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Isolation, Screening and Identification of PCP-Degrading Bacterial Strains from Sludge

Kumar Sunil[1], Tandan Neeraj[2] and Bharadwaj Uma[1]

[1]*Department of Microbiology, Maharaj Vinayak Global University, Jaipur, Rajasthan, India.*
[2]*Scientific and Applied Research Centre, Meerut, India.*

A B S T R A C T

A significant number of organic compounds present in pulp-paper mill effluent have been classified as mutagenic and clastogenic thereby turning these effluents into 'a Pandora's box of waste chemicals'. Over last decades continuous discharge of the organic pollutants from pulp paper mill into the environment has increasing the levels of these pollutants in water system. In this thesis we assessed, physico-chemical analysis of collected effluent. The collected effluent was found slightly alkaline (pH-8.2) in nature and dark brown in colour with high level of chlorophenols PCP (220 mg/l); dichlorophenol (18.65) and trichlorophenol (10.73). Other pollution parameters like COD (15766±260 mg/l), BOD (6033±155 mg/l), color 18534 CU, nitrates (35.3±3.0 mg/l) and sulphate (405±10.2 mg/l) were also found in high level. The low BOD/COD ratio (approx 0.34) and high color of effluent are due to presence of high molecular weight compounds, i.e. PCP and lignin is complex compounds, which contribute high COD instead of BOD. Moreover, this study tended to evaluate the removal of PCP by bacterial system. In this study we were isolate 31 bacteria through enrichment culture technique. Three strains PPS-S6, S7 and S8 were screened on the basis of various enzymatic activity (peroxidises and laccases enzymes) and identified as *Pseudochrobactrum sp*, *Providencia rettgeri* and *Klebsiella pneumoniae*, respectively on the basis of their biochemical properties.

Keywords: PCP, HPLC and GC-MS, COD.

A R T I C L E I N F O

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***Corresponding Author**
Kumar Sunil
Department of Microbiology, Maharaj Vinayak Global University, Global University in Rajasthan, Jaipur, India
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1. Introduction

There are more than 406 pulp paper industries as per record in 2002 producing 1.9 million tons paper per annum **(Singh and Thakur, 2006).** The pulp and paper mills utilizes huge amount of lignocellulosic components of plants and chemicals during manufacturing processes and are generally regarded as polluting industries owing to discharge of huge amount of waste material which enter into the environment. For every ton of paper production 220-380 m^3 of highly colored and potentially toxic wastewater are generated. Regardless of the manufacturing process used, pulp mill effluents are complicated mixtures consisting of several hundred compounds, among which chlorinated phenolics are an important class of toxicant.

The major problems due to the pulp and paper mill industry are presence of high colored and chlorinated compounds. The major known environmental toxicants are pentachlorophenol, tetrachloroguaiacol, tetrachlorohydro-quinone, trichlorophenol and dibenzofuran **(Thakur et al., 2001; Thakur, 2004)**.

These toxicants are generated either in bleaching of pulp or inadvertently during the disposal of pulp paper mill effluent. These toxicants not only damage to environment but also to animal including human as potent carcinogen and mutagen derived either through lignin and chlorolignin interaction of various chemicals. However, role and interaction of different pollutants with lignin or phenol is scanty. Hence, there is need to investigate detail of different pollutants present in effluent and their interaction is necessary prior to bacterial degradation and decolourisation of pulp paper mill effluent for safe disposal. The color of effluent is mainly due to the presence of lignin and its derivatives. Color not only is aesthetically unacceptable but also leads to chain of adverse effect on the aquatic ecosystem. Similarly, pentachlorophenols from category of chlorophenols, one of the most hazardous classes of environmental pollutants, have been produced in thousands of tons annually by the pulp and paper and agrochemical industries (**USEPA, 2000; Singh et al., 2008**).

2. Materials and method

The pentachlorophenol (PCP), di, tri and terta chlorophenols were purchased from Sigma. All solutions were prepared in double distilled water**.**

Culture Media and Glasswares:

All the culture media used in the work were procured from Hi-Media (Mumbai), Qualigens (Mumbai were), Sd-fine Chemicals (Mumbai)] or abroad [Sigma-Aldrich (USA), E. Merck (Germany)]. All the glassware's used in the study were purchased either from prominent dealers of Duran (Germany) or Borosil (India).

Mineral salt medium:

Mineral salts medium (MSM) was used throughout the study with the following composition (g/l de-ionised water): D-glucose, 1%; peptone, 0.5%; K_2HPO_4, 1.8; Na_2HPO_4, 2.0; NH_4NO_3, 0.2; $MgSO_4$, 0.05; $CaCl_2$, 0.02 and pH 8.0.

Sampling site and sample collection:

The effluent was collected from M/s. Modi Nagar Pulp Paper Mill, located at Meerut, Uttar Pradesh, India. The collection was taken into container. For bacterial isolation the sludge sample was collected in pre sterilized (at 121°C for 15 min) cotton plugged test tubes.

