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RESEARCH ARTICLE

KRAFT BLACK LIQUOR DECOLORIZATION BY FUNGI ISOLATED FROM CONTAMINATED PULP AND PAPER MILL SLUDGE

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ABSTRACT

Chemical Pulping process generates kraft black liquor which consists of highly alkaline solution. This study investigates the capability of three native fungi to decolorize kraft black liquor on solid and liquid medium under different concentrations. Qualitative assessment of fungal decolorization was observed by plate assay method. Out of the three fungi studied, *Nigrospora sp*. showed maximum growth (1.2 cm day^{-1}) and decolorization halo (0.9 cm day^{-1}) in malt extract agar medium containing 10 % black liquor. On the other hand, relatively slow fungal growth was observed in 20 % black liquor agar medium. In liquid medium, decolorization of black liquor was mostly influenced by the concentration of black liquor and fungal strain. The highest decolorization (61%) and COD removal (58.7 %) was observed by *Nigrospora sp*. Furthermore, mixed fungal culture enhanced the efficiency of COD and color removal upto 71.5 % and 73 %, respectively. A positive correlation was observed between color and COD removal (r = 0.99). These results indicate that the fungal strain *Nigrospora* has huge potential for treatment of kraft black liquor.

INTRODUCTION

The pulp and paper industry is highly energy and water demanding industry, categorized as sixth largest water polluting sector in the world (Pokhrel and Viraraghavan, 2004). As compared to the other industries, the total fresh water consumption of this industry is high (150-200 m3 per ton of paper produced) (Yadav and Garg, 2011), which produces 150 m^3 of effluent (Pokhrel and Viraraghavan, 2004), containing lignin and its derivatives, released from delignification of wood pulp which is an essential process to recover the paper quality. Lignin and its derivatives are recalcitrant in nature (carbon-to-carbon biphenyl linkages), which impart color to the effluent (Ali and Sreekrishnan, 2001; Crooks and Sikes, 1990). The effluent generally called black liquor is highly alkaline and has high COD and BOD, pH, organic content and dark brown color (Deilek and Bese, 2001; El-Bestawy *et al*., 2008). Beside this, it also contains chlorinated lingo-sulphonic acids, chlorinated phenols, chlorinated resin acids and chlorinated hydrocarbons (Kumar and Chopra, 2011). The bleaching (Chlorobleaching) process of wood pulp generates highly colored and toxic wastewater (Catalkaya and Kargi, 2008), which generates chloro-organics. Chlorination of the residual lignin after pulping process results in formation of chlorophenolics (Savant *et al*., 2005), however, it depends on the nature of residual lignin after pulping and bleaching process (Voss *et al*., 1980). Being hydrophobic in nature these compounds get accumulated in aquatic organisms. The highly colored effluent inhibits the photosynthesis process in the receiving water bodies due to absorbance of sun light. This leads to the detrimental effects on the aquatic organisms (Makris and Banerjee, 2002; Akhan, 2008, Orrego *et al*., 2011).

Over the years, attempts have been adopted to remove the toxicity and dark color from the effluents by using physical, chemical and biological methods. Physical and chemical methods remove high molecular weight chlorinated lignin, color, toxicants, suspended solids and COD but BOD and low molecular weight compounds are not properly removed. Moreover, chemical oxidation/precipitation methods are dreary and provide additional environmental load (Chandra and Singh, 2012). Biological color removal has been considered as a cost effective approach which include fungi, bacteria and actinomycetes.

Many researchers have studied black liquor degradation using bacterial strains. These include Bacillus sp. (Mishra and Thakur, 2010), Pseudomonas plecoglossicida (Paliwal *et al*., 2014), Pseudomonas putida (Srivastava *et al*., 1995) and

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Aeromonas formicans, (Gupta *et al*., 2001). In addition to bacterial degradation, white-rot fungi have received widespread attention due to their potential ligninolytic enzymes. A variety of fungi (white-rot, soft-rot, and brown-rot) possess oxidative enzymes i.e., lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac), for degradation of recalcitrant compounds (Linger *et al*., 2014). One of the most studied fungi is Phanerochaete chrysosporium, has high potential to decolorize kraft pulp bleaching effluent and various dyes (Wu *et al*., 2005). Treatment of bleached kraft mill effluent (black liquor) with other fungi (individually and in consortia) has been well reported by many researchers (Da Re and Papinutti, 2011, Wu *et al*., 2005; Malaviya and Rathore, 2007). Decolorization using fungal consortium is an effective association of multiple group of fungi which establish strong enzyme system and has been used by many researchers for enhanced degradation (Arantes *et al*., 2007).

