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Bioremediation of Pulp and Paper mill Effluent by Dominant Aboriginal Microbes and Their Consortium

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ABSTRACT: Paper mills are characterized as polluting industries, these use pulping process for paper manufacturing, due to which toxic chemicals released into environment. Therefore, the biological oxygen demand (BOD), chemical oxygen demand (COD) of the emanating stream is high and dissolved oxygen (DO) is low. To resolve this predicament, two dominant bacteria *Bacillus subtilis* and *Micrococcus luteus*, and one fungi *Phanerochaete crysosporium* was isolated from pulp and paper mills effluent (PPME) soils. These microbes were individually and in consortium were inoculated in (PPME) without diluting and no addition of carbon or nitrogen sources. Experiment was conducted under shaking and stationary conditions for nine days. These microbes were found competent of reducing BOD up to 87.2 %, COD up to 94.7% and lignin content up to 97% after 9 d under shaking conditions and brought down pH of raw PPME to neutral and increased DO from 0.8 mg/L to 6.8 mg/L.

Key words: Biodegradation, Bacteria, Fungi, lignin, Pulp wastewater

INTRODUCTION

The pulp and paper mill generates black effluent with very high biological oxygen demand (BOD), chemical oxygen demand (COD), toxic substances, recalcitrant organics, turbidity and high temperature. The colouring body present in the wastewater from pulp and paper mill is organic in nature and is comprised of wood extractives, tannin resins, synthetic dyes, lignin and its degradation products formed by the action of chlorine on lignin. The untreated effluents from pulp and paper mills discharged into water bodies, damages the water quality and brown colour imparted to water due to addition of effluents is detectable over long distances. The dark brown colour is due to the formation of lignin degradation products during the processing of lignocellulosics from paper and pulp manufacture. The undiluted effluents are toxic to aquatic organisms and exhibit a strong mutagenic effect. Furthermore some compounds in the effluents are resistant to biodegradation and can bioaccumulate in the aquatic food chain (Pokhrel & Viraraghavan, 2004). Several methods have been attempted for the removal of colour from the pulp and paper mill effluents. These can be classified into physical, chemical and biological methods. Physical and chemical processes are quite expensive and remove high molecular weight chlorinated lignins, colour, toxicants, suspended solids and chemical oxygen demand. But BOD and low molecular weight compound are not removed efficiently (Singh & Singh, 2004). The use of chlorine based chemicals in the bleaching process generates chlorophenol compounds which are completely resistant to microbial attack and remain as recalcitrants (Ghoreishi & Haghighi, 2007). The pollution load in terms of biological oxygen demand (BOD) from small paper mills is 2.5 times higher than that of large paper mills, which employ the soda recovery. Biological methods of the effluent treatment have the advantage of being cost effective and in addition to colour removal, they can also reduce both the BOD and COD

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of waste water (Christov & Driessel, 2003). Heavy metal removal by yeast from PPME has been achieved by (Thippeswamy et al. 2012). Multi-metal tolerant yeast with high concentration gradient of Cu, Zn, Ni, Cr, Cd and Pb was isolated. Result showed high Cd (52%) accumulation followed by Pb (45%), Ni (43%), Cr (41%), Zn (38%) and Cu (37%) in paper effluent treated with *Saccharomyces* sp.

Conventional anaerobic and aerobic treatment techniques are competent enough to partially reduce the COD and BOD in PPME. The anaerobic digestion and activated sludge process have also been used for treating PPME (Catalkaya & Kargi, 2008). The combinations of biological and physico-chemical methods were also done to find out the practical feasibility. Nakamura *et al*. (1997) were able to treat Kraft paper effluent to some extent using a combination of activated sludge process and ozonation, while Chandra *et al*. (2009) carried out aerobic treatment enclosed with activated sludge processes, aerated lagoons and biological reactors have also been used for bioremediation of paper mill effluent. The microorganisms reported for aerobic treatment were belonging to *Pseudomonas putida*, *Citrobacter* sp. and *Enterobacter* sp.

