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Original Research Article

Synergistic Bacterial Detoxification of Rayon Grade Pulp Paper Mill Effluent

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Abstract

The rayon grade paper (RGP) manufacturing industry discharges a huge amount of effluent containing high level of colr, COD, BOD, resins, tannins and many potentially hazardous compounds released during various processing stages. Microbes especially bacteria are Mothers nature's innovative recyclers, converting toxic compounds into harness by product. In this study, bacterial strains were first isolated from sludge sample by nutrient enrichment technique and then subjected into two rounds of screening. The first screening was based on the bacterial efficiency to decompose lignin + PCP in the presence and absence of a simpler form of carbon and nitrogen and second on the basis of their COD, color and lignin reduction ability. Two bacteria designated as XPB4 and XPB6 showed highest ability to remove pollution parameters and were identified as S. marcescens, and Bacillus megaterium, respectively. These selected bacteria were used for further RGP treatment studies. Overall, the maximum reductions of COD, color and BOD after bacterial co-culture treatment were 91.6%, 76% and 85.8%, respectively than single culture. We also evaluate the BOD/COD ratio during bacterial treatment of RGP effluent, with this ratio increasing from 0.4 to 0.7 (42%) higher. This increase of BOD/COD indicates that the complex compound contributing to COD was decreasing more in RGP effluent. In addition, HPLC, GC-MS and Genetoxicity assessment showed that most of the toxic organic pollutants from RGP effluent have been removed by this develop bacterial co-culture.

Keywords: Effluent, rayon grade paper, genotoxicity, toxic compound, microbes.

Introduction

In the manufacture of paper pulp, wood of trees are first harvested and then cooked in a solution of sodium hydroxide and sodium sulphite at high pressure and temperature to break down the fibre into mass. In general, manufacturing of paper in paper industries involve two basic steps: pulping process for producing brown pulp and the chemical bleaching process for producing white pulp. For the manufacture of high quality rayon grade pulp (RGP), an additional chemical process involving extensive prehydrolysis of wood chips at high pressure and temperature followed by alkaline digestion[9]. This additional process ensure that the removal of residual lignin or other coloring agent with > 90% of cellulosic fibre. In the process, it also

generates a huge amount of colored effluent. However, high color of RGP effluent is due to the presence of lignin and its chloroderivatives. Due to its heterogeneous structure, lignin is somewhat resistant toward microbial degradation. This RGP effluent also contains high level of COD, BOD, resins, tanines and several potential chlorinated compounds[2,26].

Even after conventional treatment, a significant number of recalcitrant compounds such as 2 methoxyphenol, benzene carboxylic acid, phthalate etc remain in this effluent[1]. For the past 25 years, there have been reported that effluent released from various paper industries have the potential to negatively affect different flora and fauna[1,20,21,26]. Several authors reported that compounds such a chlorophenols, phthalate and its derivatives can cause toxicity at genetic level (genotoxic)[14]. Along with this, some authors also point out that these chemicals also have Endocrine disrupting ability[12,22] and this degree of toxicity turns this RGP effluent into Pandora box of waste chemicals. Therefore due to the complex and toxic nature of this RGP effluent, it is not so easy to completely eliminate it by traditional biological treatment methods.

In recent years many physical (microfiltration, photo-degradation, adsorption) and chemical methods (ozonation, oxidation, sedimentation) have been developed and adapted to reduce the pollution parameters from pulp paper mill waste. Further improvement have been observed in the removal of various organic pollutants from paper effluent by incorporating a hybrid method such as biological process followed by physic-chemical methods. On other hand, these systems require modification in existing treatment facilities available with paper industries. Therefore, any approach that proposes to look at existing effluent treatment services with better effectiveness would be more preferable and suitable for the paper industries.

Microbes are Mothers nature's innovative recyclers, converting toxic compounds into harness by product. In attempts so far in bioremediation methods, most of the literature has been limited to a few species of white rot fungus due to the presence of non specific extracellular enzymes (LiP, Laccase)[19]. But bacteria appear to be more effective than fungal strains at reducing pollution parameters from alkaline paper mill effluent, as they have greater environmental adaptability and biochemical versatility then fungal strains[7]. In addition, fungi typically grow at acidic pH and lowering the pH of entire effluent prior to apply a fungus system may not be cost-effective. Several bacterial strains belonging to verity of genera degrade different components of paper effluent. However, most of the studies were limited to the removal of toxic chemical by pure /single bacterial culture with model lignin[10,13,25]. The results of such studies are not relevant to the field because in nature microorganisms mostly grow in mixed culture. Some authors emphasize that, when mixed culture are used, substrate competition and cross inhibition can severely affect microbial growth and time lag of biodegradation. During lignin degradation, one strain produce toxic intermediate compound that are reducing syntropic manner by and other bacterium. Therefore, the present investigation focuses on removal of different toxic compounds from RGP effluent at high pollution loads. This work has been extended to toxicity assessment of bacterial treated and untreated RGP effluent that would be useful for the management of pulp paper mill effluent.

Material and methods:

Description of sample and sampling methods

The rayon grade pulp paper mill effluent samples were collected from main outlet channel of *M/S* Century Paper mill, lalkuan, Nainital, Uttarakhand, India in pre-sterilized container (10 litter capacities). Samples were brought to the laboratory and keep under 4°C until analysis. This RGP effluent sample was used for physic-chemical analysis, biodegradation studies and characterization of organic pollution before and after degradation experiment. For isolation of aerobic bacteria, the sludge sample was collected in a 500 mL beaker at the same location.

Chemical texture of pulp paper mill effluent

The physic-chemical analysis such as total solid, dissolve solid of RGP effluent was done according to the standard protocol given in[3] (American Public Health Association).The pH and electrical conductivity (EC) of effluent sample was analysed with the help of selective electrode (Thermo Orion Model 960). Before EC measurement the electrode was calibrated at room temperature (25°C) with the standard (1413 µS/cm and 12900µS/cm). The alkalinity of sample was done by titration method[3] (Method number: 2320B) with the help of standard solution of sulphuric acid solution (0.1N). Other parameters such as color by Cobalt Platinum method, Phosphate by Stannous chloride method, Sulphate by Turbidimetric method, Biological oxygen demand (BOD) by 5-day test and Chemical oxygen demand (COD) by Open reflex method were analyzed.

