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JOURNAL OF ENVIRONMENTAL SCIENCES
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# Syntrophic co-culture of *Bacillus subtilis* and *Klebsiella pneumonia* for degradation of kraft lignin discharged from rayon grade pulp industry

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ARTICLE INFO

*Article history:*
Received 30 September 2014
Revised 17 December 2014
Accepted 27 January 2015
Available online 14 May 2015

*Keywords:*
Degradation
GC–MS
Kraft lignin
Seed germination test
Syntropic

ABSTRACT

In order to search the degradability of kraft lignin, the potential bacterial strains *Bacillus subtilis* (GU193980) and *Klebsiella pneumoniae* (GU193981) were isolated, screened and applied in axenic and co-culture conditions. Results revealed that mixed culture showed better decolorization efficiency (80%) and reduction of pollution parameters (COD 73% and BOD 62%) than axenic culture. This indicated syntrophic growth of these two bacteria rather than any antagonistic effect. The HPLC analysis of degraded samples of kraft lignin has shown the reduction in peak area compared to control, suggesting that decrease in color intensity might be largely attributed to the degradation of lignin by isolated bacteria. Further, the GC–MS analysis showed that most of the compounds detected in control were diminished after bacterial treatment. Further, the seed germination test using *Phaseolus aureus* has supported the detoxification of bacterial decolorized kraft lignin for environmental safety. All these observations have revealed that the developed bacterial co-culture was capable for the effective degradation and decolorization of lignin containing rayon grade pulp mill wastewater for environmental safety.



## Introduction

Kraft lignin is a polymer by-product of kraft pulping process. In order to manufacture the rayon grade pulp (RGP), only high quality fiber containing wood chips are preferred with an extra chemical process involving extensive pre-hydrolysis of wood chips at elevated temperature and pressure followed by alkaline digestion. Under these conditions the semi-solid pulp is collected and washed. At this point the pulp is dark brown in color and known as brown stock due to solubility of lignin and other cellulosic material. The effluent generated from this pulping stage mainly contains lignin fragments, hemi-cellulose, phenolics, resins, fatty acids, sodium carbonate, sodium sulfate and other inorganic salts which mixed together that are soluble in strongly basic medium (Zaied and Bellakhal, 2009). An average of 60,000–95,000 gallons wastewater is generated per ton by-product of such pulping operations (Pokhrel and Viraraghavan, 2004). In most cases, this effluent (row or treated)

*Abbreviations:* BOD, biological oxygen demand; Co.pt, cobalt–platinum unit; COD, chemical oxygen demand; GC-MS, gas chromatography–mass spectrometry; HPLC, high performance liquid chromatography; Lip, lignin peroxidase; MnP, manganese peroxidase; MSM, mineral salt media; RGP, rayon grade pulp; RT, retention time.

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http://dx.doi.org/10.1016/j.jes.2015.01.018
1001-0742/

is discharged into the rivers, stream or other water bodies; resulting in negative social and environmental impacts. Besides toxic load, another form of pollution occurring due to excessive load of organic matter and inorganic nutrients, which can trigger eutrophication within the receiving watercourses. The lignosulfonante component of pulp paper effluent may inhibit the growth of phototrophic planktons, algae and plants by reducing the transmission of sunlight in water (Karrasch et al., 2005). In addition, many developing countries including India due to the non-availability of alternative sources of irrigation, farmers irrigated their crop plants with industrial effluents containing high level of several toxic compounds including heavy metals. This may cause adverse effect on human through food chain.

Although several physical and chemical methods are available for the treatment of effluent, but they are less desirable, hence the researchers have focused on environmental friendly technologies for the treatment of wastewater. Therefore, the use of biological methods for the removal of contaminants from the effluent has been advocated (Yang et al., 2008). Among biological methods tried so far, most of the literature focused only on few genera of white rot fungi because of their broad range and non-specific extracellular ligninolytic enzymatic system (manganese peroxidase (1.11.1.13), lignin peroxidase (1.11.1.14) and laccase (1.10.3.2)). But, bacteria seem to be more effective than fungi for the bioremediation of environmental pollutants due to their immense environmental adaptability and biochemical versatility. The residual lignin from pulping section, chlorophenolics and chlorinated lignin derivatives (originating due to the reaction of bleaching agents such as $Cl_2$ and $ClO_2$, with lignins and phenols) are the major contributors of toxicity in paper mill effluents. They are highly toxic and persist in water and soil for longer time and adversely affect flora and fauna. Though, fungi are able to remove coloring materials and lignin compounds, but they are not efficient for the chloro-organics. However, bacteria viz. *Aeromonas*, *Bacillus subtilis*, *Pseudomonas* and *Xanthomonas* are reported to utilize lignocellulosic and chloro-organic components of pulp paper effluent (Vora et al., 1988; Jain et al., 1997; Gupta et al., 2001). Besides, bacteria isolated from compost soil viz. *Azotobacter* and *Serratia marcescens* were found capable of the degradation and decolorization of lignin (Morii et al., 1995). Similarly, three potential bacterial strains of *Panibacillus* sp. *Aneurinibacillus aneurinilyticus* and *Bacillus* sp. were also reported for degradation and decolorization of synthetic lignin isolated from pulp paper sludge and characterized their metabolic products by GC–MS (Chandra et al., 2007; Raj et al., 2007). However, all the above studies have been carried out on synthetic/model compounds which do not directly explain the degradation process of kraft lignin; present in pulp mill wastewater due to the presence of several other complex co-pollutants. Lignin degradation is well reported by pure culture (Jain et al., 1997; Gupta et al., 2001), but in nature, microorganisms exist in mixed condition due to presence of wide range of compounds. The role of co-culture for degradation of various environmental pollutants has been reported by some workers (Park et al., 1999; Tran et al., 2010; Jeon et al., 2011). However, the literature available on the bacterial decolorization of kraft lignin discharged from RGP manufacturing industry is lacking. Hence, the present study has been focused on the decolorization and detoxification of kraft lignin discharged from RGP plant in syntrophic manner which will be useful for the management of high concentration of lignin containing pulp paper mill wastewater.