Many studies have been reported for black liquor biodegradation with fungi but less reported the effect of alkaline black liquor on fungal biomass and simultaneous color degradation. Therefore, the main objective of the study was to evaluate the potential of selected fungi to decolorize kraft black liquor in solid and liquid medium under varying black liquor concentrations and to assess their growth potential and enzyme activities.

MATERIALS AND METHODS

Kraft black liquor collection and dilution

Kraft black liquor generated from hardwood pulping was collected from wood based pulp and paper mill and stored in clean plastic containers at 4°C for further experimental purpose. The black liquor was highly alkaline solution and contained high pH, organic and inorganic solids. Black liquor sample was diluted with distilled water to obtain the required concentration (10 % and 20 %) prior to use.

Isolation of fungi and culture conditions

Soil samples from contaminated site of pulp and paper mill were taken as a source for isolation of fungi. The soil samples (1 g) were serially diluted upto 10^{-9} in sterilized distilled water and these diluted suspensions (10^{-2}, 10^{-4} and 10^{-6}) were spread over malt extract agar (MEA) medium supplemented with black liquor (1%). MEA plates were incubated for 7 days at 28ºC and growing colonies were purified by repeated plating. The purified cultures were maintained on commercial MEA slants and sub-cultured every month. The batch experiments were carried out in minimal-salt medium (MSM) containing (g L^{-1}) the following: $Na_2HPO_4.2H_2O$, 7.8; KH_2PO_4, 6.8; $MgSO_4$, 0.2; $NaNO_3$, 0.085; $NH_4(CH_3COO)_3Fe$, 0.01; Ca $(NO_3)_2.4H_2O$, 0.05, and trace element solution, 1 ml L^{-1}.

Ligninolytic enzyme assay

The clear zone producing fungi were screened for ligninolytic enzyme production using suitable chromophoric substrate. Lignin modifying enzyme (LME) basal media was used for this purpose. The composition is as follows (g L^{-1}- KH_2PO_4 1.0, Yeast Extract 0.01, $C_4H_{12}N_2O_6$ 0.5, $CuSO_4.5H_2O$ 0.001, $MgSO_4.7H_2O$ 0.5, $Fe_2(SO_4)_3$ 0.001, $CaCl_2.2H_2O$ 0.01, $MnSO_4.H_2O$ 0.001). For laccase and peroxidase enzyme LME media was supplemented with 0.005% w/v α-naphthol and 0.01% w/v Azure-B dye, respectively (Pointing, 1999). The active fungal colony (1 cm) was cut, inoculated in the agar plate and incubated at 28ºC. The halo around the fungal colony was observed which may vary depending on the enzyme and substrate reaction. All enzymatic test were performed in triplicates and ligninolytic enzymes producing fungi were further taken for decolorization study.

Analysis of color and COD

The initial concentration of color (Co-Pt) in wastewater was measured using the method proposed by Bajpai *et al* (1993). The measurement was carried out by using a UV–Vis spectrophotometer (Virion Bio50) at 280 nm. The COD was determined as per standard method of wastewater analysis (Greenberg *et al*., 1995).

Fungal growth and decolorization on solid agar medium

To assess the growth rate of fungi, the agar media was prepared using different concentrations (10 % and 20 %) of kraft black liquor diluted with distilled water. The media contained the following per litre- glucose 10 g and malt extract 13 g. highly alkaline pH of kraft black liquor is not suitable for fungal growth, as fungi prefer to grow at acidic pH. So final pH of the medium was adjusted to 5 using 1 N HCl to make it acidic and sterilized by autoclaving at 121ºC for 15 min. A control media was also taken without adding black liquor to it. The selected fungi were grown on agar plates supplemented with kraft black liquor at 30ºC. Daily measurement of mycelial growth and decolorization halo was recorded by measuring radial extension of fungal mycelia at regular intervals till mycelia covered the whole plate. The measurement (cm day^{-1}) denotes the mean of triplicate assays with a standard deviation of less than 5%. The growth percentage was calculated by measuring the growth disc diameter of each fungus, according the formula-

$$\% \text{ growth} = \left(\frac{c - s}{c}\right) \times 100\ \%$$

Where, ϕs denotes fungal diameter of black liquor treated sample (cm), ϕc denotes fungal diameter of control sample (cm). 2.6 Estimation of fungal potential (individual and mixed culture) for kraft black liquor treatment in liquid media

