Bacterial degradation of synthetic and kraft lignin by axenic and mixed culture and their metabolic products

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Abstract

Pulp paper mill effluent has high pollution load due to presence of lignin and its derivatives as major colouring and polluting constituents. In this study, two lignin degrading bacteria IITRL1 and IITRSU7 were isolated and identified as Citrobacter freundii (FJS81026) and Citrobacter sp. (FJS81023), respectively. In degradation study by axenic and mixed culture, mixed bacterial culture was found more effective compared to axenic culture as it decolourized 85 and 62% of synthetic and kraft lignin whereas in axenic conditions, bacterium IITRL1 and IITRSU7 decolourized 61 and 64% synthetic and 49 and 54% kraft lignin, respectively. Further, the mixed bacterial culture also showed the removal of 71, 58% TOC; 78, 53% AOX; 70, 58% COD and 74, 58% lignin from synthetic and kraft lignin, respectively. The ligninolytic enzyme was characterized as manganese peroxidase by SDS-PAGE yielding a single band of 43 KDa. The HPLC analysis of degraded samples showed reduction as well as shifting of peaks compared to control indicating the degradation as well as transformation of compounds. Further, in GC-MS analysis of synthetic and kraft lignin degraded samples, hexadecanoic acid was found as recalcitrant compounds while 2,4,6-trichloro-phenol, 2,3,4,5-tetrachloro-phenol and pentachloro-phenol were detected as new metabolites.

Key words
Bacteria, Degradation, Lignin, Manganese peroxidase, Metabolite

Introduction

Lignin is a complex biopolymer, which is of plant origin and forms the integral part of secondary wall of plants and one of the most abundant organic polymers on earth. There are different types of lignins, the properties and composition of which depend upon the source and method of isolation. Moreover, kraft lignin (KL) is a waste byproduct formed during alkaline sulfide treatment of lignocellulose in pulp and paper industries. It differs from synthetic lignin as it undergoes a variety of reactions including aryl-alkyl cleavages, strong modification of side chains, and various ill-defined condensation reactions causing the polymer to fragment into smaller water/alkali-soluble fragments (Chakar and Ragauskas, 2004). The kraft lignin containing effluents discharged from pulp and paper industries cause serious environmental pollution along with aesthetically unacceptable intense colouring of soil and water bodies, which block the passage of sun light to the lower depths of aquatic system resulting in cessation of photosynthesis, leading to anaerobic conditions, which in turn result in the death of aquatic life causing foul smell and toxicity of waters bodies (Ali and Sreekrishnan, 2001).

Hence, before final disposal of wastewater from pulp and paper mills into environment, adequate effluent treatment is required to reduce the pollution parameters within the safe limit and also for environmental safety. Though, various physico-chemical treatment methods such as electro-coagulation, ultrasound, acid precipitation and ozonation have been reported for the degradation and detoxification of pulp and paper mill wastewater (Ugurlu et al., 2007; Pokhrel and Viraraghavan, 2004). But, these methods could not be implemented specially in small scale industries due to high installation and operational cost as well as generation of huge amount of sludge and secondary...
pollutant (Xiong et al., 2007). However, biological treatment methods are another practical choice for the treatment of pulp and paper mill effluent and various micro-organisms have been also reported for its treatment. Among biological treatment methods tried so far, most of the literature is confined to a few genera of white rot fungi because of their powerful enzymatic system (Hammel and Cullen, 2008). But, the stability of fungi is not good in practical treatment under extreme environmental and substrate conditions, such as higher pH, oxygen limitation, and low lignin concentrations. On the other hand, the bacterial ability to use low molecular weight fractions of lignin indicated that bacteria have many unique and specific enzymes with the ability to catalyze the production of various useful compounds (Masai et al., 1999). Due to their productivity, bacterial enzyme systems are expected to serve as useful tool for the bioremediation of lignin from effluent and its conversion into useful metabolites. In lignin degradation studies, synthetic/model lignin has been used widely as an experimental/model lignin (Ko et al., 2009; Raj et al., 2007; Morii et al., 1995), but above studies have provided only indirect evidence for lignin degradation, which can’t be directly correlated with the degradation of lignin present in pulp and paper mill wastewater.

