Pharo100). COD and BOD were determined by open reflux titrimetric method and Winkler's iodometric method, respectively. TSS was determined by filtration method. Hexavalent chromium and sodium concentration were determined by atomic absorption spectrometry (Shimadzu, Model AA-7000) and flame photometery (ELICO CL378 flame photometer), respectively.

To analyze the tolerance of fungal isolates towards tannery effluent, radial growth of fungi was estimated on MEA amended with 0%, 30%, 60% and 100% of tannery effluent and incubated at 28°C for 8 days. After incubation, mean radial growth diameter (cm) was measured and mean tolerance index (TI) was calculated. Seed germination test was performed for toxicity testing of treated and untreated tannery effluent (Ahsan *et al.,* 2007). Sterile Whatman No.1 filter paper was placed in the Petri dishes and was appropriately moistened with 10 ml of treated and untreated tannery effluent. Filter paper moistened with tap water served as control. Mung bean (*Vigna radiata*) seeds were surface sterilized with 8% sodium hypochlorite (NaClO) for 10 min and washed three times with sterile distilled water. A total of 10 seeds were placed in the moistened filter paper and plates were incubated at 27°C in dark with 50% air humidity. Each set of experiment was conducted in triplicate and the mean value was taken. Visible protrusion of radicle from seed coat was considered as criterion of seed germination and mean germination rate was calculated.

Results and Discussion

A total of 28 fungal isolates, *i.e., Aspergillus* spp. (n=6), *Fusarium* spp. (n=8), *Rhizopus* spp. (n=5), *Cladosporium* spp. (n=4), *Bipolaris* spp. (n=3) and *Alternaria* spp. (n=2) were isolated from bark samples. Among these isolates, only two were positive for laccase production and they were identified as *Cladosporium* sp. and *Bipolaris* sp. based on the colony morphology and microscopic characteristics. Laccase assay revealed that these isolates, *i.e., Cladosporium* sp. GRDBF21 and *Bipolaris* sp. GRDBF23 had the mean laccase activity of 193.75 UI^{-1} (11^{th} day of incubation) and 334.0 UI^{-1} (9^{th} day of incubation), respectively. Upon molecular characterization by the sequencing of LSU 28S ribosomal RNA gene, these isolates were identified as *Cladosporium uredinicola* GRDBF21 (GenBank accession number: KJ913698) and *Bipolaris maydis* GRDBF23 (GenBank accession number: KJ913699). *C. uredinicola* GRDBF21 and *B. maydis* GRDBF23 belong to the Phylum Ascomycota which comprise of members with effective lignolytic activity. Tapia-Tussell *et al.* (2011) reported that *Bipolaris* spp. isolated from wood decay was able to exhibit only 7.50 UI^{-1} of laccase activity. Laccase producing *C. cladosporioides* was reported with a maximum laccase activity of 190 U ml^{-1} (Aslam *et al.,* 2012). Jin *et al.* (2012) stated that *C. cladosporioides* isolated from soil was able to show a laccase activity of 241 U l^{-1}. Laccase producing psychrotolerant *C. tenuissimum* from cold desert in Indian Himalayas has also been reported with a laccase activity of 15.10 U l^{-1} (Dhakar and Pandey, 2016).

The study revealed that the colour of untreated effluent was black and the odour was much offensive. But after the treatment with the test fungal isolates, the colour of the effluent diminished to light yellow and also the effluent reached to an odourless state. The colour intensity of *C. uredinicola* GRDBF21 and *B. maydis* GRDBF23 treated effluents dropped to less than 8000 and 5000, respectively, against untreated effluent colour intensity of 15225 (Table1). This may be attributed to higher production of laccase enzyme by the isolate. A white rot fungus, *Trametes versicolor* was able to remove 86-89% of tannery dye, black dycem (Baccar *et al.,* 2011). Various lignolytic and other

Table 1 : Physico-chemical characteristics of untreated and treated effluent from leather tanning industry in Chennai

Parameters	Untreated effluent$^{\Phi}$	Treated effluent$^{\Phi}$	
		***Cladosporium uredinicola* GRDBF21**	***Bipolaris maydis* GRDBF23**
pH	4.45	6.10	6.02
Colour	15225	<8000	<5000
Turbidity	6750	<10	<15
BOD (mg l^{-1})	3120	2950	3100
COD (mg l^{-1})	11180	11100	10100
Total suspended solids (mg l^{-1})	4232	113	104
Electrical conductivity (µmhos cm^{-1})	19000	12620	12410
Hexavalent chromium (mg l^{-1})	0.28	0.011	0.022
Sodium (mg l^{-1})	3465	1235	1432