Media composition and source of bacteria

The Final effluent amended with 0.5% glucose and 0.1% peptone having following characteristics: lignin: 2283 mg/l, Color: 1995 copt, BOD: 1288 mg/l, COD: 3092 mg/l, sulphate: 750 mg/l, nitrate: 20.77, iron: 9.08, nickel: 2.01, lead: 1.01 and Phosphate: 35 was used for final screening and further bacterial degradation study.

Isolation, screening and identification of potential bacteria

One gram sludge sample was transfer aseptically to final effluent. Flasks were kept on shaking incubator at 120 rpm for ten days at 30°C (New Brunswick, Innova-4230 USA). When sample showed reduction in color and COD then we took 0.1 ml sample and spread on lignin amended solidified plates with 15 gm/L agar (HiMedia, India). Plates were kept in incubator at 36 °C until colonies developed. Twelve purified bacterial strains designated as XPB1, XPB2, XPB3, XPB4, XPB5, XPB6, XPB7, XPB8, XPB9, XPB10, XPB11 and XPB12 were screened first on the basis of presence and absence of carbon and nitrogen source and second on the basis of their COD, color and lignin reduction potential. Further, identification of final screened bacteria was further subjected into identification process by classical biochemical methods. **Two bacteria designated as** XPB4 and XPB6 showed highest ability to remove pollution parameters and were further identified by biochemical method as *S. marcescens*, and *Bacillus megaterium*, respectively. These selected bacteria were used for further bacterial treatment studies.

Removal of toxic pollutants by axenic and co-culture condition

Transfer a 4% (v/v) overnight grown bacterial suspension of constructed co-culture (inoculum size 2.9 x 105 and 2.6 x 105 for *S. marcescens*, and *Bacillus megaterium*, respectively) aseptically to the 250 ml flask containing a 95 ml RGP final effluent. Samples were taken every day and end of experiment and analyzed for pollution parameters (colour, lignin, COD and BOD) and toxicity assessment. During the treatment of RGP effluent, the dynamics of cell growth (by cfu/ml), pH

and enzyme activity[9] of bacterial strains were analysed. The BOD/COD ratio was also calculated by method given by[16].

Metabolite characterization by HPLC and GC-MS

After bacterial treatment, degraded samples with their respective control were centrifuged at 10,000 rpm and acidified (pH 1-2) with 0.1 HCl. for qualitative estimation of organic compounds from RGP effluent was done according to method of[1] with minor modification. In brief, 50 ml of was taken degraded samples with their respective control in separating funnel (100 ml) extracted with 50 ml ethyle acetate by shaking it at room temperature (35 °C) for 15 minutes. Organic layer was pooled together, collected and dried by using vacuum evaporator. Sample was reconstituted to 1ml prior to injection and ready for further studies (HPLC and GC-MS). For HPLC samples were analyzed using a (Waters, 515 HPLC), equipped with 2487 UV/VIS detector, via millennium software. A 10 µl Sample was njected followed by implementation of HPLC grade acetonitrile/ water (70:30) at a rate of 1 ml/min. Reverse phase C-18 column at 27 °C were used to analyze lignin at 280 nm.

For GC-MS analysis dry residues of ethyl acetate extracts were analyzed as trimethylsilyl (TMS) as described (Lundquist and Kirk 1971). The identification of low molecular weight lignin-related compounds derived after bacterial treatment was done by comparing their mass spectra with that of the NIST library available in the instrument and by comparing the retention time (RT) with those of authentic compounds available.

Genotoxicity evaluation after bacterial treatment

Cell Line and their maintenance

The main goal of this study was to assess the induced toxicity of RGP effluent on human epidermal keratinocyte cell line. This cell line is mainly used for evaluation of toxic potential for different chemical compounds. The toxicity profile of RGP effluent is very important for the evaluation of induced degree of toxicity on human, because ultimately human will affected by this effluent in different way. Hence, in this study toxicity test was performed with this cell line [1].

Genotoxicity assessment

Cells were grown in 96-multiwell black plates (2 x 10^5 cells/well). This 96 well plate were treated with four types of samples (CM-control/DMSO alone; untreated rayon grade paper mill effluent: UT; bacterial treated sample after 5^{th} day: BT5 and bacterial treated sample after 9^{th} day BT9). After completion of treatment time, the exposed cells were mixed with low melting agarose and spread on slides. The slides were incubated in lysis solution for overnight at 4 °C. They were washed with electrophoresis buffer and further incubated in the same buffer for 20 minute. Then electrophoresis was performed for 25 min at 22 Volt. After electrophoresis the gels were neutralized in neutralization buffer. Finally, the slides were stained with EtBr (15 µg/ ml) for 5 min and dipped in chilled distilled water to wash off excess EtBr and cover slip placed in dark humidified box to prevent drying of the gel. Slides were scored using an image-analysis system (Kinetic Imaging, Liverpool, UK) attached to a fluorescence microscope (Leica, Germany) equipped with appropriate filters (N2.1 excitation wavelength of 515-560 nm and emission wavelength of 590 nm). The comet parameters recorded were olive tail moment (OTM, arbitrary unit) and tail DNA (percent %).

Statistical Analysis

Numerical data were presented in the form of mean and standard deviation. One way ANOVA with post hoc test were applied to

analyze the significant difference in among physico-chemical parameters in screening and genotoxicity. Statistical analysis was carried out using the statistical package for the social science, version 22 (SPSS-22, IBM, Chicago, USA). The level of significance that means, p value was set as <0.05. Graphics were prepared by the help of SPSS and Prism Software.