Hence, there is a need to make a comparative study of bacterial degradation of synthetic and kraft lignin to explore the inhibitory factors responsible for the less degradation of pulp and paper mill effluent lignin. Therefore, in present study a potential bacterial strain isolated from sludge of a pulp and paper industry was examined for its ability to decolorize the synthetic and kraft lignin as well as to characterize the ligninolytic enzyme and the low molecular weight aromatic compounds from lignin degradation by bacterium by HPLC and GC-MS analysis.

Materials and Methods

Sample collection: The effluent samples were collected from M/s Yash Paper Mill Ltd., Darsanagar, Faizabad, (U.P.), India. The effluent samples were collected in sterile plastic containers, brought to laboratory and kraft lignin was extracted from pulp paper mill effluent following the precipitation method (Gidh et al., 2006). The synthetic lignin was purchased from Sigma Aldrich (USA).

Isolation, screening and identification of bacterial strain: For the isolation of lignin degrading bacteria, one gram sludge sample collected from contaminated site was serially diluted and spreaded on lignin amended mineral salt medium (composition in g/l: NaHPO₄, 2.4; K₂HPO₄, 2.0; NH₄NO₃, 0.1; MgSO₄, 0.01; CaCl₂, 0.01; D-glucose, 10.0; Peptone, 3.0; Agar, 15) agar plates. After 48 hr of incubation period, colonies with different morphological properties were selected as lignin degrading bacterial strains and purified by repeated plate streak method on MSM-agar plates. Further, the isolated bacterial strains were screened on the basis of lignin and colour removal capability in MSM-broth amended with synthetic and kraft lignin (600 ppm, w/v) assessed at the regular interval of 72, 96 and 144 hr with respect to control.

For the molecular characterization of bacterial isolates, the total DNA was prepared from overnight grown pure cultures by alkaline lyses method (Kapley et al., 2001). The PCR reactions were performed under the following conditions: 30 cycles of denaturation at 94°C (1 min), annealing at 55 °C (1.5 min), and a final extension at 72 °C (1 min). A 1466 bp product was amplified using forward primer 5'-AGAGTTGTATCMTGGCTCAG-3' and reverse primer 5'-TACGCGTACCTTGTACGACTT-3'. The partial sequences obtained were subjected to BLAST analysis using the online option available at www.ncbi.nlm.nih.gov/ BLAST suggesting the closest homolog of isolates. These sequences were also deposited in a publically accessible GenBank database at (http://www.ncbi.nlm.nih.gov/) under the accession number FJ581026 and FJ581023 for IITRL1 and IITRSU7, respectively. The phylogenetic tree was constructed by using the Bootstrap Tree method from Clustal X software. The method involves the aligning of sequences using Neighbor Joining (NJ) method. The sequences were aligned and distances were calculated (percent divergence) between all pairs of sequences from multiple sequence alignment.

Degradation of synthetic and kraft lignin by axenic and mixed bacterial culture: For the degradation study, 5% (v/v) overnight grown suspension of IITRL1 and IITRSU7 having inoculum size 3.2x10⁶ and 2.9x10⁸, respectively was transferred aseptically to 250 ml flask containing 95 ml MSM supplemented with synthetic and kraft lignin at the concentration of 600 ppm (w/v) in terms of optical density (0.564) at 465 nm (pH 8.0) (Parkas et al., 2007; Pearl and Benson, 1940). Similarly, in mixed condition 2.5% (v/v) of inoculums from each culture was added to obtain final inoculum size 5% (v/v) in same condition. The inoculated flasks were incubated at 34±2 °C under shaking flask condition (140 rpm) for 144 h. Samples from flasks were taken and analyzed for bacterial growth, pH, colour, TOC, AOX, COD and lignin at the regular interval of 24 h up to 144 h (Chandra et al., 2011; Pearl and Benson, 1940).