## 1. Material and methods

### 1.1. Sample collection and isolation of bacteria

Century Pulp and Paper is a unit of Century Textile and industries Ltd., India. Company was established in 1984 with an installed capacity of 20,000 TPA of writing printing paper and 20000 TPA of Rayon Grade Pulp. Now production capacities have raised up to 31300 TPA Rayon Grade Pulp, 37250 TPA paper (wood based plant). Thus, the samples were collected in sterile container from rayon grade pulp washing section of M/s. Century Pulp Paper Mill, Lalkuan, Nainital, Uttrakhand, India located (79°10′E longitude and 29°3′N latitude) at the foot hills of Himalayas. For the isolation of potential lignin degrading bacterial strains, sludge samples were collected from the disposal site of same containing decomposed wood. The autochthonous (native) bacteria were isolated by serial dilution method and purified by plate streak method on lignin amended MSM (mineral salt media) agar plates containing (g/L): lignin 0.5; D-glucose 10; peptone, 5; $Na_2HPO_4$ 2.4; $K_2HPO_4$ 2.0; $NH_4NO_3$ 0.1; $MgSO_4$ 0.01; and $CaCl_2$ 0.01 as described previously (Chandra et al., 2007).

### 1.2. Physico-chemical analysis of pulp paper mill effluent before and after bacterial treatment

The freshly collected effluent was noted highly alkaline in nature due to presence of complex residual mixture of phenolic, lignin and other persistent organic pollutants. For the measurement of color, control (uninnoculated) and degraded samples were centrifuged at 8000 ×*g* for 30 min and absorbance was measured at 465 nm on a UV-visible spectrophotometer (GBC Cintra-40, Australia) (Chandra et al., 2007). Absorbance values were transferred into color units (CU) according to the equation:

$$CU = 500A_2/A_1$$

where, $A_1$ is the absorbance of 500-CU platinum–cobalt standard solution ($A_{465}$ = 0.129) and $A_2$ is the absorbance of the wastewater sample.

For the measurement of residual lignin, samples were centrifuged at 8000 ×*g* for 30 min. Supernatant (1 mL) was diluted by adding 3 mL of phosphate buffer (pH 7.6) and absorbance was measured at 280 nm (Chandra et al., 2007). The pH of medium was also analyzed with the selective ion electrode (9172 BN) of Thermo Orion (Model 960). The biological oxygen demand was measured by a 5 day-test, chemical oxygen demand by open reflux method, total nitrogen (Micro kjeldahl), sulfate (gravimetric method), color (visual color comparison method), total dissolved solids as per methods described in APHA (2005). Nitrate was done with ion meter by their respective electrode (Ion meter, Orion 960). Heavy metals

(ICP Unit, CBC Scientific Equipment Pvt Ltd, Plasma Lab, Australia).

### 1.3. Screening and characterization of potential bacterial strains

The isolated bacterial strains were screened on the basis of their COD, color and lignin removal potential. MSM broth amended with 800 ppm (Lignin Alkali, Sigma) of lignin having COD 16,500 mg/L and initial color 2500 Co.pt color unit was inoculated with individual isolates and incubated at (34 ± 1)°C, in shaking flask condition (rpm 120) for 144 hr. Two potential bacterial strains (IITRC12 and IITRC13) showing rapid growth and reduction of pollution parameters were selected for further study.

The identification of screened strains was established by 16S rRNA gene sequence analysis. For 16S rRNA gene sequence analysis, alkaline lysis method was used for the total DNA preparation from overnight grown pure cultures. The 16S rRNA gene was amplified using universal eubacterial primers (Narde et al., 2004). 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 and reverse primer. The nucleotide sequences obtained were subjected to BLAST analysis using the online option available at www.ncbi.nlm.nih.gov/BLAST suggesting the identity of isolated bacterial strains.

### 1.4. Degradation studies by axenic and co-culture conditions

For the degradation study, 5% (V/V) overnight grown suspension of IITRC12 and IITRC13 having inoculum sizes $28.4 \times 10^5$ and $31.7 \times 10^5$, respectively was transferred aseptically to 250 mL flask containing 95 mL effluent amended with glucose (1%, w/V) and peptone (0.25%, w/V) (pH 8.0). Similarly, in co-culture condition 2.5% (V/V) of inoculums from each culture was added to obtain final inoculum size 5% (V/V) in same conditions. The inoculated flasks were incubated at (35 ± 2)°C in a temperature controlled shaker (Innova 4230, New Brunswick Scientific (UK) Ltd, Herts, UK) at 140 rpm as described by Chandra et al. (2012). During the decolorization period, samples were taken at every 24 hr interval and analyzed for bacterial growth, color, lignin, pH and enzyme activity. Growth in terms of colony forming unit (cfu/mL) was performed by spreading the sample dilutions on the surface of nutrient agar plates.

