Investigation of Bacteria and Their Degradation Efficiency Towards Pollution Load from Industrial Effluent

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***Abstract*—Industrial pollution has continued to be a major factor causing the degradation of the environment around us, affecting the quality of water we use, the air we breathe and the soil we live on. The pulp and paper industry is playing an important role in the economic development of the Indian sub-continent; however, this industry has been categorized among 17th most polluting industries.**

The pulp and paper mill discharges effluent with a higher biological oxygen demand (BOD), chemical oxygen demand (COD), and recalcitrant organics with intense colour in the form of dissolved lignin and lignin-based compounds and absorbable organic halides (AOX). The colouring body present in the pulp and paper mill effluent is organic in nature and comprised of wood extractives, tannin, lignin and its degradation products formed by the action of chlorine on lignin.

It is estimated that 100-120m3 of water is required to produce 1 ton of paper from wood and about 100m3 of waste water is discharged in the form of liquid effluent. One of the major problems is the persistent dark brown colour in the discharged effluent from effluent treatment plant of which the major contributors are lignin and its derivatives. The colour generated from a wood based paper mill normally is in the range of 1200-1500 PCU (Platinum cobalt unit).

In view of the stringent legislation imposed by concerned authorities for discharge of effluent, Indian paper industry is making all efforts to reduce and recycle the maximum process water back to the system, so as to discharge minimum quantity of effluent into the recipient bodies. Among various approaches to overcome the problem of colour, bioremediation technique using identified micro-organisms for treatment of paper mill effluent having potential to reduce colour, lignin and other pollutants may prove an effective way to treat the discharged effluent and to make it suitable for recycling back to the process with minimum discharges with a possibility recycling about 50% of the process water back to the system using for the purpose of unbleached pulp washing and pulp dilution.

The present paper basically highlights research work on isolation and screening of prominent bacteria having potential to reduce colour, lignin and other pollutants present in paper mill effluent and to develop microbial consortia having capability to treat the paper mill effluent and to achieve prescribed discharged norms in respect of colour and other presented parameters.

Sixty eight bacterial colonies were isolated from the soil, sludge and effluent samples collected from wood based paper mill. Primary screening of selected bacterial strains for their lignolytic activity was done on medium supplemented with Gallic acid. Selected strains were acclimatized by sub-culturing on medium with increase in percentage of effluent in the medium. The outlet of primary clarifier (PC outlet) from Effluent treatment plant (ETP) of wood based paper mill was used for present study and decolourization efficiency of individual isolates and combination of isolates were evaluated at laboratory scale by shake flask method. Initially bioremediation experiments were carried out by twelve selected bacterial strains and analysed for various pollutional parameters of interest like colour, lignin, COD and BOD. Five bacterial strains were found to have better decolourization efficiency individually and selected for further study. Experiments were also carried out by inoculating different percentages of bacterial inoculums in wastewater in order to check the effect of concentration of inoculums on reduction of pollutional parameters. 15% bacterial concentration was found most effective for decolourization of effluent.

Further bioremediation experiments were continued with consortia of bacterial stains. Combination of isolated bacterial strains showed higher reduction in colour of more than 50%, lignin around 45%, and COD, BOD of approximately 45-50%. Unsterilized conditions were chosen for bioremediation study of paper mill effluent i.e. favourable for further implementation at industrial scale. These results revealed that bacterial consortia proved to be more efficient for the treatment of pulp and paper industry effluent and may be implemented on pilot and mill scale.

Keywords*— *Bioremediation, Lignolytic activity, Bacterial consortia, Effluent, Decolourization

I. INTRODUCTION

Wastewaters discharged by the industries are one of the major causes of environmental pollution, particularly in the developing countries [1]. Pulp and paper industry utilises large amount of chemicals especially sodium hydroxide, solvents and chlorine compounds during paper manufacturing processes and releases coloured effluent with high BOD, COD and also consisting of potentially toxic chlorinated compounds, suspended solids, tannins, resin acids and sulphur compounds along with lignin [2].

As per the Ministry of Environment and Forest (MoEF), Government of India, the pulp and paper sector is in the "Red Category" list of 17 industries having a high polluting potential owing to its serious pollution threat. Typically in India around 75% of total fresh water supplied to pulp and paper industries emerges as waste water [3, 4].

It is estimated that 100-120m3 of water is required to produce 1 ton of paper from wood and about 100m3 of waste water is discharged in the form of liquid effluent. Thus, it is mandatory for pulp and paper mills to take appropriate standards set by Central Pollution Control Board (CPCB).