Physico-chemical analysis of collected effluents:

The effluent sample were analyzed in laboratory for various parameter i.e. Total Solid, Total Suspended Solid, Total Nitrogen, phosphate, pH, heavy metals, Total Dissolve Solid, phenol sulphate, color, COD, BOD and lignin (APHA, 1998), chlorophenols, nitrate, potassium, chloride, sodium and carbon dioxide by following methods:

Measurement of color (CPPA, 1974):

Color estimation (CAPA standard method)

Take sample (pH-7.6) centrifuge it. Take O.D at 465 nm using D.W as a blank.

Calculation:

$$CU = \frac{500 \text{ x O.D at 465 nm}}{\text{O.D of 500 Copt at 465 nm}}$$

Note: for standard preparation of 500 copt: dissolve 1.246 gm K_2PtCl_2 and 1 gm $CoCl_2$ x$6H_2O$ in 100 ml in concentrated HCl & dilute up to 1 lit.

<u>Visual comparison method</u> (APHA, 1998) was followed for the measurement of colour intensity in the different sample of paper mill effluent during the whole experiment.

Methodology:

Observed sample color by filing a matched Nessler tube to the 50 ml make with sample and comparing it with standard look vertically downward through tubes towards a white or specular surface placed at such an angle that light is reflected upward through the columns of liquid. Since treated paper mill effluent after final treatment has very high color so it was diluted 100 times to bring the color within range of the standards. The pH of effluent was noted 7.5.

Calculation:

Calculated color units by the following equation:

$$\text{Cooler units} = \frac{A \text{ X } 50}{B}$$

Where,

A = estimated color of a diluted sample

B = ml sample taken for dilution

3. Results and Discussion

Physico-chemical analysis is basic tool that define the nature and actual pollution potential of any industrial effluent. On the basis of physico-chemical characteristics we can predict the environmental fate of the industrial effluent. Thus, before devising any strategy to abate the pollutants and toxicity of effluent it is inevitable to assess or have an idea of different physical and chemical factors, which are responsible for contributing toxicity in industrial effluent. The pulp paper industry release colored effluent with different characteristics, depending on the production process and its raw material. The final effluent samples of Modi Nagar pulp paper industry were collected and analyzed for several pollution parameters, which are listed in Table (1). The industry is a small-scale paper mill produces brown and packaging grade paper using baggase, waste paper, rags, and agriculture residue as raw materials without bleaching process and has poor treatment system. The sum of pollution in any industrial wastewater is measured in term of pollution parameters, which are generally express in COD and BOD. The BOD and COD causing chemical species are generated from pulping, bleaching and soda recovery plants. In present work, very high average values of BOD and COD were recorded.

The high COD/BOD ratio (approx 3) is due to lignin and chlorophenolic contents, which make a payment in elevation of COD and color instead of BOD (**Esposito et al., 1991; Chandra et al., 2011**). These effluent wherein very high values of color and COD were noted due to high lignin and PCP content. The adverse impact of pulp-paper wastewater with high load of BOD and COD in aquatic system have been found to cause anoxic condition in bottom sediment, resulting in the loss of stationary benthic organism and habitat degradation of mobile benthic organism **(Owen, 1990**).

Table 1: Physico-chemical characteristics of pulp-paper mill effluent

Parameters	paper mill effluent
pH	8.2±0.4
Color unit (CoPt)	18534 ±56.0
Lignin (mg/l)	1413.0±45.0
BOD(mg/l)	6033.0±55.0
COD(mg/l)	15766.0±260
TS(mg/l)	1570.0±97.0
TDS(mg/l)	1274.0±38.3
TSS(mg/l)	296.0±11.4
Sulphate (mg/l)	405.4±10.2
Phosphate (mg/l)	500.0±51.8
Total Nitrogen (mg/l)	152.9±10.4
Chloride(mg/l)	351.0±31.9
Potassium(mg/l)	26.5±5.4
Sodium (mg/l)	28.9±4.9
Nitrate (mg/l)	35.3±3.0
Heavy Metals	
Cd(mg/l)	<0.002
Pb(mg/l)	<0.025
Zn(mg/l)	0.07
Cr(mg/l)	0.06
Cu(mg/l)	0.07
Ni(mg/l)	0.02
Fe(mg/l)	2.00
Mn(mg/l)	0.04
Chlorophenols	
Total Phenol(mg/l)	72.9±7.0
PCP(mg/l)	220.31*
Dichloro phenol(mg/l)	18.65*
Trichloro phenol (mg/l)	10.73*

Single values, NA = Not analyzed, Note= values marked with <(less than) sign are the minimum detection limit for the corresponding parameters.