Bioassay for ligninolytic enzyme: The manganese peroxidase (MnP) enzyme was determined by the oxidation of phenol red (BDH Ltd) in presence of MnSO₄ and H₂O₂ as described by Arora et al. (2002). The enzyme activities were expressed as International Unit (IU), defined as the amount of enzyme required to produce 1μmol product min⁻¹. For purification, the crude extract was loaded into a column (80
x 2.0 cm) containing sephadex G-100 previously equilibrated with sodium malonate buffer, pH 4.5. Protein fraction (0.5 ml) eluted with same buffer with increasing concentration gradient from 0 to 0.1 M NaCl at the flow rate of 0.5 min⁻¹ was collected and stored at -20 °C. The fraction having MnP activity was subjected to 10% denaturing SDS-PAGE analysis and it’s molecular weight was determined by comparing with the peroxidase standard (Sigma Aldrich) and protein ladder (16-97.4 KDa) (Bangalore Genei) (Boer et al., 2006).

**Metabolite characterization:** For metabolite characterization, 100 ml of bacterial treated and untreated black liquor (BL) samples were centrifuged at 5,000 rpm for 20 min and the supernatant was acidified (pH 1-2) with the help of 0.1N HCl and extracted thrice with 3 volume of ethyl acetate. The organic layer was collected, pooled and then dewatered over anhydrous Na₂SO₄. The obtained residues were dried under a stream of nitrogen gas and dissolved in acetonitrile and acetonitrile for TLC and HPLC analysis, respectively. The dry residues of ethyl acetate extracts were derivatized with trimethyl silyl (BSTFA (N, O-bis (trimethylsilyl) trifluoroacetamide) TMCS) and applied for GC-MS analysis.

**Analysis:** The physico-chemical parameters viz: biological oxygen demand (BOD), chemical oxygen demand (COD), total organic carbon (TOC), colour, and sulphate of effluent before and after bacterial treatment were analyzed as per standard methods (APHA, 2005). The adsorbable organic halogen (AOX) analysis was done by using IDC Multi X-2000 AOX analyzer. The pH of medium was measured by Thermo Orion Ion Meter (Model 960). The bacterial growth was measured at 620 nm using uninoculated medium as blank. For the measurement of colour and residual lignin, samples were centrifuged at 8000g for 30 min and the supernatant (1 ml) obtained was diluted by adding 3 ml phosphate buffer (pH 7.6) and absorbance was measured at 465 nm for colour reduction and at 280 nm for lignin degradation on a UV–visible spectrophotometer (GBC Cintra-40, Australia). Dissolve oxygen (DO) was measured by Thermo Orion Ion Meter. In thin layer chromatography (TLC), the samples were spotted on pre-coated thin layer chromatography plates (EMerck, Germany). The solvent system used was toluene: methanol: acetic acid (90:16:24; v/v/v) and the degradation products on TLC plates were visualized under ultra-violet (UV) light.

Further in HPLC analysis, the samples were analyzed using a Waters, 515 HPLC system equipped with 2487 UV/ VIS detector, via millennium software. Samples (20 μl) were injected followed by implementation of HPLC grade acetonitrile and water in 70:30 (v/v) ratio at the rate of 1 ml min⁻¹. Reverse phase C-18 column (250 mm x 4.6, particle size 5 μm) at 27°C were used to analyze the lignin content at 280 nm. Moreover, for quantitative analysis of synthetic and kraft lignin, an aliquot of 1 μl of silylated compounds was injected into the GC-MS equipped with a PE Auto system XL gas chromatograph interfaced with a Turbomass Mass spectrometric mass selective detector. The analytical column connected to the system was a PE-SMS capillary column (20 m x 0.18 mm i.d., 0.18 μm film thickness). Helium gas with flow rate of 1 ml min⁻¹ was used as carrier gas and the column temperature was programmed as 50 °C (5 min); 50–300 °C (10 °C min⁻¹, hold time: 5 min).