To develop sustainable bioremediation process, researcher also developed the fungal bioremediation along with some bacterial strains. Fungal bioremediation by *Phanerochaete chrysosporium* (Garg & Modi, 1999; Tang *et al*., 2005), *Lentinus edodes* (Durán *et al.*, 2002), *Trametes* (Coriolus) *versicolor* (Couto & Herrera, 2006), *Aspergillus niger* and *E.coli* (Suri & Sharma, 2012) has been reported. The main problem associated with fungal bioremediation is that fungi require additional supplements and other growth factors so that they can successfully grow on effluent. Also, the incubation time required for fungal bioremediation is more as compared to bacterial bioremediation (Malaviya & Rathore, 2007). Selvum et al. (2011) isolated three wood rot fungi *Polyporus hirsutus*, *Daedalea flavida*, *Phellinus* sp from Western Ghats of India, for bioremediation of PPME. Maximum decolourization of 62.2 % was achieved by *Phellinus* sp on 10th day of treatment. The chemical oxygen demand (COD) was also reduced to 3010 mg l^{-1} (42.1%) by *Phellinus* sp. In pilot scale, a maximum decolourization of 66.2% was achieved by *Polyporus hirsutus* on 10th day, inorganic chloride 582mg l^{-1} (105%) was liberated on the 10th day by *Phellinus* sp and the chemical oxygen demand (COD) was reduced to 3260mg l^{-1} corresponding to 37.3 % by *Polyporus hirsutus*. Employing enzymes like Laccase, xylanases, peroxidise, catechol dioxygenase, Mn peroxydase, cellobiose dehydrogenasein bioremediation of paper mill wastewater has been reviewed by Rao et al (2010). Mushroom (*Pleurotus florida*) has been used by Kulshreshtha et al. (2010) for bioremediation of solid sludge and effluent of both cardboard and handmade paper industries.

Earlier work focussed mainly degradation of lignins and PPME decolorization. Information about BOD and COD removal lignin degradation by indigenous microbes and their consortium using bacteria and fungi is scanty. Therefore, the main objective of current problem was to isolate dominant bacteria and fungi from contaminated soil sites and to investigate their individual and consortium effect on decreasing BOD and COD, increasing DO and lignin biodegradation under shaking and stationary conditions.

MATERIALS & METHODS

The dominant bacterial and fungal strains were isolated from the soil samples collected from the pulp and paper mill effluent (PPME) sites. It was hypothesized that the microbes isolated from their natural habitat have capability of surviving in harsh conditions by developing some catabolic enzyme systems, specific for particular components present in the natural habitat. Soil samples (from Meerut region, latitude 29°01'N, longitude 77°45'E, Uttar Pradesh, India) were collected in December, 2012 from the various contaminated sites of PPME, in clean sterile wide mouthed glass bottles and were shipped to the laboratory and kept at 4°C until analysed. Isolation of bacterial and fungal strains was done using nutrient agar (NA) and potato dextrose agar medium (PDA), respectively. Appropriate serial dilution of PPME soil was placed on the respective medium and incubated at 25°C for fungi and 28°C for bacteria. The dominant bacterial colonies were isolated, purified, identified on the basis of morphological, biochemical (Holt et al. 1994) and by further API kits (Biomerieux), while the fungal strain were identified on the basis of morphological characters (Yao *et al*., 2009).

Total 23 bacteria and 7 fungal strains were initially isolated from the soil samples. The isolated bacterial colonies were diverse in their morphologies, ranging from small pin-pointed to large sized, fluorescent to whitish, cream, yellow, orange. Total 9 bacteria out of 23 isolates and two fungi were selected, on the basis of their fast growth in the Nutrient medium and Potato dextrose medium containing PPME, not distilled water. The selected isolates were further screened for PPME bioremediation. To achieve this, NA and PDA medium were prepared using PPME and not by distilled water. The selected dominant soil isolates were streaked on Petri plates containing respective medium and incubated; based on the decolorization of medium, two

bacterial isolates *B. subtilis* and *M. luteus* and one fungal isolate *P. chrysosporium* were selected for final bioremediation progression. Further, to screen for BOD and COD remediation, selected 9 bacterial isolates and two fungal strains were again tested for their reduction potential using PPME under shaking (200 rpm) and stationary conditions. Based upon their rate of degradation of COD and BOD after 48 h at 28°C for bacterial strains viz. *Bacillus subtillis* and *Micrococcus luteus* and one fungal strain *Phanerochaete chrysosporium* were selected for final studies.