### 1.5. Ligninolytic enzyme activity in potential bacterial strains

The LiP assay was done by monitoring the oxidation of dye Azure B in the presence of $H_2O_2$. The reaction mixture contained sodium tartrate buffer (50 mmol, pH 3.0), Azure B (32 mM), 500 mL of culture filtrate, 500 mL of $H_2O_2$ (2 mmol). OD was taken at 651 nm after 10 min (Arora et al., 2002). MnP assay was performed by the method as described by de Oliveira et al. (2009) which is based on the oxidation of phenol red. Reaction mixture (4 mL) contained 1 mL of potassium phosphate buffer (pH 7.0), 1 mL of enzyme extract, 500 mL of $MnSO_4$ (1 mmol), 1 mL of phenol red (1 mmol) and 500 mL $H_2O_2$ (50 mmol). 1 mL sample was removed from reaction mixture and 40 mL of 5 mol NaOH was added to stop the reaction. OD was taken at 610 nm at every 1 min interval. The absorption at 610 nm was measured against a blank without any manganese in the reaction mixture. ΔAbs per min was converted to U/L using an extinction coefficient of the oxidized phenol red that is 22 m/mol/cm. Moreover, laccase activity was detected by taking the absorbance at 450 nm. Reaction mixture was prepared by 3.8 mL of acetate buffer (10 mmol, pH 5.0), 1 mL of guaiacol (2 mmol) and 0.2 mL of enzyme extract. Then reaction mixture was incubated at 25°C for 2 hr (Arora et al., 2002). One international unit of enzyme activity was defined as activity of enzyme that catalyzed the conversion of 1 mmol of substrate/min.

### 1.6. Confirmatory analysis of degradation by HPLC and GC–MS

For confirmatory analysis, 100 mL of bacterial degraded and undegraded sample were centrifuged at 5000 ×*g* for 20 min. Supernatants were acidified (pH 1–2) with the help of 0.1 mil/L HCl with consistent agitation and extracted thrice with 3 volume of ethyl acetate. The organic layer was pooled and dewatered over anhydrous $Na_2SO_4$. Residues were dried under a stream of nitrogen gas and dissolved in acetonitrile for HPLC analysis. The samples were analyzed using a Waters, 515 HPLC, equipped with 2487 UV/Vis detector, via millennium software. Samples (20 μL) were injected followed by implementation of HPLC grade acetonitrile/water (70:30) at the rate of 1 ml/min. Reverse phase C-18 column (250 mm × 4.6, particle size 5 μm) at 27°C were used to analyze the lignin at 280 nm (Chandra et al., 2007).

For GC–MS analysis, the dry residues of ethyl acetate extracts were derivatized with trimethyl silyl (BSTFA (N, O-bis (trimethylsilyl) trifluoroacetamide) TMCS). 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-5MS capillary column (20 m × 0.18 mm i.d., 0.18 μm film thickness). Helium gas with a flow rate of 1 mL/min was used as carrier gas. The column temperature was programmed as 50°C (5 min); 50–300°C (10°C/min, hold time: 5 min). The metabolic products were identified by comparing their mass spectra with that of National Institute of Standards and Technology (NIST) library available with instrument and by comparing the retention time with those of available authentic organic compounds.

### 1.7. Toxicity assessment through α-amylase activity for environmental safety

The toxicity evaluation of treated effluent samples was analyzed as per guidance of Environmental Protection Agency (EPA), US, using the seed germination bioassay tests. The bioassay of acute toxicity test was performed to evaluate the detoxification of effluent using seed germination method of *Phaseolus aureus* (Wang, 2003). For toxicity evaluation the samples (untreated control and bacterial treated) were diluted with tap water at different concentrations *i.e.*, 10%, 20%, 40%, 60%, 80% and 100% (v/v). The toxicity was expressed in terms

of % inhibition of their amylolytic activity (Bharagava and Chandra, 2010).

For α-amylase assay, twenty seeds from each treatment were homogenized with 0.1 mol sodium acetate buffer (pH 4.8); filtered through two layers of cheese cloth to remove large particles and the supernatant obtained was centrifuged at 15,000 ×*g* for 20 min. All the preparations were carried out at 4°C. The supernatant obtained was used as crude enzyme extract for α-amylase assay. For enzyme assay, the reaction medium (3 mL) contained 1 mL of 0.1 mol acetate buffer, pH 4.8, 0.5 mL of enzyme extract diluted to 1 mL using acetate buffer, and 1 mL of 0.1% soluble starch solution. The enzyme extract was diluted to obtain an absorbance range of less than one during the enzyme assay. The reaction medium was incubated for 10 min at room temperature and then the reaction was stopped by adding 1 mL of 0.1% iodine reagent and 3 ml of 0.05 mol/L HCl. The absorbance was measured at 620 nm and decreased in absorbance was expressed in terms of amylase activity (Beri and Gupta, 2007). The enzymes was purified, concentrated and SDS-PAGE was performed according to the methodology explain by Bharagava and Chandra (2010).

### 1.8. Statistical analysis

All the data were reported as means ± SD for triplicate samples. Tukey's test using the Graph Pad software (Graph Pad Software Inc., Fay Avenue, Suite, La Jalla, CA 92037, USA) was used for statistical analysis (Ott and Longnecker, 1984).