**Results and Discussion**

**Physico-chemical characteristics of synthetic and kraft lignin:** The physico-chemical characteristics of synthetic and kraft lignin were as follows pH: 8.0, 8.5; sulphate: 3.5, 117; colour: 1600, 1610 Co.pt (cobalt platinum); COD: 12045, 15300; BOD: 4060, 4646; TOC: 4600, 5800; AOX: 0.93, 3.6; lignin: 600, 643, respectively. The pulp and paper industry has been considered as one of the biggest consumers of natural resources (wood, water) and energy (fossil fuels and electricity) and as a significant taxpayer of discharge of pollutants to the environment (Chandra and Singh, 2012). The high pH and sulphate value of kraft lignin was due to the presence of sodium hydroxide and sodium sulphide which is used in pulping process (Chandra et al., 2011). Beside it, the compounds with high molecular weight, such as lignin and its derivatives do not affect BOD, but would contribute high COD and colour (Esposito et al., 1991).

**Bacterial isolates and their role in lignin degradation:** Among thirteen bacterial strains isolated from sludge samples, the most potential bacterial strains found were IITRL1 and IITRSU7. Both the strains could reduce 41, 45 and 31, 33% lignin and 39, 43 and 32, 36% COD in synthetic and kraft lignin, respectively at 144hr of incubation period. Further, these lignin degrading bacterial strains were identified as *Citrobacter freundii* (FJ581026) and *Citrobacter* sp. (FJ581023), respectively on the basis of 16S rRNA gene sequence analysis (Fig. 1). Similar to our results, many authors have also reported that *Citrobacter* sp. has good potential for the degradation of various azo, anthraquinone and other toxic pollutants (Wang et al., 2009; Khalid et al., 2009).

Further, during the bacterial degradation of synthetic and kraft lignin, the axenic and mixed bacterial culture showed optimum growth at 96 hr of incubation period. Results revealed that mixed bacterial culture was found to be more effective for lignin degradation compared to axenic culture as mixed culture has resulted 85 and 62% decolorization of synthetic and kraft lignin, respectively whereas axenic culture IITRL1 and IITRSU7 decolourized 61, 64% synthetic and 49, 54% kraft lignin, respectively.
(Fig. 2A and B). In addition, mixed bacterial culture was also found capable for the removal of 71, 58% TOC; 78, 53% AOX; 70, 58% COD and 74, 58% lignin from synthetic and kraft lignin, respectively. However, low decolorization at initial phase in spite of fast bacterial growth might be possible due to the utilization of simpler form of carbon (glucose) and nitrogen source (peptone) available in media but, depletion of these C and N sources from media compelled bacteria to utilize lignin as co-substrate. Similar to our observation, a co-metabolism mechanism in bacteria and fungi during the degradation of kraft pine lignin and synthetic lignin (DHP) has been also reported (Raj et al., 2007a and b). In addition, the enhanced reduction in AOX, colour, lignin and other pollution parameters during degradation by mixed culture of IITRL1 and IITRSU7 was also observed.

In addition, the fluctuation in pH during degradation did not affect the bacterial growth, it might be related with the metabolic activity of bacterial strains in growth medium supplemented with glucose and peptone. Similar observation during the microbial degradation of alkali lignin
and pulp paper mill effluent has been also reported (Hernandez et al., 2001; Diez et al., 2002). Furthermore, the DO content after 144 h becomes 0.21 and 0.20 mg l⁻¹ for strain IITRL1 and 0.1, 0.12 mg l⁻¹ for strains IITRSU7 in synthetic and kraft lignin, respectively as compared to control (3.62 mg l⁻¹) (Fig. 2C). However, in present study, the decolourization was not apparently affected by low O₂ concentration and the inhibition is more likely to be a metabolism dependent event. The decrease in DO may be attributed to the glucose fermentation, which contributes to decrease its concentration in medium. However, the decolourization at low oxygen concentrations (< 0.2 mg l⁻¹) can be considered as an indication of the facultative microaerophilic nature of the bacterial strains (Isik and Sponza, 2003).