Conventional procedures to treat these effluents involve physical and biological techniques with no complete degradation of the recalcitrant organic matter. In addition, the

chlorophenolic compounds formed in chlorine bleaching are toxic, persist, bioaccumulate, and transform into other compounds which are more hazardous. Government agencies mark the standards for the discharge of wastewater into the environment; the BOD standard of 30 mg/L for discharge on inland surface water and 100 mg/L for disposal has been notified under the environment protection [5]. Therefore it is required to find out alternative treatment mechanisms, which enable the release of effluent to the environment or the reuse of effluent at some point along the same process [6]. In contrast, bioremediation process exploits the machinery of microorganisms to breakdown complex organic compound to simpler one without effecting the environment and it also capable of efficiently removing low molecular weight compounds [7].

Biological methods are often preferred since it has many advantages like rapid biodegradation rates, low sludge yield and excellent process stability. Biological methods are of particular interest because they can also reduce chemical and biological demands (COD and BOD), which are also significant problem in pulp wastewater [8, 9].

The first steps in generating an efficient microbial bioremediation process are the identification of a suitable microorganism and the optimisation of the culture conditions necessary for its rapid growth and the production of the relevant enzymes [10].

In view of these problems, recent research has been reported on biotechnological a with white-rot fungi (WRF) due to their powerful lignin-degrading enzyme system [1]. The white rot fungi have found to possess good lignolytic activity because of production of enzyme like lignin peroxidase (LiP), manganese-dependent per-oxidase (MnP) and laccase [2, 8].

Several fungal strains have proved effective in decolourization, and decontamination of effluent. Fungal bioremediation by Phanerochaete chrysosporium [11, 12], Lentinus edodes [13], Trametes (Coriolus) versicolor [14] has been reported by many workers [15].

However the use of fungal systems for effluent treatment purpose has been constrained due to their requirement of narrow pH 4-5 for their growth and enzyme production. Generally, pH values of pulp and paper mill are high (pH 7-9) and requirement to reduce the pH prior to the application of fungal system adds additional cost. In contrast to fungi, bacteria growing at neutral to alkaline pH may play important role in decolourisation of pulp and paper mill effluents without any need of pH adjustment [2]. The main problem associated with fungal bioremediation is that fungi require additional supplement 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. More persistent types of pollutants still do not have a successful degradation by fungi within the reasonable time period [1, 7].

Several species of bacteria have been evaluated for lignin degradation and for pulp and paper mill effluent treatment [7, 3]. However, not much work has been undertaken towards lignin degradation by bacteria. Karn et. al., 2010 [16] reported pentachlorophenol degradation by Pseudomonas stutzeri. Raj et. al., 2007 [17] studied decolourisation and treatment of pulp and paper mill effluent by lignin-degrading Bacillus sp.

The aim of this research study is to isolate the predominant bacteria having potential to reduce pollutants viz. colour, lignin and COD from wood based paper mill effluents and evaluating their degradation efficiency of individual isolates and combination of isolates at laboratory scale.

II. MATERIAL AND METHODS

A. *Collection of Soil and effluent sample*

For isolation of bacteria and effluent decolourisation studies, Soil, sludge, and effluent samples were collected from effluent treatment plant of wood based paper mill located in Saharanpur District of U.P., India. Samples were collected in sterilized zip lock bags and bottles and stored at 4◦C till further use.

B. *Characterisation of effluent sample*

The pH of the effluent was measured using pH meter. Chemical oxygen demand (COD), biological oxygen demand (BOD) and lignin were measured according to guidelines prescribed by American Public Health Association. Colour of effluent was measured using CPPA, 1974 [18].

Isolation of Bacteria: For isolation of bacteria, serial dilution technique was performed. Bacterial strains were isolated by continuous enrichment of mineral salt medium(MSM) (L-1) containing: K2HPO4 0.87g, KH2PO4 0.68g,MgSO4.7H2O 0.25g, CuSO4.7H2O 0.01g, EDTA 0.116g, FeSO4.7H2O 0.027g, NaMoO4.2H2O 1.0g, (NH4)2SO4.2H2O, ZnSO4 0.029g and Agar 20g supplemented with 500mg/l lignin. The different colonies appeared on MSM agar plates after 24-48 hrs at 340C, were sub-cultured on MSM-agar plate containing increasing concentration of lignin i.e. 1000, 1500 and 2000 mg/l. The isolates showing growth at 2000 mg/l lignin were purified by repeated streaking on nutrient agar plates.