## 2. Results and discussion

### 2.1. Screening and identification of bacteria

The collected effluent was dark brown in color with high COD, BOD, nitrates and sulfate because of various dissolved organic compounds present in effluent (Table 1). The high COD/BOD ratio (approx 2.8) in effluent is due to presence of high molecular weight compounds, *i.e.*, lignin and its derivatives which contribute high COD and color instead of BOD (Esposito et al., 1991). The source of sulfate ions in effluent might be sodium sulfite, which is used in pulping process and the nitrates detected in effluent indicated the presence of nitrogen in lignin (Singhal and Thakur, 2009). The concentration of metals in kraft effluent was found 10.22, 5.03 and 9.83 mg/L for Fe, Ni and Zn, respectively which are very high than their permissible limit as recommended by EPA (USEPA, 2000). These metals might be added in effluent because of corrosion of digestion vessels and possibly due to bioaccumulation of these metals by plants itself grown on contaminated site which are used as raw material in industry.

Out of fifteen bacterial isolates isolated from sludge, the most efficient bacterial strains were IITRC12 and IITRC13, which could reduce 36%, 39% lignin and 44%, 41% color and 51%, 48% COD, respectively. The pulp paper mill sludge was rich in lignin and other chemicals. The areas for collecting the sludge samples were selected by assuming the presence of delignifying bacteria in that sector with recalcitrant compounds and genetic potency to degrade these compounds.

**Table 1 – Physico-chemical parameters of effluent discharged from RGP manufacturing industry.**

| Sample no. | Parameters | Wash machine effluent | Permissible limits (USEPA, 2000) [a] |
|---|---|---|---|
| 1 | pH | 8.5 ± 1.0 | 5–9 |
| 2 | TDS (mg/L) | 977 ± 7.2 | – |
| 3 | COD (mg/L) | 16550 ± 507.2 | 120 |
| 4 | BOD (mg/L) | 7250 ± 123.0 | 40.0 |
| 5 | Color (CU) | 2538 ± 53.3 | Colorless. |
| 6 | Lignin (mg/L) | 800 ± 18.4 | – |
| 7 | Total nitrogen (mg/L) | 126 ± 32.8 | 25.0 |
| 8 | Sulfate (mg/L) | 1003 ± 5.3 | – |
| 9 | Phosphate (mg/L) | 7.3 ± 0.4 | – |
| 10 | Nitrate (mg/L) | 193.30 ± 6.1 | 10.0 |
| 11 | Heavy metals | | |
| | Cd (mg/L) | BDL | 0.01 |
| | Cr (mg/L) | BDL | – |
| | Cu (mg/L) | 0.09 ± 0.14 | 0.20 |
| | Fe (mg/L) | 10.22 ± 9.02 | 5.00 |
| | Ni (mg/L) | 5.03 ± 1.02 | 0.20 |
| | Zn (mg/L) | 9.83 ± 1.13 | 2.00 |
| | Pb (mg/L) | BDL | 0.05 |
| | Mn (mg/L) | 0.04 ± 0.04 | 0.20 |

BDL: below detection limit.
[a] Permissible limit of trace elements in wastewaters (USEPA, 2000).
(–): not specified.

Isolation was done by using such sludge samples because it is easy to acclimatize those bacteria in the paper industry effluent due to their existence in particular environment and these bacteria were enriched in the presence of toxic compounds therefore, the strains could be evolved with the process of acclimatization. These strains also showed extracellular linginolytic activity which is essential for degradation and detoxification of complex compounds. Therefore, these two isolates were selected for degradation study. According to the common accepted theory, the initial attack of aromatic pollutants is made by the extracellular ligninolytic enzymes. The ligninolytic enzymes perform a one-electron oxidation, thereby generating cation radicals of the contaminants. The cation radicals may undergo spontaneous chemical reactions such as C–C cleavage or hydroxylation resulting in more hydrophilic products. Those products are taken up by the microbial cells and co-metabolized in the presence of a proper carbon source to carbon dioxide. Further, the screened bacterial strains IITRC12 and IITRC13 were identified as *B. subtilis* (GU193980) and *Klebsiella pneumoniae* (GU193981), respectively by 16S rRNA gene sequence analysis.

### 2.2. Bacterial degradation of effluent

During the time course of degradation, a marked increase in growth revealed that the optimum growth of axenic and mixed culture was at 96 hr of incubation period. In mixed condition these bacteria had much faster growth rate and decolorization as compared to axenic. These findings showed that the co-culture grows well in medium and showed cumulative enhancing effect on growth and color reduction (Fig. 1a). This finding is corroborated with the observation of other studies (Singh et al., 2008; Chandra et al., 2009; Kumar et al., 2012).