Lignin degradation also involve the production of extracellular H₂O₂ and peroxidase enzymes because peroxidase activity requires H₂O₂, which is produced during the glucose oxidation and thus establishing the necessity to add glucose as extra carbon source (Miyata et al., 2000). Therefore, during the synthetic and kraft lignin degradation by mixed culture, the enzyme activity was recorded 4.3 and 3.1 U ml⁻¹, respectively at 96 hr of incubation period (Fig. 3A). The low peroxidase activity in kraft lignin in comparison to synthetic lignin might be due to the presence of different heavy metals and other pollution parameters which have negative effect on bacterial cells. Further, the denaturing

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**Fig. 2**: Bacterial growth and decolorization of synthetic lignin (A) and kraft lignin (B); IITRL1 (○), IITRSU7 (■), mixed culture (X) and control (x); (C) the comparative DO (mg l⁻¹) and pH of culture media in control and bacterial degraded lignin at zero hr and 144 hr. synthetic lignin (SL) and kraft lignin (KL).
and kraft lignin has been broken down into smaller fractions during the bacterial degradation process because a clear fragmentation pattern was observed for bacterial degraded synthetic and kraft lignin samples compared to control samples (Fig. 4a, Table 1 and 2). In addition, the HPLC analysis of degraded samples of lignin after 144 hr of incubation period showed the clearcut reduction in peak area compared to control samples for axenic as well as mixed culture (Fig. 4a) whereas kraft lignin degraded samples by axenic culture exhibited shifting of peaks compared to control (Fig. 4b). The decrease in colour intensity in bacterial treated samples has clearly indicated the degradation of lignin by bacterial peroxidase enzymes (Lara et al., 2003). Moreover, the treatment with mixed bacterial culture has revealed that presence of each bacterial strain in culture medium has cumulative enhancing effect on growth, degradation and decolourization of lignin rather than inhibition.

Further, to confirm the degradation of synthetic and kraft lignin, the samples were also processed for GC-MS analysis and the total ion chromatogram (TIC) of degraded samples of synthetic lignin by axenic and mixed culture showed significantly less peaks compared to control samples (Fig. 5). The low molecular weight compounds released from lignin and its phenolic units due to the bacterial degradation process are presented in Table 1 and 2. In addition, some chlorophenols such as 2,4,6-trichlorophenol (RT-16.0), 2,3,4,5-tetrachlorophenol (RT-18.8) and pentachlorophenol (21.8) was also found in kraft lignin degraded by axenic cultures, while some compounds such as hexadecanoic acid in case of synthetic lignin remain undegraded (Table 2). Besides, there were many compounds like RT-27.7 in synthetic lignin and RT-19.7 and 23.3 in kraft lignin that could not be identified by mass spectrometry. Most of studies have been focused only on optimizing the processes itself (Singhal and Thakur, 2009) while few reports have been emphasized on the compositional changes occurs during the biological treatment process.

However, Camarero et al. (2001) have used GC-MS pyrolysis to evaluate the compositional changes of wheat lignin treated by a fungal peroxidase enzyme. It has provided a useful guidance to investigate the capability of P. eryngii peroxidase in situ modification of wheat-straw lignin. In present study, phenolic units derived from lignin and some natural compounds were detected by GC-MS analysis. The TIC of control synthetic lignin has shown more peaks than control of kraft lignin after 144 hr of incubation period. This might be due to presence of kraft lignin in pulp and paper mill effluent, which becomes more complex due to the association of various other organic and inorganic compounds during the effluent treatment process in industries. These complex compounds can’t be fractionated.
Fig. 5: (a) Total ion chromatogram of ethyl acetate extracts from untreated and (1) treated synthetic and (2) kraft lignin; (b) with IITRL1; (c) IITRSU7 and (d) Mixed bacterial culture
Table 1: Compounds identified as TMS derivatives in ethyl acetate extract from control and bacterial degraded samples of synthetic lignin