C. *Screening of lignin degrading bacteria*

Bacterial isolates were screened for their ability to degrade lignin and phenolic compounds present in effluent. The MSM supplemented with gallic acid (0.5%w/v) was used to screen ligninoytic activity of bacteria.

D. *Biological treatment of effluent*

For Bioremediation experiment, nitrogen and phosphorous sources were added into effluent and acclimatized bacterial strains were quantitatively inoculated into effluent. Control was also maintained without inoculation of bacterial strain for comparison. All flasks were incubated at room temperature for different durations in a rotary shaker.

E. *Characterization of selected bacterial strain*

Characterization of bacterial strains was carried out in respect of Gram staining, oxygen demand and for catalytic activity [19].

III. RESULT AND DISCUSSION

A. *Characterization of paper mill effluent*

The physico-chemical characteristics of pulp and paper mill effluent are detailed in Table 1. Values are mean ± SD of triplicate samples.

Table 1: Physicochemical characterization of pulp and paper mill effluent

Parameters	Parameters Values
pH	8.1
Colour, PCU	1512 ± 27 PCU
Lignin, mg/l	316 ± 18 mg/l
COD, mg/l	845 ± 58 mg/l
BOD, mg/l	172 ± 14 mg/l

The effluent had a dark brown colour and was slightly alkaline in nature. The analysis of effluent showed presence of high colour (1512 PCU) and lignin (316 mg/l) in effluent. Apart from this the various parameters were: COD (845mg/l) and BOD (172 mg/l). Lignin and phenolics are plant constituents and major pollutants in pulp and paper industry effluent. Lignin causes high COD and colour in pulping effluent.

B. *Isolation of bacteria*

Sixty eight bacterial strains were isolated from the soil and sludge samples collected near Effluent treatment plant (ETP) of wood based paper mill. It was hypothesized that bacteria 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. The isolated bacterial colonies were diverse in their morphologies, ranging from small pin-pointed to large sized, fluorescent to whitish, smooth margined to wrinkled periphery.

C. *Screening and acclimatization of lignolytic bacterial strain:*

Screening of selected bacterial strains for their lignolytic activity was done on medium supplemented with Gallic acid. After 48 hours of incubation, the plates were observed for the appearance of brown colour respectively.

Out of 68 strains, only 12 strains showed positive results for the lignolytic enzyme (poly phenol peroxidase) by showing brownish colour around the growth. Selected Bacterial strains from secondary screening were sub cultured in Nutrient Agar (NAM) and were preserved at 4˚C for future use. Table 2 showing screening of isolated bacterial strains in MSM supplemented with Gallic acid.

Table 2: Screening of isolated bacterial strains

S. No.	Sample and Sources	Isolated bacterial Colonies	Screened positive strains	Code of screened positive strains
1.	Aeration tank sludge	12	3	BR1(A), BR1(B), BR1(C)
2.	Effluent from primary clarifier	8	3	BR2(A), BR2(B), BR2(C)
3.	Soil nearby aeration tank sludge	11	2	BR3(A), BR3(B)
4.	Soil nearby final discharge	9	1	BR4(A)
5.	Soil nearby unbleached wood storage	10	3	BR5(A), BR5(B), BR5(C)

All 12 bacterial strains selected in screening were subjected to acclimatization study. Acclimatization study was carried out by growing the bacterial strains on Nutrient Agar Medium supplemented with effluent to test the survival of bacterial isolates in paper mill effluent.

D. *Biological treatment of effluent using individual strains*

To determine the efficiency of pollutant removal using isolated bacterial strains, bacterial strains were inoculated in effluent supplemented with nitrogen and phosphorous sources [K2HPO4 and (NH4)2SO4]. Un-inoculated flask was treated as control. The inoculums was prepared by inoculating one loopful of all the 4 individual bacterial isolates separately in 25ml sterilized nutrient broth. The inoculated broths were incubated in an orbital shaker at 34◦C for 16–24 hours so as to obtain actively growing mother cultures. After achieving the desired growth (1.0 optical density), the cultures were centrifuged at 8000 rpm for 15 min at 4◦C. The 250mL of flasks containing 100mL of effluent sample were inoculated with the 10% bacterial inoculums and incubated in shaker at 150 rpm at ambient temperature (34◦C). The samples were with-drawn after 24 h for the analysis for reduction in colour, lignin and COD. Experiment was conducted in triplicates and mean value was represented here. Table 3 shows the colour, lignin and COD of effluent after treatment of isolated bacterial strains individually.