Results and Discussion

Description of Study area

In India, Century Pulp Paper Mill is one of the largest paper manufacturing industries exclusively in manufacturing of rayon grade paper (RGP). Apart from RGP, this paper mill is the largest manufacturer and exporter of boards, tissues and pulp in India with a capacity of about 1500 metric tonnes per day from a single location. It is continuously in operation since 1984 and has been a major player in the domestic and export market for this paper industry. This paper mill uses a variety of raw materials like wheat straw, bamboo, eucalyptus and bagasse to make paper.

Chemical texture of collected effluents

Physico-chemical analysis is the basic tool that defines the nature and actual pollution load or condition of any industrial wastewater. The chemical texture and heavy metals data along with their statistical measures such as mean, median with standard deviation for the collected effluent are presented in Table 1. The pH and EC were 7.28 and 1639.7±85.6 found in RGP effluent. Usually the dissolved solids in natural water are mainly inorganic salts such as sulfates, chlorides, phosphates and nitrates of calcium, magnesium, sodium, potassium, iron, etc., and in addition to all these compounds, small amounts of organic matter and Dissolved gases[15]. The values of total dissolved solids (TDS in mg/L) in RGP flows were 1207.8 ± 18. According to various natural and international environmental agencies, water with TDS above 500 mg/l is not considered desirable for drinking water supply, however where better water is not available, more mineralized water is also used. Water containing TDS > 500 mg/L causes gastrointestinal irritation in humans, so it was not recommended for drinking purposes in our Indian subcontinent.

A high mean value of color (1995.08±27.3 copt), BOD (mg/L) (1288±86.2) and COD (Mg/L) (3092±96.43) and lignin (2283.5±18.58mg/L) were showed in the RGP effluent. In fact, the color of pulp paper mill effluent is mainly due to lignin and its derivatives. The very high average values of sulphate (750.61±22 mg/l) and nitrogen in the form of nitrate (4.61±0.58mg/l) and nitrite (20.77±1.1 mg/l) were recorded in RGP effluent, respectively. The source of sulfate ions in the effluent is sodium sulfite which is used in the process of making brown pulp and the nitrogen present in the lignin contributes to the nitrate. In fact, the sodium sulfite used in pulp during paper making is the major source of ions, and the nitrogen in this effluent comes from the skeleton of lignin[26]. As we know, the discharge of inorganic matter such as sulfate, phosphate and nitrogenous wastes into the aquatic system causes many environmental problems like eutrophication in the aquatic ecosystem, especially in summer[24]. It is this same eutrophication that causes significant hammering of native species and the assault of exotic and opportunistic natives more tolerant to this kind of pollution. It may be noted that sulphate is responsible for the odor but has comparatively less effect on the taste of water than chloride. This result was consistent with an earlier report for fish life in the aquatic environment surrounding paper mill flows[5]. In addition, RGP effluent was found to have a low BOD/COD ratio (approximately 0.3-0.4) which is due to the lignin and chlorophenolic content which

offer higher COD and color payoff than BOD[9]. The adverse effects of pulp-paper wastewater with high loads of BOD and COD in aquatic systems have been reported by many authors[5].

The data obtained from heavy metal analysis are also present in Tables 1. The mean values of heavy metals (mg/L) of RGP effluent were recorded as 1.83 for Zn, 0.68 for Pb, 7.19 for Ni, 1.08 for Cr, 0.85 for Mn. Compared to other metals, Fe was found to have reasonably the highest concentration of heavy metals. The iron present in water after a level can be very harmful for any kind of household chores. Various international agencies and the Bureau of Indian Standards have recommended 0.3 mg/l as the desirable limit and 1.0 mg/l as the maximum permissible limit of iron in drinking water[4]. The metals tested in this study except Fe, Ni and Pb were below the detection limit which may come from corrosion of the digestive vessels and possibly due to the bioaccumulation of these metals by the treatment plants.

Table 1: Chemical Texture of RGP effluent samples

Characteristics	Unit	Mean.	Median	Std.Dev.
pH		7.28	7.2	0.32
Conductivity	(µS/cm)	1639.72	1600.49	85.67
COD	(mg/l)	3092	3010.8	96.43
BOD	(mg/l)	1288	1300	86.16
TDS	(mg/l)	1207.83	11.36	18.09
K	(mg/l)	6.847	6.654	1.08
Lignin	(mg/l)	2183.5	2280.19	18.6
Color	(CoPt)	1995.08	2000.1	27.3
Cl^-	(mg/l)	138.08	140.12	15.7
T. Hardness.	(mg/l)	406.59	332	6.66
Ca	(mg/l)	32.04	34.09	5.04
Mg	(mg/l)	72.08	75.41	1.53
T. Alkalinity.	(mg/l)	267.91	270.88	4.33
NO_2^-	(mg/l)	20.77	23.64	1.06
SO_4^{2-}	(mg/l)	750.61	736.45	22.04
PO_4^{3-}	(mg/l)	35.07	34.06	1.77
F^-	(mg/l)	0.86	0.912	0.05
NO_3^-	(mg/l)	4.61	4.80.5	0.58
Heavy metals				
Cu	(mg/l)	0.01	0	0.03
Fe	(mg/l)	9.08	8.86	0.87
Mn	(mg/l)	0.85	0.94	0.14
Cr	(mg/l)	1.08	1.37	0.06
Ni	(mg/l)	7.19	8.1	0.07
Pb	(mg/l)	0.68	0.71	0.013
Zn	(mg/l)	1.83	1.9	0.04

Isolation and screening potential bacteria for reduction of pollution parameters of RGP effluent

Microorganisms can develop the ability to genetically modify themselves and remove xenobiotic compounds if they have been in their harsh environment for long periods of time. A variety of bacteria have been claimed over the years for pulp-paper effluent remediation, isolated from soils contaminated with waste released by the pulp-paper mill[1,17,11]. But the problem is that most of the works are on synthetic and model lignin and their model fails on real practical[11]. At the same time, reports on bacterial species are inconsistent as well as little compared to other organisms that can remove pollution from this RGP effluent[9]. These grounds require an ongoing search for additional efficient bacterial species that can be used to treat actual pulp-paper mill wastewater rather than model lignin.