In a sterile 250ml Erlenmeyer flask, 100 ml of PPME was added and inoculated with bacterial inoculum, 1ml containing 10^8 cells and mycelial agar plugs of *P. chrysosporium* (~5 mm^2) were cut approximately 5 mm from the colony margin. The flasks were kept in two sets of stationary and shaking conditions (200 rpm) at room temperature of 26±1°C for nine days. The experiment was conducted in six sets with individual microbes and with their consortium. The samples were collected periodically after 3, 6 and nine days for measuring DO, BOD, COD, pH and lignin content. A control set was also kept at stationary and shaking conditions without inoculation of microbes for comparison.

In the present study, PPME samples were collected from a paper mill located in outskirt of Meerut city, India. The COD load of waste water varies from time to time due to use of various raw materials used in pulping and paper making process. COD of effluent of paper making unit (back water) varied from 935-1005mg/L, the BOD ranged from 130-136, dissolved oxygen (DO) 0.7-0.9 mg/L and the pH ranged from 8.9 to 9.2.

Physicochemical parameters including pH was analyzed on site at the time of sample collection by water analysis kit (Model LT-61, Labtronics, Guelph, Ontario, Canada) as per manufacturer instruction. Other parameters i.e. dissolved oxygen (DO), biological oxygen demand (BOD) and chemical oxygen demand (COD) were performed in laboratory by standard titrimetric method (APHA *et al.*, 1999). The data were analyzed statistically by using analysis of variance (ANOVA) to find out significance at 5% levels. In figures, error bars indicate standard error of the mean, where error bars are not visible; they are smaller than the marker.

Laccase activity was measured by using syringaldazine as a substrate as per the method of (Valmaseda *et al.*, 1991). The activity was assayed using using 1.0 ml of 0.2 M sodium phosphate buffer (pH 5.7) and 0.2 ml syringaldazine (1.6 mg/ml) in absoluted ethanol, (4.47 Mm). Reactions were initiated by the addition of syringaldazine and after mixing; incubations were conducted at 30°C for 1h, because after 1h highest enzyme laccase activity was observed. The absorbance was measured in a spectrophotometer (ELICO SL 150) before (0 time) and after incubation (60 min) at 526 nm and the increase in absorbance was calculated. One unit activity was defined as the enzyme producing one absorption unit/min at 526 nm.

RESULTS & DISCUSSION

The inoculation of PPME with microbes like *P. chrysoporiium*, *M. luteus* and *B. subtilis* individually and their consortium till 9 days led to decrease in pH from 9 (control uninoculated) to neutral and near neutral under shaking and stationary conditions (Fig. 1).

Increase in DO was found after inoculation of PPME with microbes individually and their consortium till 9 days. *P. chrysosporium*, *B. Subtilis*, *M. luteus* and consortium leads to increase in DO from 0.8 (control uninoculated) to maximum 6.4, 6.3, 6.5 and 6.8 after 9 days under shaking conditions (Fig. 2).

The reduction in BOD and COD of PPME with microbes individually and by their consortium generated encouraging results. *Phanerochaete chrysosporium* resulted in reducing BOD up to 87.2%, consortium 85.2%, *B. Subtilis* 61.5% and *M. luteus* 56.1% under shaking conditions (Fig.3). *Phanerochaete chrysosporium* and *B. subtilis* rectified COD up to 94.4 and 94.7%, while *M. luteus* and consortium decreased COD up to 89.6 and 92.7% respectively, again under agitation conditions (Fig. 4). The reduction of BOD and COD under stationary conditions was also appreciable but values were low compared to shaking.

