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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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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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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