Table 3: Characterization of effluent after treatment of isolated bacterial strains individually

Bacterial Strain code	Effluent characterization					
	Colour		Lignin		COD	
	PCU	% R	mg/l	%R	mg/l	%R
Blank	1510	-	299	-	833	-
BR1(A)	**978**	**35**	**197**	**34**	**508**	**39**
BR1(B)	1260	16	240	19	666	20
BR1(C)	**944**	**37**	**194**	**35**	**483**	**42**
BR2(A)	1160	23	220	26	591	29
BR2(B)	**1000**	**33**	**199**	**33**	**487**	**41**
BR2(C)	1110	26	229	23	550	34
BR3(A)	1003	33	204	31	516	38
BR3(B)	1206	20	231	22	650	22
BR4(A)	**1140**	**31**	**208**	**30**	**475**	**43**
BR5(A)	1330	30	210	29	625	25
BR5(B)	**998**	**33**	**198**	**34**	**459**	**45**
BR5(C)	1295	32	205	31	591	29

Table no. 3 represents the higher reduction in colour, lignin and COD was found 33%, 34% and 45% by BR5(B) and 37%, 35% and 42% by BR1(C) bacterial strain. Above experiment indicated that BR1(A), BR2(B) and BR4(A) were also effective for COD and colour reduction. These five bacterial strains BR1(A), BR1(C), BR2(B), BR4(A) and BR5(B) were selected for further study on the basis of more efficiency for reduction in colour, lignin and COD than other bacterial strains.

E. Optimization of inoculums dose for bioremediation of paper mill effluent

Experiment were carried out by inoculating different percentages i.e. 10% 15%, 20%, 25%, 30% and 35% of five selected bacterial inoculum in paper mill effluent in order to check the effect of concentration of inoculum on colour and COD reduction. Flasks were incubated in shaker at 140rpm. After 24hrs, colour and COD were performed and degradation was calculated. Figure 1 represents the effect of inoculums dose of five selected bacterial strains on decolourization and COD reduction of paper mill effluent.

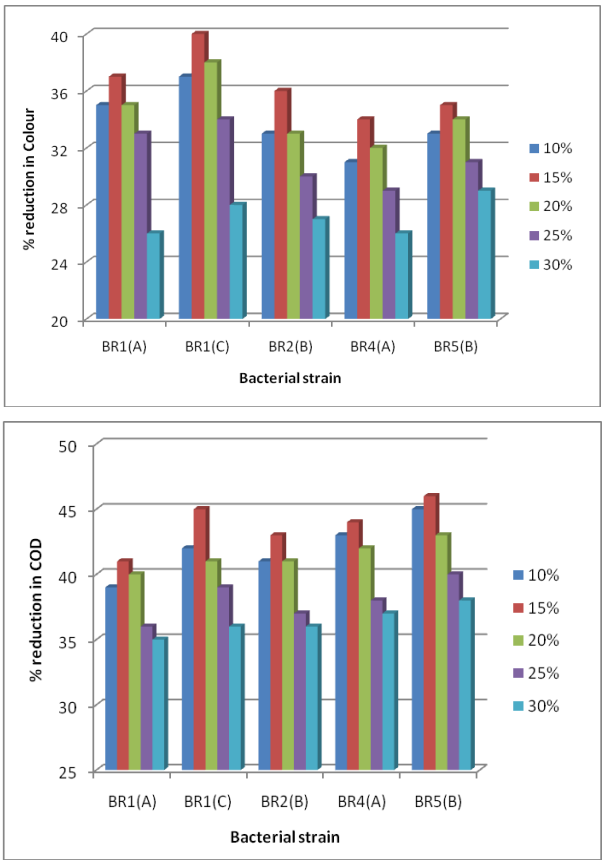

Figure 1: Effect of inoculums dose of five selected bacterial strains on decolourization and COD reduction of paper mill effluent

From the results shown in fig. 1, 15% inoculums dose was found better reduction capabilities than other inoculums doses of all five selected bacterial strains.