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Synthetic lignin</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>R.T    a    b    c    d</td>
</tr>
<tr>
<td>Propanoic acid</td>
<td>10.3   -     +     -     -</td>
</tr>
<tr>
<td>2-methoxy phenol</td>
<td>12.9   +     -     +     -</td>
</tr>
<tr>
<td>Benzene acetic acid</td>
<td>13.3   -     +     -     -</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>13.8   -     +     -     -</td>
</tr>
<tr>
<td>Benzylic benzoate</td>
<td>14.4   -     +     +     +</td>
</tr>
<tr>
<td>Phthalic anhydride</td>
<td>15.5   +     -     -     -</td>
</tr>
<tr>
<td>Homovanillic acid</td>
<td>16.7   -     +     -     -</td>
</tr>
<tr>
<td>Butylated hydroxytoluene</td>
<td>17.1   -     +     -     -</td>
</tr>
<tr>
<td>Gloxyllic acid</td>
<td>17.9   -     -     +     +</td>
</tr>
<tr>
<td>Glycerol</td>
<td>19.2   +     -     -     -</td>
</tr>
<tr>
<td>Vanillin acid</td>
<td>20.5   +     -     +     -</td>
</tr>
<tr>
<td>Dibutyl phthalate</td>
<td>21.5   -     +     -     -</td>
</tr>
<tr>
<td>Tetradecanoic acid</td>
<td>23.1   -     +     -     -</td>
</tr>
<tr>
<td>Hexadecanoic acid</td>
<td>24.8   +     +     +     +</td>
</tr>
<tr>
<td>Octadecanoic acid</td>
<td>26.4   +     +     +     +</td>
</tr>
<tr>
<td>Unidentified compound</td>
<td>27.7   -     -     -     -</td>
</tr>
<tr>
<td>Benzyl butyl phthalate</td>
<td>28.1   +     -     -     -</td>
</tr>
<tr>
<td>1-phenanthrene carboxylic acid</td>
<td>29.4   -     +     +     +</td>
</tr>
<tr>
<td>Bis (2-ethylhexyl) phthalate</td>
<td>30.4   -     +     +     +</td>
</tr>
</tbody>
</table>

a: Control (Untreated); b: IITRL1; c: IITRSU7; d: mixed culture; RT: Retention Time; +: Present; -: Absent

Apart from these aromatic compounds, more acid-type compounds than aldehyde and ketone-type were also detected due to degradation of lignin while on the other hand, compounds such as hexadecanoic acid in case of synthetic lignin remain unaltered. Unfortunately, there were also many compounds like RT-27.7 in synthetic lignin and RT-19.7 and 23.3 in kraft lignin that could not be identified by mass spectrometry. Some chlorophenols found in kraft lignin degraded by axenic culture were not found in mixed bacterial culture treated samples because it might be utilized as carbon, nitrogen and energy source. However, chlorophenols are not the fragment of lignin, it is a priority pollutant that has been used widely as a general biocide in commercial wood treatment (Lee et al., 2006) and it probably comes during the extraction of lignin from pulp and paper mill effluent because at low pH, lignin and chlorophenols both get precipitated. Hence, the obtained data has indicated that the developed bacterial strains were capable for the degradation of synthetic lignin more efficiently compared to kraft lignin because chlorophenols and other pollution parameters inhibit the oxidative pathway (Yang et al., 2006).

On the basis of results obtained, it was concluded that the developed bacterial strains was capable efficiently to degrade synthetic as well as kraft lignin and could be useful for the decolourization and degradation of pulp paper mill effluent.

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