$^{\Phi}$ Values are mean of duplicate determinations

Table 2 : Effect of tannery effluent from leather tannery in Chennai on the growth of fungal isolates by plate assay

Fungal isolates	Effluent concentration (%)	Mean€ radial growth diameter (cm)			Tolerance index
		Day 2	Day 6	Day 8	
Cladosporium uredinicola GRDBF21	0	0.5	1.8	2.5	--
	30	0.8	2.4	2.8	1.12
	60	1	2.6	3	1.2
	100	0.8	2	2.5	1
Bipolaris maydis GRDBF23	0	0.8	2.3	2.8	--
	30	0.5	2.7	3.8	1.35
	60	1.3	3.3	4.2	1.5
	100	0.9	2.5	3.2	1.14

€Values are mean of triplicate determinations

fungal species *i.e., Phanerochaete* sp., *Trametes* sp., *Cladosporium* sp., *Pleurotus* sp., *Aspergillus* sp., and *Ganoderma* sp., have shown their ability to remove colour from various industrial and wastewater effluents (Srikanlayanukul *et al.,* 2006; Tehrani *et al.,* 2015; Ravikumar *et al.,* 2011).

The pH of untreated tannery effluent was found to be 4.45. Discharge of untreated tannery effluents with low pH to nearby water bodies and soil may be detrimental to aquatic and soil system. *C. uredinicola* GRDBF21 and *B. maydis* GRDBF23 effectively increased the pH of tannery effluent to 6.10 and 6.02, respectively, from an acidic to almost neutral pH range (Table 1). Turbidity of untreated effluent was 6750. After treatment with *C. uredinicola* GRDBF21 and *B. maydis* GRDBF23, the turbidity was reduced to <10 and <15 NTU, respectively. This indicates that the lignolytic fungi grown in tannery effluent had either used or degraded the molecules causing turbidity. Similar to the present study, Sharma and Malaviya (2016) stated that *Aspergillus flavus* isolated from a tannery container exhibited 98.02% reduction in the turbidity of treated tannery effluent.

Tannery wastewater with high BOD and COD is characterized by substantial organic, inorganic matter content and refers to high oxygen demand through biological organisms and chemicals, and thus treatment of tannery effluent poses a major challenge. BOD and COD level of untreated effluent was 3120 mg l^{-1} and 11180 mg l^{-1}, respectively. Treatment with *C. uredinicola* GRDBF21 and *B. maydis* GRDBF23 brought down the BOD level to 2950 and 3100 mg l^{-1} and COD level to 11100 mg l^{-1} and 10100 mg l^{-1}, respectively. In a study, *Botryosphaeria rhodina* use for treating undiluted crude tannery effluent with high BOD and COD levels couldn't support the fungal growth and was not able to give satisfactory results in the reduction of BOD and COD level (Hasegawa *et al.,* 2011).

TSS level of tannery effluent was 4232 mg l^{-1}, but after treatment with *C. uredinicola* GRDBF21 and *B. maydis* GRDBF23 it was reduced to 113 mg l^{-1} (% reduction-97.32%) and 104 mg l^{-1} (% reduction-97.54%), respectively. These solid impurities may cause turbidity in the receiving water bodies and lead to poor penetration of light in aquatic system. *Trametes hirsuta,* a white

Table 3 : Effect of untreated and treated effluent from leather tanning industry in Chennai on mung bean (*Vigna radiata*) seed germination

Treatment of seeds	No. of seeds germinated*	Mean germination rate (%)
Tap water	9.66±0.577	96.6
Untreated tannery effluent	0	0
Cladosporium uredinicola GRDBF21 treated tannery effluent	6.66±0.577	66.6
Bipolaris maydis GRDBF23 treated tannery effluent	7.66±0.577	76.6

*Values are mean ± SD of triplicate determinations

rot fungus isolated from decaying wood samples was able to reduce the suspended solids in the industrial effluent and tannery effluent by 41% and 68%, respectively (Bisht and Harsh, 2014). Reduction in TSS and turbidity is ascribed to entrapment of suspended solid particles by the filamentous fungi (Fakhru'l-Razi and Molla, 2007). The untreated effluent also showed a higher level of electrical conductivity of 19000 µmhos cm^{-1}, which reflects high concentration of ions, salts and organic and inorganic substances. The electrical conductivity of the effluent decreased to 12620 µmhos cm^{-1} (% reduction – 33.5) and 12410 µmhos cm^{-1} (% reduction – 34.68) after the treatment with *C. uredinicola* GRDBF21 and *B. maydis* GRDBF23, respectively. Results show that the performance of both fungi in the reduction of electrical conductivity was almost similar.