A total of 18 bacterial species based on different colony morphologies were isolated from sludge on PCP and lignin amended agar containing 0.5% glucose and 0.1% peptone as co-substrate in medium. However, purification of these bacterial species by microscope revealed that only 12 of the 18 strains were pure. First screening of potential bacterial strains was based on the bacterial efficiency to decompose lignin + PCP in the presence of a simpler form of carbon (glucose) and nitrogen (peptone). Various concentrations of carbon and nitrogen such as 0%, 0.5% and 1.0% were added to the culture medium prior to sterilization. The bacterial growth pattern and color reduction dynamics are shown in Table 2. These results suggest that most bacterial strains were unable to grow without additional carbon and nitrogen sources. The bacteria XPB1, XPB2, XPB3, XPB7, XPB8, XPB9, and XPB12 showed spectacular growth and discoloration up to 1000 ppm, but declined significantly thereafter. In addition, only a significant bacterial increase was observed at 1% glucose and peptone at all concentrations of lignin and PCP, but not reduced color. Because of this we found 0.5% glucose and peptone to be optimal for further study.

The RGP effluent having following characteristics lignin 2283 mg/l, BOD 1288 mg/l, COD 3092 mg/l, sulphate 750 mg/l, nitrate 20.77, iron 9.08, nickel 2.01, lead 1.01 and Phosphate 35 was used for final screening and further degradation study**.** Twelve pure isolated bacterial strains were screened for their COD, color and lignin reduction potential. The most efficient bacterial strains XPB4 and XPB6 were obtained from 12 bacterial isolates, which reduced 46, 42% lignin and 55, 48% color and 48, 41% COD, respectively. In addition, this study was extended to evaluate the most efficient bacterial strains XPB4 and XPB6 in mixed condition. The results show that mixed culture of two bacteria (XPB4 and XPB6) reduces 49% lignin, 60% color and 53% COD from pulp paper mill effluent. Therefore, these two isolates were selected for further identification studies. This study also shows that the mixed culture of these two bacteria was more efficient than the single one.

Table 2: Screening of tolerance pattern of bacterial isolates with and without glucose

Strains codes	Different concentration of Lignin and PCP														
	500 ppm			1000 ppm			1500 ppm			2000 ppm			2500 ppm		
	Different concentration of glucose and Peptones (%)														
	0%	0.5%	1%	0%	0.5%	1%	0%	0.5%	1%	0%	0.5%	1%	0%	0.5%	1%
XPB1	-/-	+++/***	+++/***	-/-	+++/***	+++/**	-/-	+++/**	++/*	-/-	+/*	++/-	-/-	-/-	-/-
XPB2	-/-	+++/***	+++/***	-/-	+++/***	+++/**	-/-	+++/**	++/*	-/-	++/**	++/*	-/-	-/-	-/-
XPB3	-/-	+++/***	+++/***	-/-	+++/**	+++/**	-/-	+++/***	++/*	-/-	+++/**	+/-	-/-	-/-	-/-
XPB4	**++/****	**+++/*****	**+++/*****	**++/****	**+++/*****	**+++/****	**++/****	**+++/*****	**+++/****	**++/****	**+++/*****	**+++/****	**++/****	**+++/*****	**+++/***
XPB5	-/-	+++/***	+++/***	-/-	+++/***	+++/**	-/-	+++/**	+/*	-/-	+/*	+/-	-/-	-/-	-/-
XPB6	**++/****	**+++/*****	**+++/*****	**++/****	**+++/*****	**+++/****	**++/****	**+++/*****	**+++/****	**++/****	**+++/*****	**+++/****	**++/***	**++/****	**++/***
XPB7	-/-	+++/***	+++/***	-/-	+++/**	+++/**	-/-	++/*	++/*	-/-	+/*	+/*	-/-	-/-	-/-
XPB8	-/-	+++/***	+++/***	-/-	+++/**	+++/**	-/-	+++/***	++/*	-/-	++/-	+/-	-/-	-/-	-/-
XPB9	-/-	+++/***	+++/***	-/-	+++/***	+++/**	-/-	+++/**	+++/*	-/-	+/-	+/-	-/-	-/-	-/-
XPB10	+/*	+++/***	+++/***	+/*	+++/***	+++/**	+/-	+++/***	+++/**	+/-	++/*	++/*	-/-	-/-	-/-
XPB11	+/*	+++/***	+++/***	+/*	+++/***	+++/**	-/-	+++/**	+++/*	-/-	++/*	++/*	-/-	-/-	-/-
XPB12	-/-	+++/***	+++/***	-/-	+++/***	+++/**	-/-	+++/***	+/*	-/-	+/-	+/-	-/-	-/-	-/-

*+: poor growth; ++: moderate growth; +++luxuriant growth; *: poor decolorization (<20%); **: moderate decolorization (20-50%); ***Good decolorization (50-70%); -/-: no growth no declolorization*

Biochemical identification of screened bacteria:

An interesting thing has emerged for the bacteria code XPB4. The colony of XPB4 bacterium appears red on nutrient agar medium but the color of this bacterium was not visualized on screening media containing lignin and phenol. This might be due to the selective pressure of lignin and PCP on the bacteria. Furthermore, this XPB4 strain was motile, rod-shaped, and passed a Gram-negative test. The second bacterium coded XPB6 is showing a creamy nature in the colony and no sign of any pigmentation. This XPB4 strain was non-pigmented that produced motile, rod-shaped bacteria. The details of the biochemical testing of bacteria XBP4 and XBP6 are given in Table 3**.** Finally on the basis of biochemical test both bacteria XBP4 and XBP6 were identified as *Serratia marcescens* and *Bacillus megaterium* respectively.