The PPME inoculation with microbes individually and their consortium leads to commendable decrease in lignin content from 1.958 mg/L (control uninoculated) to minimum 0.057 mg/L after 9 days by consortium i.e. reduction up to 97%, while the *P. chrysosporium* reduced 95.8%, *B. subtilis* 95.1% and *M. luteus* 89.3% under shaking conditions (Fig. 5).

In pulp and paper industry, wood and wood materials are used in the production of chemical pulp, where in, lignin is degraded and dissolved almost completely in black liquor. If not removed from treated wastewater, the lignin presents a serious pollution and toxicity problem in aquatic ecosystem, owing to its biodegradability and high range of colour (Berryman *et al.*, 2004; Tišma *et al.*, 2010). Environment friendly manner for reducing COD, colour and BOD of pulp and paper effluent is in demand. The method should not be much time consuming and less expensive. Several physico-chemical methods were used they are efficient but more expensive.

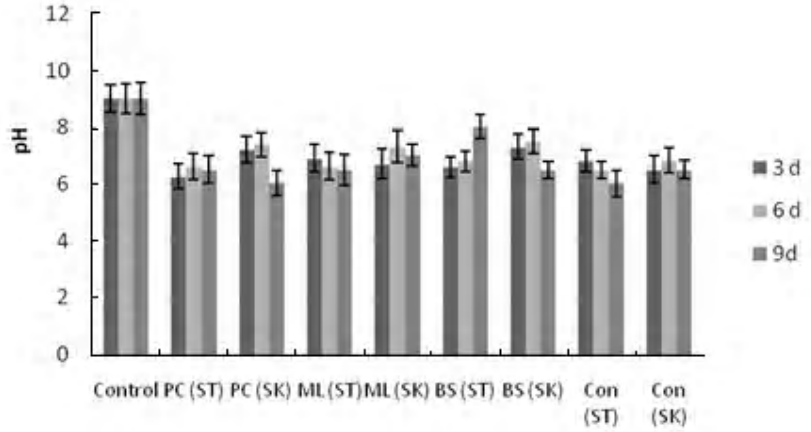

Fig. 1. Change in pH of PPME after different days under shaking and stationary conditions with individual microbes and consortium. (PC = *P. chrysosporium*; BS = *B. subtilis*; ML = *M. luteus*; ST = Stationary; SK = Shaking; Con = Consortium)

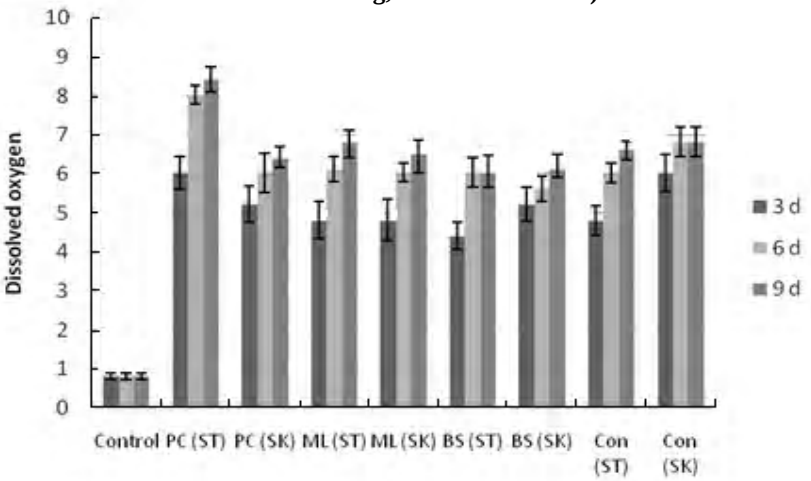

Fig. 2. Change in DO of PPME after different days under shaking and stationary conditions with individual microbes and consortium. (PC = *P. chrysosporium*; BS = *B. subtilis*; ML = *M. luteus*; ST = Stationary; SK = Shaking; Con = Consortium)