In this study, a slight alkaline pH 8.2 was recorded for collected effluents. Table 4.1 shows very high average values of color (18534 Copt), PCP (220 mg/l) and lignin (1413 mg/l) in effluent, respectively. This high value of different pollution parameters is due to absence of proper treatment methods in this paper industry. The very high average values of sulphate (405.4 mg/l), phosphate (500 mg/l) and total nitrogen (152.9 mg/l) were recorded in paper mill effluent, respectively. In pulp-paper effluent sodium sulphite which is used in pulping is the major source of sulphate ions and nitrogen present in this effluent comes from skeleton of lignin (**Singhal and Thakur, 2009**). The discharged of sulphate, phosphate and nitrogen rich effluent into aquatic system causes several environmental problems in aquatic ecosystem such as eutrophication, especially in warmer months (**Pokhrel and Viraraghavan, 2004**). This eutrophication causes in loss of native species and an invasion of exotic and opportunistic native more tolerant to pollution. It could be noted that the sulphate comparatively have less effect on taste of water than chloride but responsible for foul smell. Similar findings were also reported in the aquatic environment in the vicinity of paper mill effluent for fish life (**Milestone et al., 2012).**

In addition, result it can be seen that very high average values of TS, TDS and TSS were presence in effluent. The effluent of pulp and paper mill was found to be contains considerable amount of suspended solids mainly left over lignin, fiber and presence of a range of chemicals like soda, sodium sulphate, calcium, powder, alum, chlorine, resin, dye and preservative used during the manufacturing process of paper **(Kreetachat et al., 2007; Pokhrel and Viraraghavan, 2004**).

The high-suspended solids containing effluent may affect aquatic biota; particularly fishes and benthic organism **(Orrego et al., 2009; Chamorro et al., 2010**). The average values of heavy metals were recorded as, Cu 0.07, Fe 2.00, Ni 0.05 and Zn 0.07mg/l for paper mill effluent (Table 1). Out of recorded heavy metals, Fe was found comparatively in higher amount.

On the basis of PCP reduction and enzymatic activity strain AB1, AB12 and T7 were selected as PCP-degrading bacterial strains and used for further study. That's why these three isolates were further identified by different biochemical tests.

Three bacterial strains found potential for reduction of PCP were identified by biochemical test and results are shown in Figure 1. All the strains were motile, rod shaped Gram-negative bacteria. The detail of biochemical test was in Table 4.4. On the basis of the biochemical properties strains, the bacterial strains which were manually coded in our lab by AB1, AB12 and T7 indicated a very secure similarity to *Pseudochrobactrum sp*, *Providencia rettgeri* and *Klebsiella pneumoniae*, respectively.

Table 2

S no.	Designation	Growth	Reduction of PCP (%)	Enzyme activity		
				Li. P.	Mn. P.	Laccase
1.	**AB1**	**++++**	**37**	********	********	***
2.	AB2	+	16	-	-	-
3.	AB3	+++	16	-	**	-
4.	AB4	+++	21	-	-	-
5.	AB5	+	-	**	-	-
6.	AB6	+	-	-	-	-
7.	AB7	+	-	-	-	-
8.	AB8	+	7	-	-	-
9.	AB9	++	13	*	*	-
10.	AB10	+++	24	*	**	-
11.	AB11	+++	22	-	**	-
12.	**AB12**	**++++**	**34**	********	*******	****
13.	AB13	++	14	-	-	-
14.	AB14	+++	12	-	-	-
15.	AB15	+	-	-	-	-
16.	T1	+	-	-	-	-
17.	T2	++	13	-	-	-
18.	T3	+++	12	-	-	-
19.	T4	+	-	-	-	-
20.	T5	++	-	-	-	-
21.	T6	++	-			
22.	**T7**	**++++**	**31**	********	********	****
23.	T8	++	5	-	**	-
24.	T9	+	-	-	*	-
25.	T10	+++	9	-	*	-
26.	T11	+	5	*		*
27.	T12	++	-	-	-	-
28.	T13	+	-	-	-	-
29.	T14	+++	-	-	-	-
30.	**T15**	**++++**	**29**	******	******	***
31.	T16	+++	-	-	-	-

*++++: luxurious growth; +++: moderate growth; ++slow growth; +: weak growth; NG: No growth; **** high enzyme activity: ***: moderate enzyme activity; **slow enzyme activity; * weak growth; -: No enzyme activity*

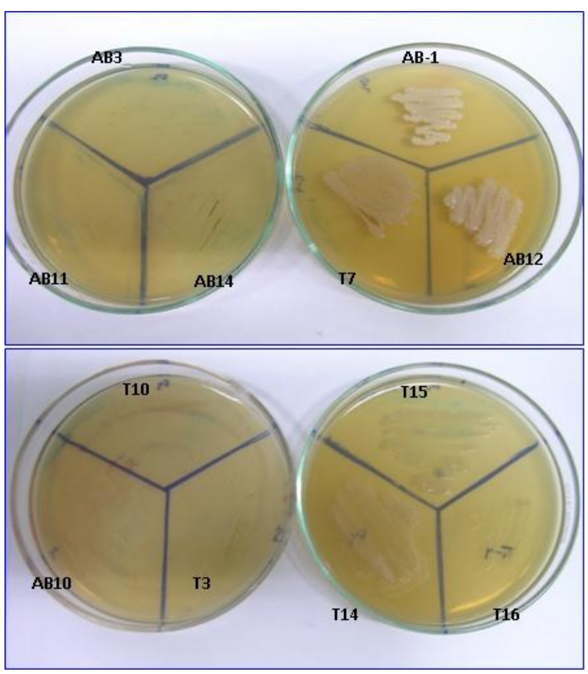

Figure 1: screening of bacterial strains on PCP amended MSM medium.

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