Degradation experiment:

During the course of degradation, both bacterial strains showed steady growth up to the fourth day. However, the cfu number of the co-culture was dominated over single bacterium. Initially, *Serratia marcescens* showed a slow growth rate but a continuous log phase that came to dominate *Bacillus megaterium* after the 5th day (Figure 1A). The basis for the success of these investigated bacteria may be due to the production of laccases and peroxidases that are used for ligninolytic activity. *S. marcescens* is known to production of laccase enzyme and its activity is positively related with mineralization or degradation of lignin and its derivatives[23]. Several authors have reported that *Bacillus* sp. has shown good potential for the degradation of paper effluent and different toxic pollutants[25]. However, the possibility of using these two organisms as co-cultures for bio-treatment of paper mill effluents forming rayon grades paper has not been explored. In this study, the results showed that enzyme activities and bacterial growth were directly related, the maximum LiP activity was observed on day 4 when *Bacillus* was dominantly growing and the highest activity of laccases was observed on day 6 and 7 where growth of *S marcescans* was high (Figure 1B). Therefore, in this study, enzyme activity was found to be related to growth with peroxidase activity first followed by laccase. Data obtained from co-culture treatments, the presence of each bacterial strain in the culture medium showed a cumulative growth effect on bacterial growth and reduced by various pollution parameters rather than inhibition. Despite the rapid growth of bacteria in the early stages of decay, there was less bleaching. This may be due to the use of a simpler form of carbon and nitrogen source available in the growth medium. But as soon as these simpler forms are depleted, bacterial starvation leaves no choice but to use lignin as a co-substrate. Hence lignin and color start to decline after 2 to 3 days. This study was consistent with the co-metabolic mechanism in fungal degradation of pulp paper mill effluent, which has also been reported by various authors[26].

A change in pH towards acidic conditions was noted during the initial 1 to 2 days of degradation, indicating the utilization of the simpler form of carbon sources present in the medium by bacterial co-culture. Because simpler carbon sources give a different acidic by-product at the end of the carbon cycle, the pH is lower in the early days[25]. As the carbon source is depleted, the pH of the medium will shift towards higher, facilitating lignin mineralization because lignin is soluble at higher pH. This is the reason that the reduction of lignin starts after 2 days (Figure 2 A). Overall, the reductions of COD, color and BOD after bacterial co-

culture treatment were 91.6%, 76% and 85.8%, respectively. This reduction after bacterial co-culture treatment can be attributed to the removal of complex colored compounds and other organic and inorganic substances present in the pulp paper mill effluent to meet nutritional requirements.

The BOD/COD ratio was also evaluated in this study (Figure 2B). The lower BOD/COD ratio is basically due to the presence of higher molecular weight compounds, i.e. lignin and its derivatives that contribute more to COD and color rather than BOD[16]. A low BOD/COD ratio (about 0.4) was observed in untreated (control) paper mill effluents. In this study we evaluate the BOD/COD ratio at the end of the experiment, with this ratio increasing to 42% (0.4 to 0.7). This increase of BOD/COD indicates that the complex compound contributing to COD was decreasing more. This means that industrial effluents, which were partly or at low levels earlier, can now be easily degraded.

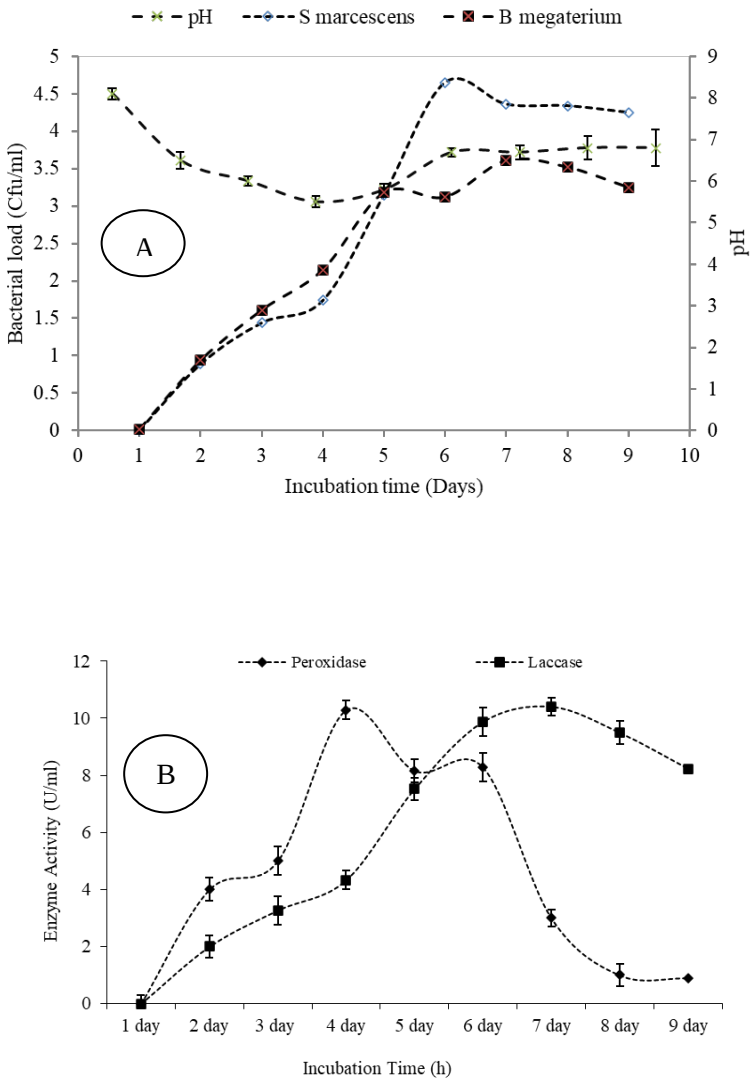

Figure 1: Bacterial growth dynamics (A) and Enzyme activity (B) (Peroxidase and Laccase) during time course of bacterial treatment study.