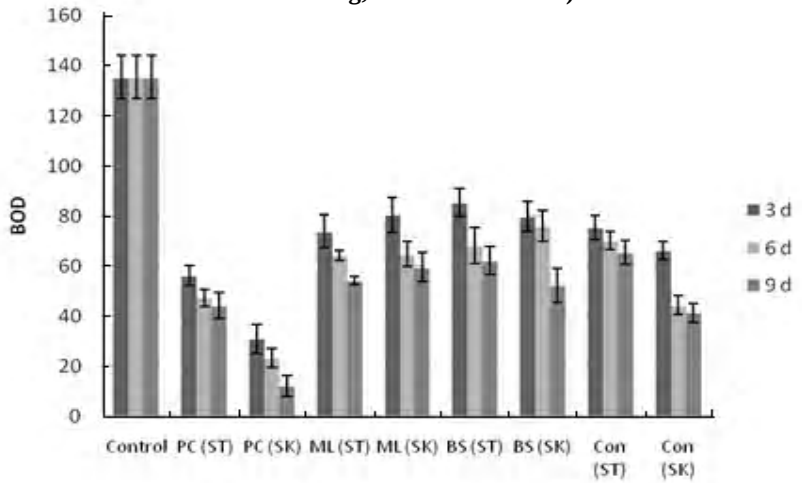

Fig. 3. Change in BOD of PPME after different days under shaking and stationary conditions with individual microbes and consortium. (PC = *P. chrysosporium*; BS = *B. subtilis*; ML = *M. luteus*; ST = Stationary; SK = Shaking; Con = Consortium)

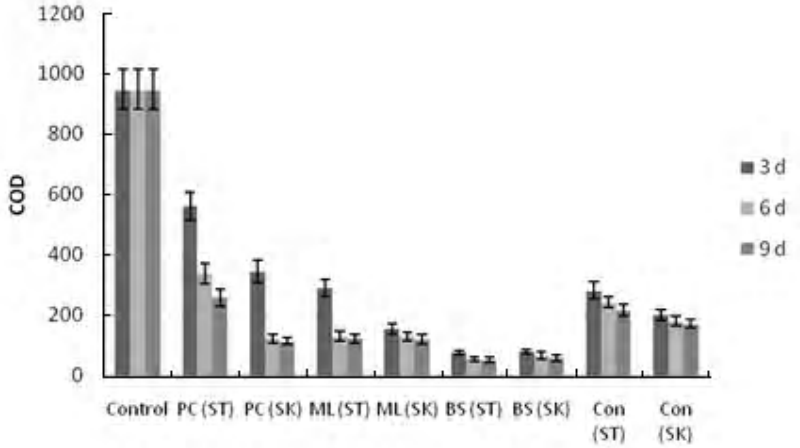

Fig. 4. Change in COD of PPME after different days under shaking and stationary conditions with individual microbes and consortium. (PC = *P. chrysosporium*; BS = *B. subtilis*; ML = *M. luteus*; ST = Stationary; SK = Shaking; Con = Consortium)

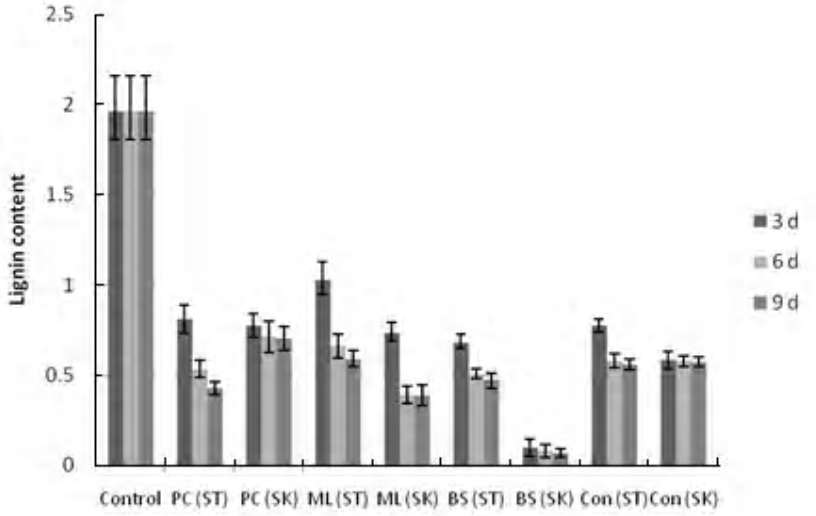

Fig. 5. Change in lignin (PPM) of PPME after different days under shaking and stationary conditions with individual microbes and consortium. (PC = *P. chrysosporium*; BS = *B. subtilis*; ML = *M. luteus*; ST = Stationary; SK = Shaking; Con = Consortium)