The concentration of hexavalent chromium (Cr^{6+}) in tannery effluent was 0.28 mg l^{-1} and was reduced to 0.11 mg l^{-1} and 0.22 mg l^{-1}, after treating with *C. uredinicola* GRDBF21 and *B. maydis* GRDBF23, respectively. The results suggest that both the isolates efficiently removed hexavalent chromium (Cr^{6+}) from tannery effluent. Toxic effects and penetration rate of hexavalent chromium to biological membranes are higher when compared to trivalent chromium (El-Kassas and El-Taher, 2009). Many researchers have reported that acidic pH supports the reduction of hexavalent chromium (Cr^{6+}) effectively (Gochev *et al.,* 2010; Shoaib *et al.,* 2013; Saranraj and Sujitha, 2013). These reports justify the findings of the present study using fungi for treating tannery effluent with acidic pH. Microbes assisted technologies provide an alternative or addition to conventional methods of metal removal (Saranraj and Sujitha, 2013). Similar to the present study, the cellular biomass of *C. neoformans* was also able to remove 98% of chromium (Acosta *et al.,* 2004; Ahluwalia, 2014). It was found that *Aspergillus oryzae* was able to grow in a chromium concentration of 120-1080 mg l^{-1} and also removed 94% of chromium from tannery effluent at 3.3 pH (Igwe and Abia, 2006). In a shake flask culture study, *Aspergillus niger* and *Fusarium chlamydosporium* reduced the chromium concentration by 70 and 64.68% at pH 6 and 5.3, respectively (Srivastava and Thakur, 2006; Sharma and Malaviya, 2014). The living mycelium of *Phanerochaete chrysosporium,* a widely used lignolytic fungi for bioremediation purposes, has been reported reduce hexavalent chromium in artificial wastewater (Nikazar *et al.,* 2008).

The concentration of sodium in tannery effluent before treatment was 3465 mg l^{-1} and after treatment with *C. uredinicola* and *B. maydis* reduced to 1235 mg l^{-1} (% reduction-64.36) and 1432 mg l^{-1} (% reduction-58.68). Two different studies using *Fusarium chlamydosporium* and *Aspergillus flavus* for the treatment of tannery effluent showed 11.69% and 32.79% reduction in sodium level, respectively (Sharma and Malaviya, 2014, 2016). The tolerance index (TI) of test fungal isolates showed that *C. uredinicola* GRDBF21 and *B. maydis* GRDBF23 exhibited a maximum TI of 1.2 and 1.5 TI at 60% effluent concentration (Table 2). Tolerance index analysis indicates the growth potential and resistance of fungal isolates at high effluent concentration.

The effluent toxicity test showed that the untreated tannery effluent completely inhibited the process of germination in mung bean seeds. Germination of mung bean seeds treated with tap water as control showed a mean germination rate of 96.6% whereas *C. uredinicola* GRDBF21 and *B. maydis* GRDBF23 treated effluents exhibited a mean germination rate of 66.6% and 73.3%, respectively (Table 3). Seed germination was completely inhibited in all the mung bean (*Vigna radiata*) seeds treated with 100% untreated tannery effluent and none of the green gram seeds germinated even after incubation period. This may be due to the toxicity caused by high BOD, COD, TSS, EC, chromium and acidic nature of the effluent. It is also reported that inhibition of seed germination at higher effluent concentration may be due to high level of solids which enhances salinity and conductivity of solute absorbed by seeds (Sundaramoorthy and Kunjithapatham, 2000). Previous study using *Bjerkandera adusta* for the reduction of acute toxicity of wastewater by decolourisation is also reported (Choi *et al.,* 2014).

Sharma and Malaviya (2018) reported that an indigenous fungal isolate, *Trichoderma viride* SPFT1 involved in the decolourisation and detoxification of tannery effluent and the seed germination test exhibited a considerable reduction in the toxicity of fungal treated tannery effluent. According to Noorjahan and Siddiqui (2017), the tannery effluent treated with *A. niger* and *Rhizopus* sp. reflected in the increase in seed germination rate. Similar to the present study, Mehta and Bhardwaj (2012) demonstrated the toxic effects of treated and untreated industrial effluents on the seed germination of *Vigna radiata* (Mung bean) and reported that untreated effluent significantly inhibited the seed germination.

The results of the present study indicates that lignolytic fungal strains, *Cladosporium uredinicola* GRDBF21 and *Bipolaris maydis* GRDBF23 isolated from tree bark could be effectively used in decolouration and detoxification of tannery effluent.

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