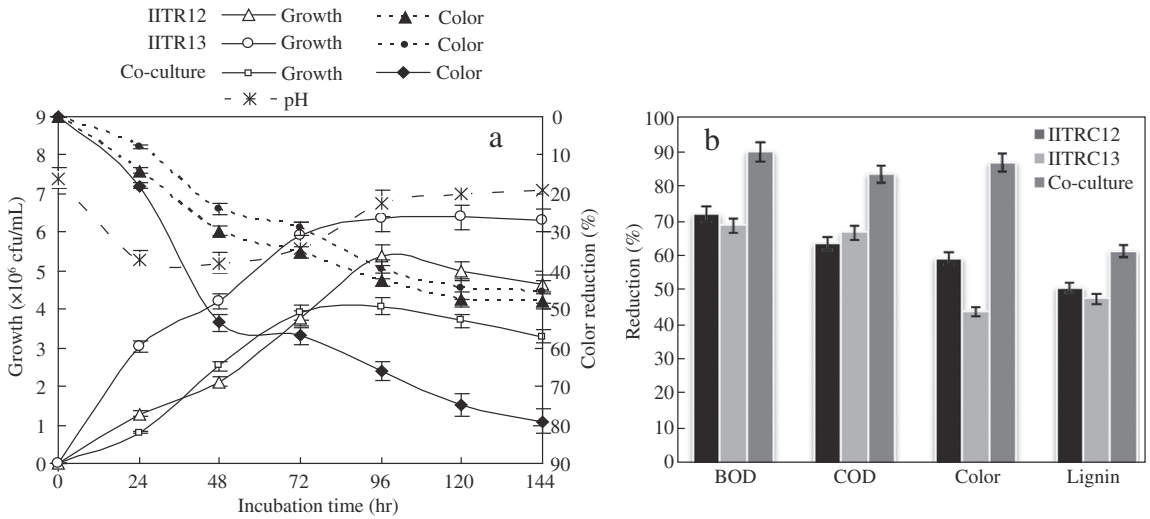


**Fig. 1 – Bacterial growth pattern (a) and reduction in pollution parameters (b) by axenic and co-culture condition.**

Low decolorization at initial phase in spite of fast bacterial growth might be due to the utilization of simpler form of carbon and nitrogen sources available in growth media. But, subsequent starvation of easily available nutritional source in media insisted the bacterial culture to utilize lignin as a co-substrate. Similar to this study, co-metabolism mechanism in fungi for lignin containing effluent degradation has also been reported by various authors (Singhal and Thakur, 2009; Zhang et al., 1999). During degradation, the shift in pH towards acidic condition was noted within initial 48 hr of bacterial growth which indicated the formation of acidic compounds through tricarboxylic acid (TCA) cycle, utilizing the simpler form of carbon sources present in medium by bacteria (Yang et al., 2008). The formations of acidic products have also been reported in previous study which corroborated with the other findings (Chandra et al., 2011a; Yadav et al., 2011). As the supplementary nutritional source depleted, the pH of medium shifted gradually towards higher side facilitating the lignin degradation, as lignins are uniformly soluble at high pH that is why the more reduction in COD, BOD and color were noted after 48 h of incubation period (Chandra et al., 2011b).

A non-specific ligninolytic enzymatic system, which consist of peroxidases (lignin and manganese peroxidase) and laccases secreted by these isolates were found to be associated with lignin degradation properties (Fig. 2). During the time course of degradation production of ligninolytic enzyme could be improved by co-culture (LiP — 3.4, MnP — 2.3 and Laccase — 4.5 U/mL) compared to axenic culture (Fig. 2) and reach optimum at 48 hr in case of peroxidase and 96 hr in case of laccase. The production of extracellular $H_2O_2$ and peroxidase involved in lignin degradation because peroxidase activity requires $H_2O_2$, which is produced during the glucose oxidation and thus, establishing the necessity to add glucose as extra carbon source (Miyata et al., 2000). Production of laccase and peroxidase activity showed direct correlation with lignin degradation. Lignin induced laccase (EC 1.10.3.2), lignin peroxidase (EC 1.11.10.14) and manganese peroxidase (EC 1.11.1.13) were identified and characterized in unicellular bacteria by various authors (Hullo et al., 2001; Singh et al., 2007; Dick et al., 2008), but it has not been reported for decolorization as well as detoxification of industrial effluent.

### 2.3. Metabolite characterization

The degradation was further conformed by HPLC analysis which showed the reduction as well as shifting of peaks compared to controls. This indicated the degradation with minor biotransformation of kraft lignin (Fig. 3). Decrease in color intensity by bacterial culture clearly revealed the depolymerization of lignin by bacterial ligninolytic action (Chandra et al., 2007; Lara et al., 2003). Several authors have reported that white rot fungus *Phanerochaete chrysosporium* secret a family of enzymes which degrade lignin and chlorlignin and reduce the COD by destroying both the color bodies and chromopheric structure and removes total organic chlorine by converting into inorganic chlorine (Kirk et al., 1978). Pulp paper effluent is a complex aqueous system and it is difficult to comprehensively to investigate the changes during the degradation process.

Many studies were focused only on optimization of the processes itself while few reports emphasized on the compositional changes during the biotreatment process. In the present work, phenolic units derived from lignin and some natural compounds were detected by GC–MS analysis. To confirm the degradation and identification of metabolic products, control and co-culture were analyzed by GC–MS analysis. Detail of compounds generated from lignin and other phenolic compounds due to the degradation process is listed in Table 2.

Gas chromatograms corresponding to the compounds extracted with ethyl acetate from the acidified supernatants obtained from the untreated and decolorized effluent are shown in Fig. 4. Several compounds *i.e.*, 2-methoxyphenol (RT = 14.50 min; guiacol); 2,6-dimethoxy phenol (RT = 15.95 min; syringol), ethanone, 1-(-4-hydroxy-3,5-dimethoxyphenyl) (RT = 19.86 min;