Researchers concentrated on bacterial degradation of the pulp and paper effluent in a less expensive and eco friendly manner. The results of the present study were compared quantitatively with those reported for bacterial bioremediation of pulp and paper mill effluents for COD and BOD and lignin reduction with respect to effective time interval of treatment. Previous reduction experiments of pulp and paper mill effluents using bacterial isolates are not much significant as compared to our results with respect to time interval of incubation; moreover early researchers used some sort of carbon and nitrogen source to sustain the proliferation of inoculated microbes, which is cost intensive.

Chandra *et al.* (2007) degraded Kraft lignin by three bacterial isolates which were able to reduce 69% color, 40% lignin and total substrate by 50% after 48 h. On the other hand, Chandra et al. (2009) used *Bacillus cereus* (ITRC-S6) and *Serratia marcescens* (ITRC-S7) for reduceding color (45-52%), lignin (30-42%), BOD (40-70%), COD (50-60%), in 7-day period supplementing the sample with glucose and peptone. Similarly, Raj et al. (2007) demonstrated that *Bacillus* sp. was able to remove 61%, 53%, 82% and 78% of color, lignin, BOD and COD within 6 days of incubation by adding co-substrate glucose and peptone. Gupta et al. (2001) employed two strains of *Aeromonas formicans* were able to remove 70% to 80% of COD, and lignin's while the color around 85% in 8 d. Also, Singh *et al.* (2008) used mixed culture of two bacterial strains and showed potential pentachlorophenol

degradation and decolourization of pulp and paper effluent which ultimately reduce high load of BOD, COD, TS, TDS and total suspended solids (TSS) after 168 h of incubation by addition of glucose and peptone as additional nutrient source. By using sulphate reducing bacteria, Hao & Man (2006) were able to remove COD up to 70 -75% after 3 weeks and increase to 82 - 88% by subsequent aerobic treatment for 48 h. Deschamps *et al.* (1980) used the bacteria and degraded lignin up to 98% after 5 days of incubation. In our efforts, the use of dominant aboriginal *B. subtilis* and *M. luteus* singly generated good results by reducing the BOD and COD in 9 days under agitation up to 61.5%, 56.1 % and 94.7%, 89.6%, respectively, without adding any additional growth factors.

Fungus and bacteria in combination were also used by some researchers to increase the degradation rate. The fungal-treated wastewater was again treated with the bacteria for the biodegradation process (Chupal *et al.*, 2005) used *Paecilomyces* sp. and *Pseudomonas syringae* and reported significant reduction in color (88.5%), lignin (79.5%), COD (87.2%) and phenol (87.7%) in two steps.

Several other treatment methods for reducing the load of pulp and paper effluent were also tried like chemical method in combination with biological agent (Ghoreishi & Haghighi, 2007). They used chemical and biological reactions in series and reported 99% of BOD and 92% of COD and 97% of TSS reduction after 6 d. In similar manner, Pedroza *et al.* (2007) used biological and photocatalytic treatment to reduce the pollutant levels in wastewater. Results showed that after whole sequential treatment 97% of COD and 99% of chlorophenol was removed after 96 h and 20 min incubation, but with addition of carbon and growth requirements of microbes, which augmented the cost of experiment.