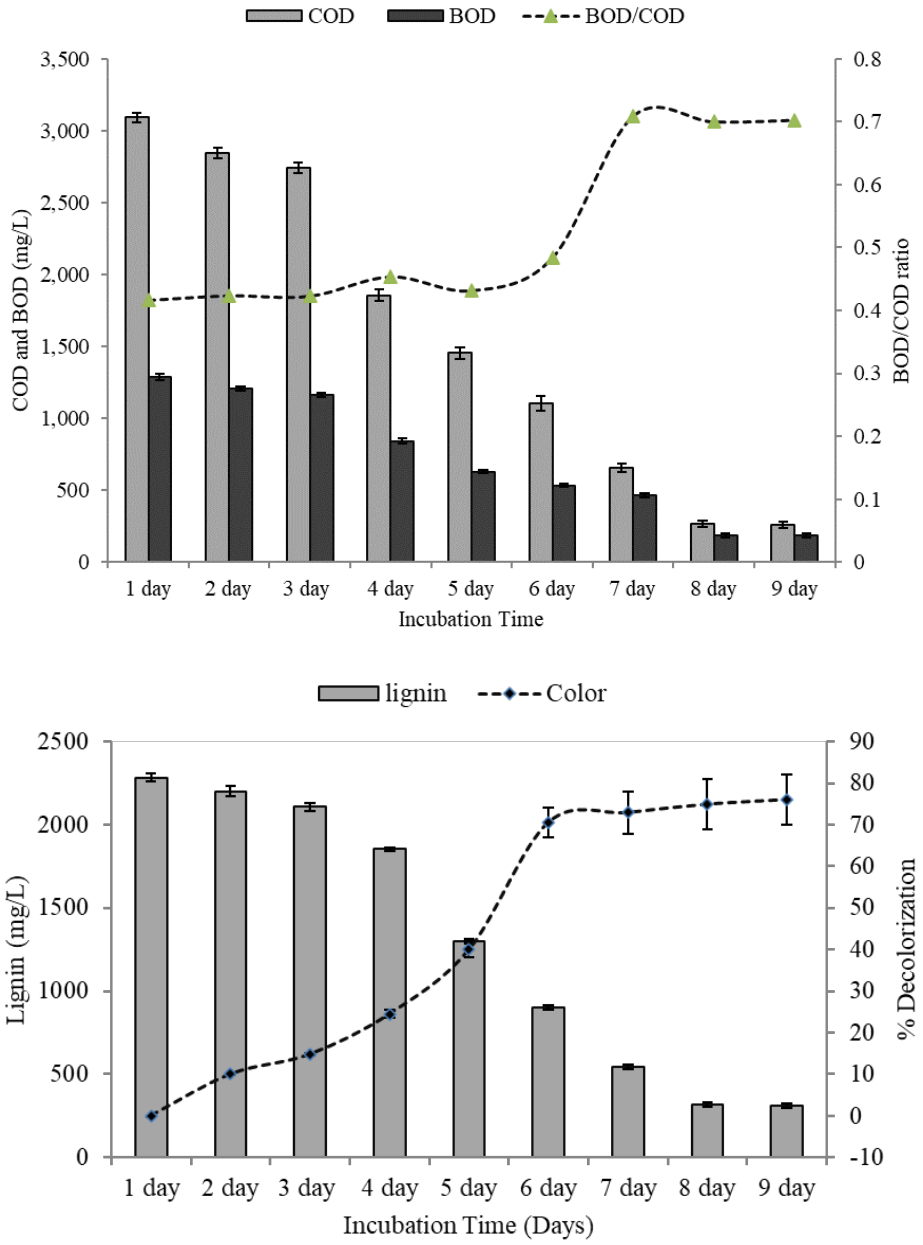

Figure 2: Reduction of pollution parameters Lignin and color (A) and COD, BOD (B) during time course of degradation study

Characterization of EDCs by HPLC and GC-MS before and after bacterial co-culture treatment:

In this study three samples were extracted from pulp paper mill effluents; the first was from untreated effluent, the second and third were from bacterial treated samples on the 5th day and the last day of the experiment, which was on the 9th day. The reduction in pollution parameters was finally confirmed by HPLC analysis. HPLC of bacterially treated samples shows reduction as well as shifting of peaks as compared to untreated control (Figure 3). This decrease of HPLC peaks indicates a reduction or degradation with minor biotransformation. Furthermore, a significant reduction in terms of color intensity of the final effluents was

observed after bacterial treatment, clearly suggesting that isolated bacteria degrade lignin and its derivatives by ligninolytic action[25].

In recent years, scientists have characterized a range of metabolic products of paper flux. Some workers claim that certain metabolic compounds have endocrine disrupting properties[18,22]. To confirm the degradation and identification of a variety of metabolic products that may have EDC properties, control and bacterial treated samples were analyzed with the help of GC-MS. GC-MS analysis of the untreated control sample showed prominent peaks between 9 and 29 retention times (RT-min) (Supplementary material), and the details of the compounds generated after bacterial treatment of the final effluent are listed in Table 4.

The GCMS results showed a significant qualitative and quantitative difference in the pattern of compounds obtained after bacterial treatment of the final effluent compared to the control sample. The total ion chromatogram (TIC) of the bacterially treated sample showed a heavier consumption of the compounds as compared to its untreated control sample. This consumption of compounds after bacterial treatment indicates that the developed co-culture has a strong potential to utilize the carbon, nitrogen and components of this final flux as the sole source of energy. Several compounds which are in aromatic in nature were detected in the untreated control sample, such as phenol 2-methoxy (guaiacol) (RT 11.40 min), 2-hydroxymethyl cyclopropane carboxylic acid (12.01), 3-methoxyphenol (RT = 14.35), phenol, 4-ethyl -2-methoxy (RT = 15.1), phenol, 2,6-dimethoxy (RT = 15.9), benzene, 1,2,3-trimethoxy-5-methyl (RT = 17.7), low-molecular-weight of phenolic units of lignin as a derivative[1,25]. In addition to this finding, other workers have also reported more acid-type compounds than aldehyde and ketone-types to cause degradation of lignin[13].