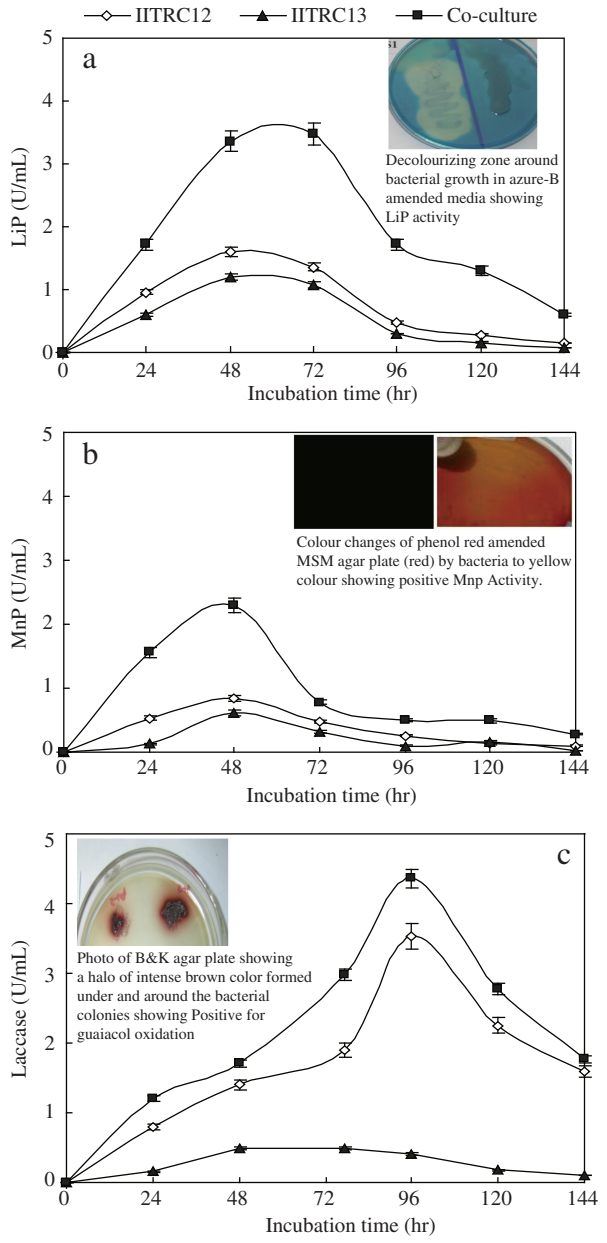


**Fig. 2 – Time course of lignin peroxidase (a), manganese peroxidase (b) and laccase activity (c) during axenic and co-culture degradation studies. Side photo shows ligninolytic activity of bacterial stains on the plate.**

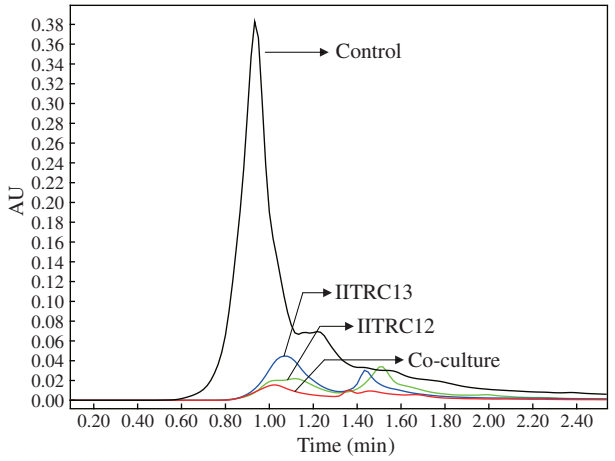


**Fig. 3 – Comparative HPLC chromatograph of control and degraded samples of effluent by axenic (IITRC12: *Bacillus subtilis* and IITRC13: *Klebsiella pneumonia*) and co-culture after 144 h of incubation period.**

acetosyringone) were detected in control sample these compounds can be easily related to the lignin because they are considered to be the basic moieties that build the lignin structure which may possibly be coming from the industrial pulping process. However, these compounds are not found in degraded sample. This showed the massive consumption or complete mineralization of compounds as compared to control which indicated that the co-culture has a strong ability to utilize its constituents as carbon, nitrogen and energy source. Apart from these, several phenolic compounds *i.e.*, 2-methoxy-4-ethyl-phenol (RT 16.30 min); 3-allyl-6-methoxyphenol (m-eugenol RT 16.3 min); 2-methoxy-4-(1-propenyl) phenol (RT 16.30 min); and 4-methoxycinnamic acid (RT 24.50 min) were also found in the control effluent. In addition, acidic compounds were also detected which are 2-hydroxymethyl cyclopropane carboxylic acid (RT 12.50 min); benzene acetic acid (RT = 17.57 min); benzoic acid (RT 20.16 min); 1,2-benzene dicarboxylic acid (RT 20.92 min); hexadecanoic acid (RT 21.68 min); and octadecanoic acid (RT 23.50 min). Some workers have also been detected with many aromatic compounds in control sample, such as 2-methoxy phenol (Guaiacol) (RT 14.5 min), benzene acetic acid (RT 17.5 min), and benzoic acid (RT 20.1 min) as low-molecular-weight phenolic units of lignin (Raj et al., 2007; Yang et al., 2008; Ko et al., 2009). However, control as well degraded sample contain intense smelling contents of low-molecular-weight fatty acids such as acetic acid. This acid is formed as metabolic products by anaerobic and facultative anaerobic micro-organisms in water and pulp systems. Octadecane (RT 23.05 min) was detected in bacterial degraded samples. Octadecane and other related similar compounds have been reported in the previous study also during bacterial degradation of pulp paper mill effluent (Chandra et al., 2012). However, the mechanism of octadecane production and role in degradation of chlorlignin waste are still not clear. Hence, there is a need to investigate the role of octadecane in the bacterial degradation of pulp paper mill pollutants in the environment. Whether, these are signal molecule produced by bacteria or a metabolic product as carbon source. Zhang and Miller, 1992 have been showed that rhamnolipid surfactant enhances the octadecane biodegradation by *Pseudomonas*. The phthalate derivative had been also detected from photodegradation of black liquor lignin (Ksibi et al., 2003) as well as fungal peroxidase degradation of lignosulfonate (Shin and Lee, 1999). However, propaonic acid (RT 10.07 min), pyridinium, 1-amino-4-methyl-, hydroxide, inner salt (RT 16.67 min), docosane, 1, 22-dibromo (RT 24.88 min) and decane, 1-bromo-2-methyl (RT 25.63 min) were detected as biotransform products. On the other hand, compounds such as trimethylsilyl (RT 26.1 min) remain unchanged because it is a derivatizing agent which is used during the derivatization process. This result are corroborated with the earlier findings that the GC–MS analysis is the suitable technique for demonstrate the metabolism of low molecular weight