The screened fungal strains designated as coded as LDF4 (*Nigrospora sp*.), LDF21 (*Alternaria sp*.) and LDF5 (*Trametes sp*.) were investigated for their efficiency to decolorize kraft black liquor, individually, as well as in combination. For this, pre grown purified fungal strains were used as seed culture. For biodegradation study, the medium contained 10 % (v/v) kraft black liquor supplemented with MSM, 1 % dextrose and 0.5 % sodium nitrate as additional carbon and nitrogen source. PH of the culture medium was adjusted to 5.0 using 1 M HCl before autoclaving. Three treatments (R1, R2 and R3) were inoculated (1 % v/v) with individual seed culture (LDF4, LDF21 and

LDF5), respectively in separate Erlenmeyer flasks (250 ml) containing 100 ml of autoclaved medium incubated in shaker at 30°C temperature and 150 rpm. Another treatment designated as R4 was carried out with mixed fungal culture, by inoculated (1 % v/v) in an Erlenmeyer flask (250 ml) containing 100 ml of autoclaved black liquor medium, incubated under the same conditions. Control treatment experiments (R5) without fungal inoculum were also run in parallel. Samples were extracted from R1, R2, R3, R4 and R5 treatments at 1, 3, 6 and 9, 12 and 15th day of incubation period and analyzed for color, COD and fungal biomass. Fungal biomass in the treatment process was estimated using the dry weight method (Dimitrokallis *et al*., 2008).

Decolorization Measurement

All treated sample and control samples were measured for color degradation. The change in absorbance of black liquor was determined at 365 nm with a UV-Vis spectrophotometer (Optizen 2120 UV). The medium containing only black liquor was used as a control. The percentage of decolorization was calculated as follows:

$$\text{Decolorization \%} = \left(\frac{Cc-Cs}{Cc}\right) \times 100\ \%$$

Where, Cc is the initial effluent concentration (ppm) and Cs is the final effluent concentration (ppm).

Statistical Analysis

The experiments were run in triplicates and data were expressed as means ± standard error (S.E.). Data was statistically analyzed by analysis of variance (ANOVA), where the statistical significance of difference among various treatments was analyzed by using SPSS software and expressed at $p<0.05$.

Table 1 Ligninolytic enzyme production by fungal strains

Fungal Strain	Laccase (α-naphthol)	Peroxidase (Azure B)
Nigrospora sp.	+++	++++
Alternaria sp.	++	++++
LDFC1	+	+++
LDFD1	+	-
LDFD2	+	-
LDFD3	-	++
LDFD4	-	++
Trametes sp.	++++	-

Decolorization strength +, (25%); ++, (25>50%), +++, (>50%) denotes clear decolorization. - denotes no decolorization.

Table 2 Decolorization by selected fungi using two concentration of black liquor (10 and 20 %) in solid medium

Fungal strain	Medium	Dilution % (v/v)	Decolorization rate (cm day^{-1})
Nigrospora sp.	MG	10	0.97 (7)
		20	0.43 (9)
Trametes sp.	MG	10	0.65 (8)
		20	0.12 (12)
Alternaria sp.	MG	10	0.58 (9)
		20	0.21 (14)

Values are means of three experiments. Value in parenthesis denotes the day at which decolorization halo was observed; MEA malt extract–glucose medium

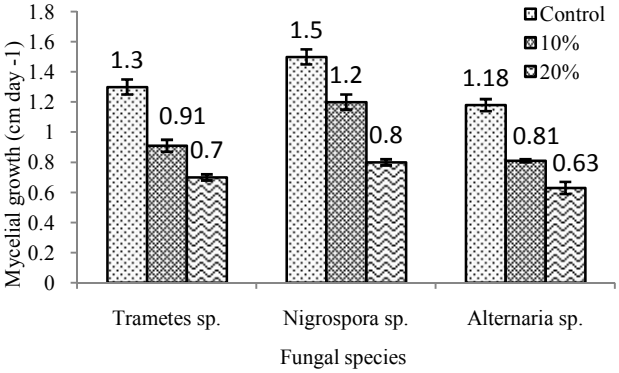

Figure 1 Fungal growth in different concentrations of kraft black liquor solid medium