In any PPME, lignin is the single most important activity in the biological cycle of carbon. The multitudes of inner unit bonds and functional groups and heterogeneity of the polymer is the main reason for the resistance of lignin for microbial attack and it is in fact one of the main reason that it is one of the most recalcitrant naturally occurring biological material. The utilization of lignin by inoculated microbes during this study period indicates that lignin has been utilized by microorganisms as carbon source and energy and for the production of hydrolytic enzymes (Abdulla *et al.*, 2000). The white rot fungi are well known for their remarkable enzymatic complex capable of degrading lignin in wood. Lignin peroxidase, Mn peroxidase and laccase are commonly found in many white rot basidiomycetes (Tein & Krik, 1983; Alessandro *et al.*, 2000). Removal of color from Kraft mill effluent was significantly good at certain amount of added carbon source, with the addition of excessive carbon source led to decrease in enzyme activity of laccase, lignin peroxydase, ß-galactosidase and Mn peroxydase, therefore, hampered the lignin removal capability and COD reduction from the waste water (Elisa *et al.*, 1999). In our study, *P. chrysosporium* also produced laccase (we have checked production of this enzyme only, because it plays key role in bioremediation and decolorization) and the efficient decolorization may be attributed to either through the ac-tion of extracellular enzymes such as laccase (Sirinivasan *et. al.*, 1995; Rodríguez *et. al.*, 1997) and/or biosorption by the fungal biomass. The decrease in pH (acidic) of PPME may also be due to conversion of complex organic compounds in simple inorganic acids and cell lysis (Singh, 2005; Sehanat *et al.*, 2009). The COD of the effluent also decreased due to removal of lignin.

The biodegradative capacities of *P. chryosporium* are remarkable. Of all the white rot fungi, this organism is the most studied and it has emerged a model in bioremediation of toxic, recalcitrant and colored effluent. The organism is unique both in terms of the number of different chemicals involved and its degradative ability. *P. chrysosporium* has been shown to be effective in removing color from textile-dye effluents of wastewater (Ashoka *et al.*, 2000). Unfortunately, due to the complex mechanisms involved in its biodegradative mechanism, the technology has been slow to emerge. Apart from the mechanisms involved in the degradation, the physicochemical parameters of the effluents are very important. In this work, we have isolated *P. chrysosporium* from the contaminated site of PPME. Use of this fungus as bioremediator did not let down the hope of researchers, as it performed excellently well under agitation conditions and utilized lignin as carbon source and reduced it to 0.081 mg/L from 1.958 mg/L after 9 days of incubation.

After 9 days of PPME incubation, the continuous decreasing trend in BOD, COD and lignin was not observed, which could be related to secondary metabolic compounds produced by these inoculated microbes (Freitas *et al.*, 2009). This observation suggested that greater time of incubation was not a positive factor for higher degradation rates of organic compounds present in the final effluent. Our study demonstrated that BOD and COD load could rapidly be reduced to 84% and 94 %; lignin to 97% and colour to 82% within 9d, using a 2% inoculum size, agitation at 200 rpm, and temperature at 26±1°C. After comparing the results with previous studies the time taken to complete the degradation process was less in an eco-friendly manner without addition of any carbon or nitrogen sources.

During the entire course of study, we did not add any carbon or nitrogen source in the medium. Selected microbes were directly inoculated in the PPME without any dilution. This was done to reduce the cost of experiment and secondly the isolated microbes and their enzymes were already acclimatized to PPME environment, and they have developed powerful lignin degrading enzyme system. These selected isolates were supposed to biodegrade PPME without adding any supplement to favour their growth and survival. However, this warrants a further investigation.

CONCLUSION

The results showed that native microbes isolated from the site of pulp and paper mill have the ability to use lignin, as carbon source and reduce the COD and BOD values after 9 days of incubation under optimized of shaking and stationary conditions. With the increased demand of paper, the treatment of effluents emerges as most pressing problem in environmental protection. Presently, bioremediation is taken to be an attractive option for reducing the pollution load from contaminated water because of its high efficiency and economical impact than the chemical remediation. The current study clearly showed that, application of native dominant bacterial and fungal isolates can be used for the treatment of large pulp and paper mills effluents. The fungal and bacterial isolates having appropriate enzyme activity with optimized proper physical conditions are playing significant role in the bioremediation process.

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