**Table 2 – Compound identified as trimethylsilyl (TMS) derivatives from control and degraded samples as given in Fig. 4.**

| Sample no. | Compounds | Retention time (min) | Control | Degraded by co-culture |
|---|---|---|---|---|
| 1 | Propanoic acid | 10.07 | – | + |
| 2 | Acetic acid | 11.40 | + | + |
| 3 | 2-Hydroxymethyl cyclopropane carboxylic acid | 12.5 | + | – |
| 4 | Benzyle benzoate | 14.36 | + | – |
| 5 | 2-Methoxyphenol | 14.50 | + | – |
| 6 | Phthalic anhydride | 15.70 | + | – |
| 7 | 2,6-Dimethoxy phenol | 15.95 | + | – |
| 8 | 2-Methoxy-4-ethyl-phenol | 16.30 | + | |
| 9 | Pyridinium, 1-amino-4methyl-, hydroxide, inner salt | 16.67 | – | + |
| 10 | Butylated hydroxytoluene | 17.10 | + | – |
| 11 | Benzene acitic acid | 17.57 | + | – |
| 12 | Gloxylic acid | 17.78 | + | – |
| 13 | 3-Allyl-6-methoxyphenol | 19.40 | + | – |
| 14 | Ethanone, 1-(-4-hydroxy-3,5-dimethoxyphenyl) | 19.86 | + | – |
| 15 | Benzoic acid | 20.16 | + | – |
| 16 | 1,2-Benzene dicarboxylic acid | 20.92 | + | + |
| 17 | Hexadecanoic acid | 21.68 | + | + |
| 18 | 2-Methoxy-4-(1-propenyl) phenol | 22.25 | + | – |
| 19 | Clionasterol acetate | 22.63 | – | – |
| 20 | Octadecane | 23.05 | – | + |
| 21 | Octadecanoic acid | 23.50 | + | – |
| 22 | 4-Methoxycinnamic acid | 24.50 | + | – |
| 23 | Docosane, 1, 22-dibromo- | 24.88 | – | + |
| 24 | Decane, 1-bromo-2-methyl- | 25.63 | – | + |
| 25 | Trimethyl-salyl | 26.10 | + | + |
| 26 | 1,1-(1,2-Ethanediyl) bis[4-methoxy] benzene | 26.40 | + | – |
| 27 | Heptacosane | 27.32 | – | + |

+: present; –: absent.

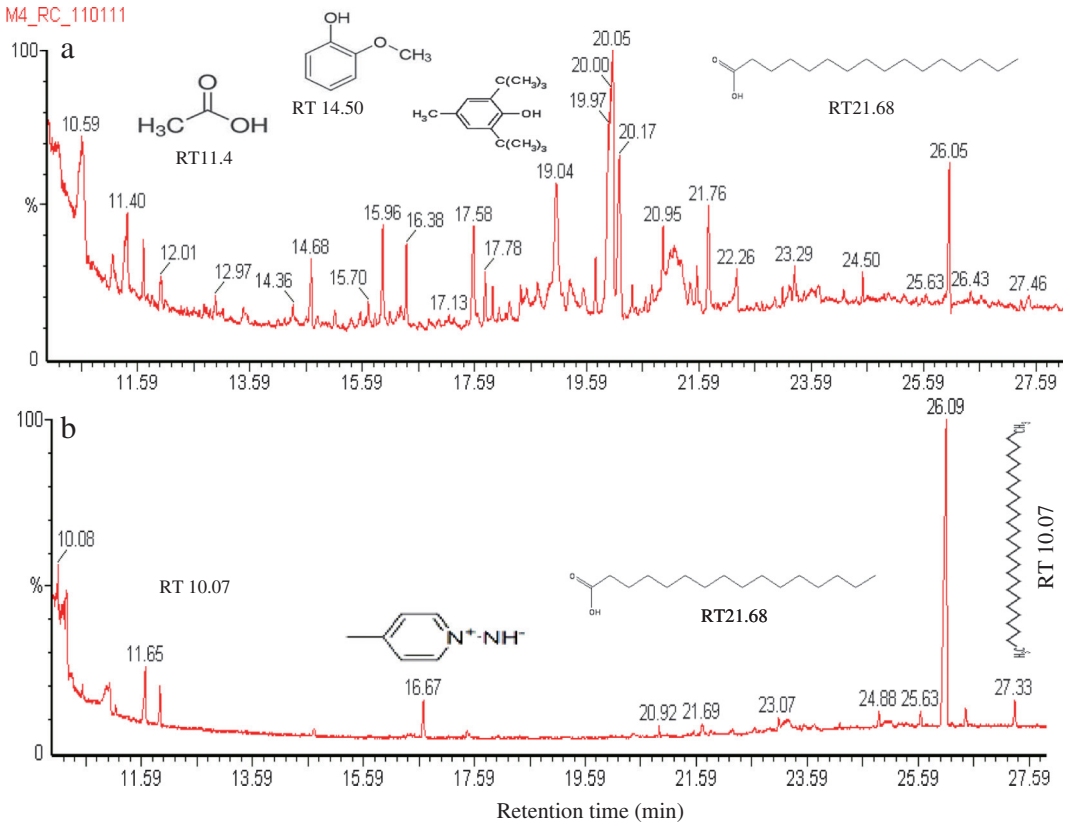


**Fig. 4 – GC–MS chromatogram of control (a); and degraded (b: with co-culture) sample of kraft lignin.**

compounds released from lignin degradation and has been a method of choice for analysis of volatile low molecular weight compounds (Hernández-Coronado et al., 1998).