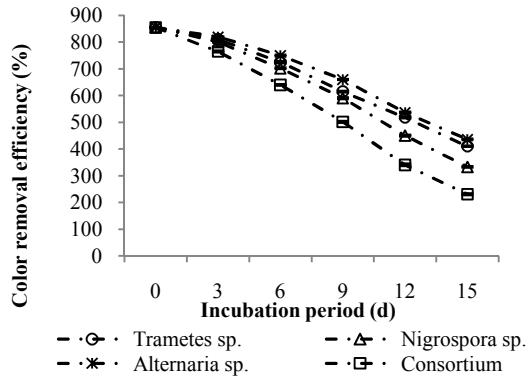

Figure 2a

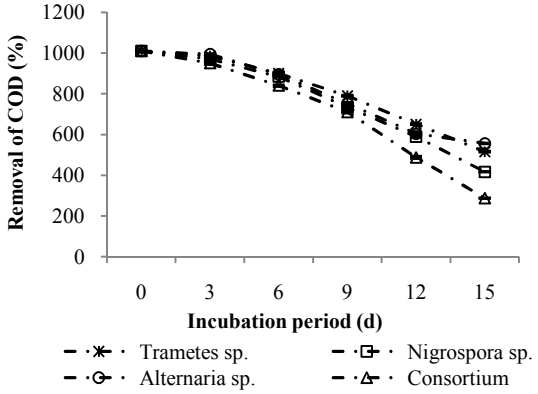

Figure 2b

Figure 2 (a) Percentage color removal of kraft black liquor by fungi (individual and mixed); (b) Percentage COD removal of kraft black liquor by fungi (individual and mixed)

RESULTS AND DISCUSSION

Ligninolytic Activity of Isolated Fungi

The ligninolytic activity of isolated fungi from pulp and paper mill sludge was assessed based on plate assay for laccase and peroxidase activity. Amongst the isolated fungal strains, LDFC1, LDFD1, LDFD2, LDFD3, LDFD4, only 3 fungi, *Nigrospora sp*., *Trametes sp*. and *Alternaria sp*. showed maximum activity of the enzymes after reacting with different substrate (Table 1). Peroxidase activity was confirmed on disappearance of blue color of LME basal media while appearance of dark violet color verified the activity of laccase. All the three fungi showed laccase activity which catalyse the oxidation of phenolic and non-phenolic compounds

(Krastanov, 2000; Bourbonnais and Paice, 1997) and has been reported in basidiomycetes, ascomycetes and deuteromycetes (Bourbonnais *et al*., 1995, Leontievsky *et al*., 1997). Peroxidase activity was shown by all the fungi except *Trametes sp*. Peroxidase enzyme oxidizes a variety of reducing substrates and has been reported in basidiomycetes and actinomycetes (Pointing *et al*., 2005; Niladevi and Prema, 2005).

Fungal growth and decolorization on kraft black liquor solid medium

The mycelial growth and halos of black liquor decolorization of selected fungi (*Nigrospora sp*., *Trametes sp*. and *Alternaria sp*.) on agar plates containing different concentration of black liquor is given in figure 1. The fast fungal growth was observed in control agar medium in which black liquor was not added, followed by 10 % concentration of black liquor, and least in 20 % concentration of black liquor. All the three fungi grew well in 10 % kraft black liquor plates, with *Nigrospora sp*. LDF00204 (LDF4) (1.2 cm day $^{-1}$) was the fastest, followed by *Trametes sp*. (LDF5) (0.91 cm day $^{-1}$) and *Alternaria sp*. (LDF21) (0.81 cm day $^{-1}$). In contrast, slow growth of *Nigrospora sp*., *Trametes sp*. and *Alternaria sp*. were observed on 20 % black liquor agar plates, which was 0.8, 0.7 and 0.6 cm day $^{-1}$ respectively. The fungal growth was comparatively slow in black liquor agar medium as compared to control medium during 15 days period. The results demonstrated that increase concentrations of black liquor hinder the fungal growth as it contains high content of sulphur, sodium, chlorine, carbon and low concentration of nitrogen (Cardodo *et al*., 2009).