Compounds such as 2-methoxy phenol, phenols, pentacholorophenol (RT=19.74), Phthalate and its derivative such as phthalic anhydride (RT=15.6) is produced after degradation of lignin. photodegradation of lignin. This result was consistent with[9] who worked on bacterial degradation of black liquor lignin. Phthalic anhydride is a phthalate derivative belonging to the class of endocrine disrupting chemicals. Some workers study on Phthalate and its derivatives and discovered that this compound is associated with decreased fertility, pregnancy loss and adverse obstetric outcomes[6,12].

GC-MS peaks showed that these EDCs compounds were removed after bacterial treatment. In addition, compounds such as ethanone, 1-(-4-hydroxy-3,5-dimethoxyphenyl) (RT = 19.8), 1,2-benzenicarboxylic acid (RT = 26.01) were produced as new metabolites. On the other hand, compounds such as cholesterol trimethylsilyl ether (RT 23.56 min) remain unchanged. Cholesterol is the trimethylsilyl ether derivative agent used during the derivatization process. Thus, this bacterial co-culture showed potential application for bioremediation of rayon grade paper mill final effluent with high pollution load.

Table 3: Biochemical characterization of screened bacterial strains

Biochemical Tests	Strains Code	
	XBP4	XBP6
Gram reaction	Gram -ve	Gram +ve
capsule	-ve	-ve

spore	-ve	+ve
Shape	Rod	Rod
Motility	+ve	+ve
Catalase	+ve	+ve
Oxidase	-ve	-ve
O/F test	Facultative anaerobes	Facultative anaerobes
Urea	+ve	+ve
H2S	-ve	-ve
Pigment	Red pigment +ve	-ve
Citrate	+ve	-ve
Indole	-ve	-ve
MR	-ve	-ve
VP	+ve	+ve
Gelatin hydrolysis	+ve	+ve
Gas production	Variable	+ve
Acid Production	+ve	+ve
Sugar fermentation		
Dextrose	+ve	+ve
Mannose	+ve	+ve
Sucrose	+ve	-ve
Mannitol	+ve	+ve
Trehalose	+ve	-ve
Dulcitol	-ve	-ve
Galactose	+ve	+ve
Arabinose	-ve	+ve
Xylose	-ve	+ve
Lactose	-ve	+ve
Raffinose	-ve	-ve
Sorbitol	+ve	-ve

+ve=positive, -ve=Negative; O-Oxidative, F-fermentative

Table 4: Identified compounds of before and after bacterial treatment of pulp paper mill effluent.

S no.	**COMPOUNDS**	**RT**	**Contro**	**After 5^{t} day**	**After 9^{t} day**
1	Acetamide, n-(beta,-merceptoethyl)-	10.52	-	+	-
2	Propane , 2,2-bis(ethylthio)-	10.59	+	-	-
3	Silane, ethoxytrimethyl-	11.01		+	
4	Triacetone triperoxide (tatp)	11.14	+	-	-
5	Acetamide,2-(2-hydroxyethoxy)-	11.40	+	+	
6	Acetic acid	11.46	-	-	+
7	Silane, (1,4-dioxan-2-yloxy)trimethyl	11.92	-	+	-
8	2-hydroxymethyl cyclopropane carboxylic acid methyl ester	12.01	+	-	-
9	2-Methoxyphenol trimethylsilyl ester	14.35	+	-	-
0	Phenol, 4-ethyl-2-methoxy-	15.10	+	-	-
11	Phthalic anhydride	15.69	+	-	-

12	Phenol,2,6-dimethoxy	15.9	+	-	-
13	Ethanol,2-propoxy	16.0	+	-	-
14	Pyridinium, 1-amino-4methyl-, hydroxide,	16.6	-	+	-
15	Ethanone, 1-(-4-hydroxy,3-methoxyphenyl)	17.5	+	-	-
16	Benzene,1,2,3-trimethoxy-5-methyl-	17.7	+	-	-
17	Pentachlorophenol	19.7	+	-	-
18	Ethanone, 1-(-4-hydroxy-3,5-dimethoxyphenyl)-	19.8	-	-	+
19	5-Amino-2-methoxyphenol,	19.9	+	-	-
20	Ethanone, 1-(-4-hydroxy-3,5-dimethoxyphenyl)	20.0	+	-	-
21	Benzeneacetic acid	20.1	+	-	--
22	1,2-Benzene dicarboxylic acid,	20.9	+	+	-
23	Hexadecanoic acid	21.6	-	+	-
24	Pyrrolo [1,2-a] pyrazine-1,4-dione	21.8	-	-	+
25	Hexadecanoic acid	22.2	+	-	+
26	Clionasterol acetate	22.6	-	+	-
27	1-Napthalenepropanol,	22.7	-	-	+
28	Octadecane-1,2-diol, bis(trimethylsilyl) ether	23.0	-	-	-
29	Octadecanoic acid	23.2	+	-	+
30	Trimethylsilyl ester	23.5	-	-	+
31	Methoxycinnamic acid	24.5	+	-	-
32	Dotriacontane	24.6	-	-	-
33	Undecane,3,8-dimethyl-	24.8	-	-	-
34	Docosane, 1, 22-dibromo-	24.8	-	+	-
35	Decane, 1-bromo-2-methyl-	25.6	-	+	-
36	1-Hexene, 3,5,5-trimethyl-	26.0	-	-	-
37	1,2-Benzenedicarboxylic acid,	26.0	+	-	+
38	Thiazole,2-(-thienyl)-	26.0	-	+	-
39	Hexadecanoic acid,2,3-bis[(trimethylsilyl) oxy propyl ester	26.1	-	-	+
40	Phthalic acid bis (7-methyloctyl) ester	26.4	-	+	-
41	Triacontane	27.3	-	-	-
42	Heptacosane	27.3	-	+	-
43	Tetradecane, 2,5-dimethyl-	28.3	-	-	-
44	Pentatriacontane	28.3	-	+	-
45	Hexadeca-2,6,10,14- tetraen-	28.5	-	-	-
46	Squalene	28.6	+	-	-