### 2.4. Toxicity evaluation test on P. aureus

Seed germination is a complex physiological and biochemical process in plants that can be affected severely by several environmental factors. Starch is the major component of most of the world's crop yield and degradation of starch is essential for seed germination. In germinating seeds, starch degradation is initiated by α-amylase producing soluble oligosaccharides from starch. These are then hydrolyzed by α-amylase to liberate maltose and finally, glucosidase breaks down maltose into glucose providing energy to germinating seeds. Two controls were used, untreated effluent and tap water. Untreated effluent was used to compare the effectiveness of treatment in reducing toxicity. Tap water was used to see the normal process of seed germination test. Results of seed germination test revealed that the untreated effluent above 10% (V/V) inhibited amylase activity and thereafter a continuous decline in α-amylase activity was observed at higher concentration (>10%) (Fig. 5). However, the seeds treated with tap water have shown lower amylase activity (0.3 U) than the seeds treated with 20% and 80% (V/V) concentration of untreated and treated effluent, respectively. This revealed that untreated effluent acted as growth promoter at lower concentration (10%) whereas after treatment it was found as growth supporter up to 80%. This indicated that toxicity has been reduced significantly after bacterial degradation. The growth promoting effects of untreated effluent on amylase at lower concentration might be due to the presence of optimum level of organic nutrients essential for plant growth (Kannan and Oblisami, 1990). The reduction in amylase activity at higher concentration of undegraded effluent might be due to the high pollution content affecting various physiological and biochemical processes during the seed germination.

Further, the denaturing SDS-PAGE of α-amylase enzyme extracted from germinating seeds treated with same concentration of untreated and treated effluent has yielded three bands of different molecular weight and intensity/concentration (Fig. 5c–d). Results indicated that the concentration of amylase enzyme (*i.e.*, the intensity of band) decreases gradually as the concentration of untreated effluent increases at 10%. *Phaseolus* seeds treated with more than 40% (V/V) concentration of untreated effluent have shown reduced amylase activity compared to control and no amylase activity or enzyme production was observed in seeds treated with 100% (V/V) concentration of untreated effluent (Fig. 5c–d).

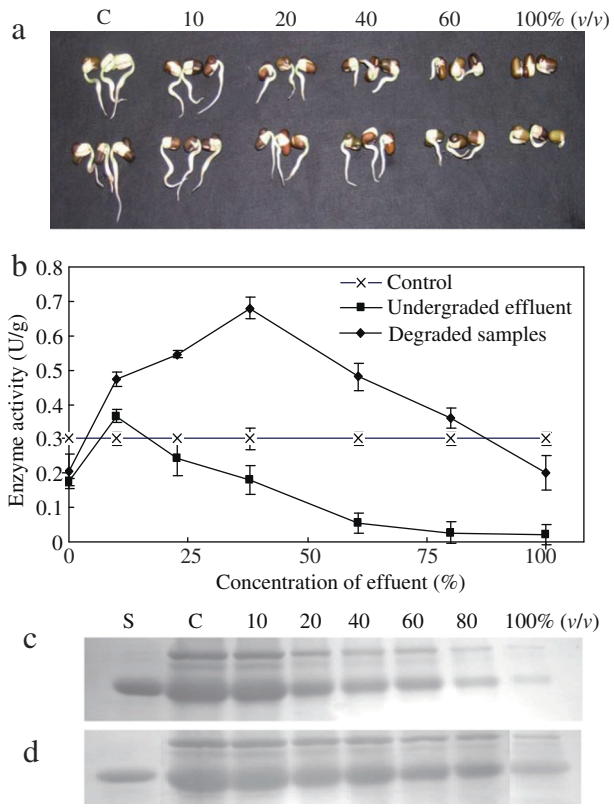


**Fig. 5 – Effect of different concentration of effluent on seedling growth (a), amylase activity (b); SDS page of amylase induction and its content in germinating seeds of *Phaseolus mungo* L. before (c) and after bacterial treatment (d). Lane S: Amylase standard; lane C: seed treated with tap water/control.**

## 3. Conclusions

*B. subtilis* (GU193980) and *K. pneumoniae* (GU193981) were found for the production of ligninolytic enzyme. These were capable to reduce 82% color, 58% lignin, 85% BOD, and 79% COD after 144 hr in shaking incubation. Further, the HPLC and GC–MS analyses of control and bacterial degraded sample showed metabolization and transformation of pollutant present in effluent and further it was noted that co-culture decolorized more efficiently than axenic culture. In support of this, toxicity of treated effluent was significantly reduced and treated effluent was able to support the growth of *P. aureus*. All these observations have revealed that the developed bacterial co-culture was capable for the effective degradation and decolorization of lignin and could be useful for the decolorization and detoxification of rayon grade pulp mill wastewater for environmental safety.

## Acknowledgments

The financial assistance from Department of Science and Technology (DST) vide sanction order No. F. No SB/50/BB-0042/2013 for project and UGC Kothari fellowship to Dr. Sangeeta Yadav is highly acknowledged.

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