F. Biological Treatment of Effluent by bacterial Consortia

On the basis of higher efficiency of pollutants reduction by 05 bacterial strains BR1(A), BR1(C), BR2(B), BR4(A) and BR5(B) were selected for preparation of bacterial consortia. Randomly ten different combinations of selected five bacterial strains BR1(A), BR1(C), BR2(B), BR4(A) and BR5(B) were prepared shown in table 4.

Table 4: Ten Different Consortia Of Five Selected Bacterial Strains

Bacterial Consortia no.	Combinations of bacterial strains
1.	BR1(A) + BR1(C)
2.	BR1(A) + BR4(A)
3.	BR1(C) + BR5(B)
4.	BR4(A) + BR5(B)
5.	BR1(A) + BR1(C) + BR2(B)
6.	BR1(C) + BR2(B) + BR4(A)
7.	BR2(B) + BR4(A) + BR5(B)
8.	BR1(A) + BR1(C) + BR5(B)
9.	BR1(C)+ BR2(B) + BR5(B)
10.	BR1(A) + BR1(C)+ BR4(A)

Different combinations of bacteria shown in table no. 3 were selected for further study. 15% inoculums of bacterial consortia are used for bioremediation of paper mill effluent and samples were with-drawn after 24 h for the analysis for reduction in colour, lignin, COD and BOD.

Table 5: Effluent characterization by treatment of bacterial consortia

Bacterial Consortia	Colour		Lignin		COD		BOD	
	PCU	% R	mg/l	% R	mg/l	% R	mg/l	% R
Blank	1498	-	297	-	830	-	155	-
1	831	44	183	38	500	40	91	41
2	842	43	195	34	508	39	100	35
3	739	**51**	169	**43**	453	**45**	83	**46**
4	835	44	181	39	511	38	94	39
5	814	45	199	33	501	39	87	44
6	809	46	172	42	483	42	89	42
7	800	46	175	41	461	44	83	**46**
8	750	**50**	169	**43**	448	**46**	80	**48**
9	721	**52**	161	**46**	441	**47**	78	**49**
10	790	47	173	42	460	**45**	84	**46**

Table 6: Results of gram staining and catalase test for selected bacterial strains

S. No.	Bacterial Strain	Gram +ve bacteria	Gram -ve bacteria	Shape	Arrangement	Catalase +ve	Catalase –ve
1.	BR1(A)	✓	_	Rods	Chain	_	✓
2.	BR1(C)	✓	_	Coccus	Chain	_	✓
3.	BR2(B)	_	✓	Rods	Cluster	✓	-
4.	BR4(A)	✓	_	Coccus	Chain	✓	-
5.	BR5(B)	_	✓	Rods	Scattered	✓	-

On the basis of light microscopy, it was concluded that the bacterial strain BR1(A), BR2(B) and BR5(B) are rod shape and other isolated bacterial strains were round in shape (coccus).The result of catalase test shown in table 6 revealed that the bacterial strains BR2(B), BR4(A), and BR5(B) were catalase positive while bacterial strains BR1(A) and BR1(C) were catalase negative. The oxygen requirement test showed that all 5 bacterial strains were aerobic.

IV. CONCLUSION

The present study regarding the treatment of pulp and paper mill effluent reveals that five isolated bacterial strains exhibit promising results. Sixty eight bacterial strains were isolated from natural sites of wood based paper mill. Among the isolated strains, five bacterial strains were individually more effective for decolourization and COD reduction. Ten randomly selected different combinations of these five bacterial strains were studied for bioremediation of pulp and paper mill effluent at lab scale. 15% bacterial inoculums have shown best bioremediation potential for bioremediation of pulp and paper mill effluent. Among ten different combinations of bacteria, bacterial consortia BR1(C) + BR2(B) + BR5(B) was able to remove 52%, 46%, 47% and 49% of colour, lignin, COD and BOD respectively from pulp and paper mill effluent while BR1(C) + BR5(B) and BR1(A) + BR1(C) + BR5(B) also showed good reduction capability for decolourization i.e. 51% and 50% and BOD reduction i.e. 48% and 46% respectively. Further studies will be continued with other consortia to improve the reduction capabilities of pollutants from paper mill effluent.

The high removal efficiency at lab scale shows that the strain has the potential for being tried on a large scale for the treatment of pulp and paper mill effluents. The study can proved to be a cost effective ways to reduce pollutant level from effluent generated in pulp and paper mill industry and maximum percentage of this treated water can be recycled back to the system, so as to discharge minimum quantity of effluent into the recipient bodies.

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