RT: Retention Time

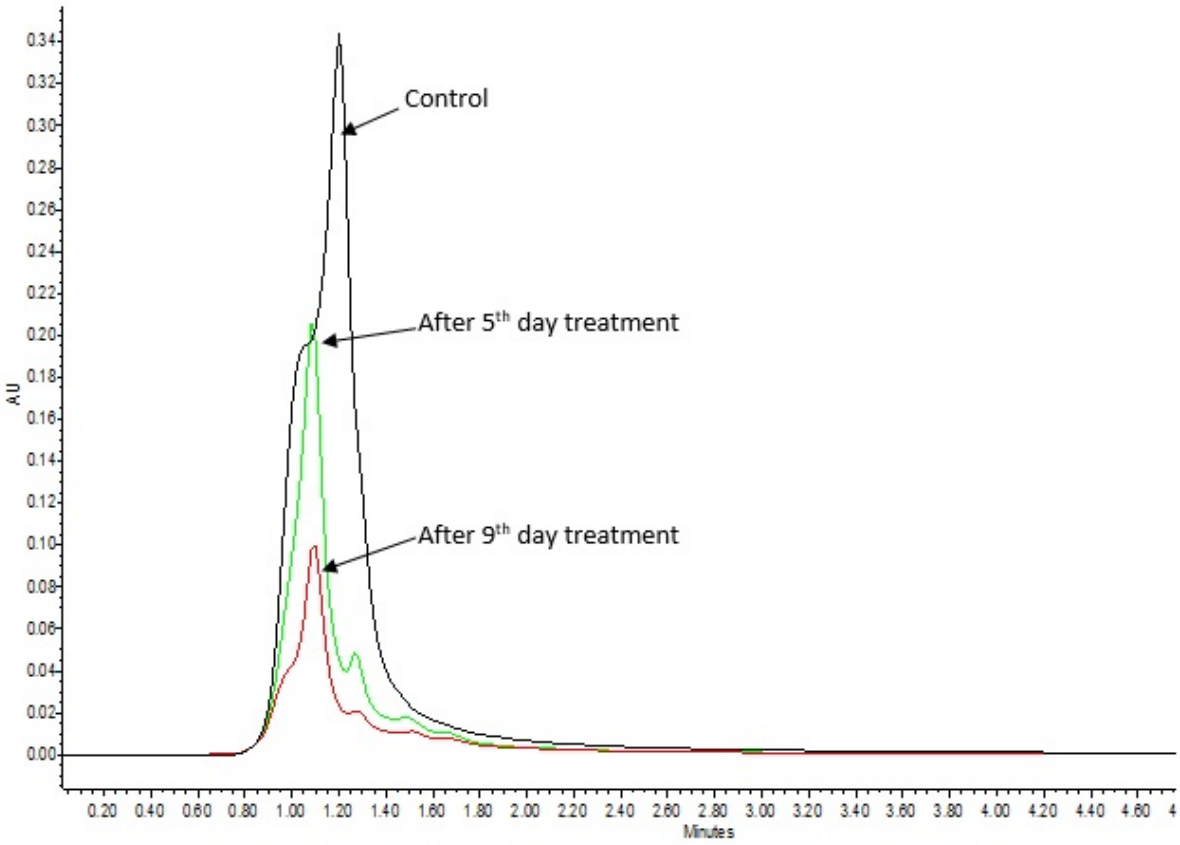

Figure 3: HPLC analysis of control and bacterial treated RGP effluent samples

Toxicity assessment (genotoxicity) for confirmation of reduction of different EDCs present in RGP effluent:

The comet assay, also known as single cell gel electrophoresis, is a simple but very sensitive technique for measuring brakeage of DNA stand. In this study, comet assay elucidated that DNA break down upon exposure to various chemicals extracted from untreated and bacterial treated pulp paper mill effluent. We have used medium control (Table 5). The medium control is used to observe the effect of process of single cell gel electrophoresis. The untreated paper mill effluent shows higher toxicity which expressed in terms of olive tail moment (OTM) and percent (%) tail DNA (6.13±0.27 and 20.31±1.08). This genotoxic nature of untreated paper mill effluent may be due to high load of EDCs which are present in untreated paper effluent. For example chlorophenols, Phthalate derivatives has high carcinogenic and mutagenic effect and may cause toxicity to living beings[18]. On other hand, heavy metals like Chromium, cadmium, arsenic also influence the toxicity at genetic level. After bacterial treatment the OTM and percent % tail DNA was reduces up to 55.4% and 43.87%. This reduction in genotoxicity indicated reduction of EDCs present in paper mill effluent.

Table 5: Genotoxicity of untreated and bacterial treated RGP effluent

Samples	Tail DNA (%)	OTM (Arbitrary unit)
Medium control	6.04±0.11	0.28^{a}±0.04
Un treated paper mill effluent	20.31±1.08	6.13^{b}±0.27
Bacterial treated sample after 5^{th} days	12.88±1.18	4.08^{c} ±0.11
Bacterial treated sample after 9^{th} days	11.4±0.82	2.73^{d} ±0.7
Total reduction	**43.87%**	**55.4%**

Different alphabets showing significant variation in separate column; significance was calculated by ANOVA followed by post hoc tukey test.

Conclusion:

Finally, this study concluded that the isolated bacteria have potential to remove different toxic pollutants in mixed condition rather than single culture. The developed bacterial strains were identified as *Serratia marcescens* and *Bacillus megaterium* on the basis of primary and secondary biochemical test. These two bacteria *Serratia marcescens* and *Bacillus megaterium* capable for more than 76% reduction of color and 91% of COD of RGP effluent. The HPLC and GC-MS analysis reveals that toxic pollutants removed by develop bacterial co-culture. Finally, the toxicity analysis of bacterial treated samples by the help of comet assay reveals that toxic pollutants present in paper mill effluent was removed after bacterial treatment.

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