These three fungal strains were able to grow in both the concentrations, although not all of them showed substantial decolorization (Table 2). Cultures on MG showed that *Nigrospora sp*., *Trametes sp*. and *Alternaria sp*. were able to decolorize the black liquor (10 %) rather than 20 % concentration, in which slow growth and decolorization was observed. Maximum decolorization zone was observed in *Nigrospora sp*., followed by *Trametes sp*. and *Alternaria sp*. which showed slower growth velocities. During decolorization experiments on plates a darker halo was observed, before decolorization occurred. This brown zone occurred due to reaction of laccase enzyme with lignosulfonates, which enabled the process of polymerization and depolymerization with simultaneous increase in darkening zone (Kim *et al*., 2009). The visible clear zone around the colony was observed after darkening zone, which suggests degradation of color producing compounds in black liquor due to ligninolytic enzymes. Fungal growth and enzyme production beyond 28°C has been reported earlier (˘Snajdr and Baldrian, 2007).

Comparison of three fungi for the removal of color and COD

The initial color and COD of kraft black liquor obtained from wood based pulp and paper mill was 855.06 mg L^{-1} and 1010.5 mg L^{-1}, respectively. The black liquor was diluted with distilled water to get a concentration of 10 % (v/v), resulting in 85.50 mg L^{-1} color and 101.05 mg L^{-1} COD for experimental study. As maximum fungal growth was observed in 10 % (v/v) kraft black liquor, this concentration was selected for decolorization in liquid medium by selected fungi. The color removal (%) was analyzed during 15 days incubation. Among the experiments, R1 treatment (*Nigrospora sp*.) demonstrated higher efficiency for color removal (61 %) than R2 (*Trametes sp*.) and R3 (*Alternaria sp*.) treatments, with removal efficiency 52 % and 49 % (Figure 2a), respectively, on day 15. For the first three days no color removal was observed as the fungal growth takes time to produce biomass than bacteria. An initial color removal was then observed from 4th day, which enhanced gradually and reached maximum on day 15 for all the three species. This result shows that there were slower changes in the removal of color with time which may be due to cell autolysis or the depletion of glucose in the culture (Frederick *et al*., 1991).

Effectual decline in COD was observed for *Nigrospora sp*., *Trametes sp*. and *Alternaria sp*. (Figure 2b). Maximum COD (58.7 %) was removed by *Nigrospora sp*. on 15th day incubation period, while for *Trametes sp*. and *Alternaria sp* it was 48% and 44 % on day 15. As demonstrated in Figure 2b, the COD removal showed a parallel trend to the decolorization. A positive correlation was observed between color and COD removal. Addition of glucose (1g L^{-1}) promoted the growth of white-rot fungi, as they needed an additional readily metabolizable carbon source for growth. Elisa *et al*. (1991) reported that the removal of color from kraft mill wastewater was significant only at an appropriate range of carbon source concentration. Excessive carbon source resulted in a decline in enzyme activity of lignin peroxidase, Mn peroxidase, laccase and b-glucosidase, and therefore reduced the removal efficiency of COD from effluent. Apart from this, in R4 treatment, which comprised of mixed culture of all three fungi, increased color and COD removal. The total color removal was 73% and COD, 71.5% respectively on day 15. This may be due to combination of fungal enzymes secreted by all three fungi in the medium. Lignin peroxidase, Mn peroxidase and laccase are common to many white rot basidiomycetes (Alessandro *et al*., 1999, 2000) which are able to remove lignin and color. For all three fungi, COD removal coincided with color removal and a positive correlation ($r = 0.99$, $p < 0.05$) was observed between color and COD removal.

CONCLUSION

Present investigation clearly reveals the potential of native soil fungi isolated from paper mill sludge to grow in highly alkaline kraft black liquor of pulp and paper mill. Out of all selected fungi, *Nigrospora sp*. showed fast growth under different concentration of black liquor and effective removal of color and COD. These findings suggest the potential of selected fungi to decolorize the wastewater and other industrial effluent even under extreme alkaline conditions.

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conflict of interest.

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