Research Paper

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Biochemical Analysis of Paper Mill Effluent & Microbial Degradation of Phenol

Environment

KEYWORDS : Paper mill effluent, physico-chemical parameters, phenol degradation, microbes

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ABSTRACT

Paper mill effluent contains organic and inorganic toxic materials, which affects the soil physico-chemical parameters and microbial population and its diversity. Phenol is one of the toxic aromatic compounds which have deleterious effects on soil micro flora as well as on vegetation in effluent affected areas. In the present study, the physico-chemical parameters of paper mill effluents obtained from Nagaon Paper Mill (NPM), Jagiroad, Assam were analysed. All total nine strains, capable of growing in phenol containing MSM medium at a concentration range of 100 to1000ppm, were isolated. Pure cultures of phenol-degrading microbial strains were obtained by growing on enrichment medium (Mineral salt medium) containing 500ppm. Three strains were found to be efficient among the nine strains, which is confirmed by measuring their capability of phenol degradation with respect to incubation time. This study provides scope for future study of identification of phylogenetically closely related species for phenol degradation in industrial wastes and their use in eco-friendly and more economic way, with the development of technology.

INTRODUCTION

In the recent past, environmental pollution caused by different industries has become a matter of great concern world-wide. Improper management of wastes produced by industries is affecting the ecological health in a great manner. Paper industry is not an exception to this, as it uses an enormous quantity of water and chemicals during processing, which are disposed to land or water bodies as wastes. Nearly 75 to 95% of the water discharged by the Paper industries as effluent contains organic, inorganic pollutants and colouring materials. Presence of these chemicals may affect soil and in turn the growth and development of plants (Baruah et al., 1996, Medhi et al., 2011). But this wastewater is also a potential water resource because it also contains considerable amount of nutrients, which may prove beneficial for enhancing the nutrient pool of soil and hence, plants growth and development (Sahai et al., 1985 Mishra & Behera, 1991). However, effluent generated from pulp and paper mills needs to be treated to reduce the pollutants load to stipulated limits before disposing it off into water bodies or using it for land application for crop growth. Out of the numerous inorganic and organic compounds as pollutants in the paper mill effluents, phenol is one of the stable organic compounds and it may be toxic even at low concentration towards living organisms. A phenol concentration at a range of 100-400µg/ml may lead to complete inhibition of photosynthesis. They may also cause unfavourable chemical changes in water and soil, often having BOD and COD levels well above the permitted level, and pose a significant wastewater treatment challenge to mill operators (Kostyeav, 1973). However, there are reports on existence of phenol degrading microorganisms growing in industrial wastes. Rigo and Alegre (2004) screened twenty two strains of microorganisms and *Candida parapsilopsis* was reported to be able to tolerate, grow and degrade phenol at 1000 ppm concentration.

Nagaon Paper Mill (NPM) situated at Jagiroad, Morigaon district of Assam, India generates about 2270 m^3 of effluent containing 1484 mgL^{-1} of total dissolved solids (TDS) daily. For last seventeen years, indiscriminate use of these effluents for irrigating agricultural land by the farmers in the nearby areas might cause shift in the functions and structure of native microbial communities (Hazarika et.al., 2007). Thus, there is a need to study the shift in microbial diversity of such soils in relation to changes in soil physico-chemical properties, which are governed by agricultural management practices. The literature survey indicates that while there are treatment methods that remove either colour and/or phenols from effluents, there is dearth of treatment processes that effectively remove both colour and phenols from the effluent (Deilek & Bese, 2001). It is therefore, necessary to isolate, identify and conserve microorganisms which are able to detoxify the toxic components present in the industrial effluents. Information on dynamics of microbial populations in wastewater treatment systems and the relationship between population dynamics and functional stability of treatment systems may help in reducing the toxic effects of paper mill effluents on the environment.

It is in this backdrop that the present study is undertaken with the following objectives of analysis of different parameters of Nagaon Paper Mill effluent, assessment and comparison of soil parameters in paper mill effluent irrigated fields and assessment of phenol degrading bacterial strains.

MATERIALS AND METHODS

Sample Collection

For the present investigation the paper mill effluents samples were collected from Nagaon Paper Mill, situated at Jagiroad, Assam. The research study was carried out during Nov. 2010-April 2012. Soil samples were collected from the fields, where paper mill effluent was used for irrigation since last two decades in succession. The effluents samples and soil samples were collected in glass containers that were previously cleaned by washing in non ionic detergent, rinsed with tap water and later soaked in 10% HNO_3 for 24 hrs and rinsed thoroughly with deionised water. For the sampling of soil, three cultivated fields were selected (i) Control field where effluent irrigation was not done at all and is being irrigated only with fresh water, (ii) Treated effluent irrigated field (TEIF) and (iii) Raw effluent irrigated field (REIF). Soil samples were collected from rhizosphere, and non rhizospheric regions of the crops growing in the region.

Analysis of soil physico-chemical properties

The physico-chemical analysis of effluent was performed using the standard methods of APHA (American Public Health Association, 1998). All the analysis was done in triplicates for each of the samples. Soil moisture, pH and total organic content of soil samples affected by paper mill was analysed for TEIF and REIF.

Table 1: Physico-chemical parameters of paper industry effluent (Mean value ±SD)

Sl. No.	Parameters	Value
1	Colour	Dark brown

2	Odour	Unpleasant
3	pH	7.65±0.33
4	Temp°C	30.2±2.1
5	EC(dS m^{-1})	2.4±0.14
6	TDS(mg/l)	1460±38.0
7	BOD(mg/l)	270±30.8
8	COD(mg/l)	438±15.5
9	Na(mg/l)	278.5±4.1
10	Ca(mg/l)	262.8±14.0
11	Mg(mg/l)	15.2±11.77
12	K(mg/l)	46.88±10.3
13	P (mg/l)	3.11±2.2
14	Phenol content(mg/l)	71.64±12.2

Table 2: Soil moisture (%), pH and total organic carbon content of soil samples affected by paper mill effluent

Treatment	Paper mill effluent contaminated soil		
	Moisture %	pH	Organic Content%
CF*	11.13	7.03	0.73
TEIF	18.62	7.17	0.81
REIF	20.16	7.54	1.21

*CF: Field away from paper mill effluent affected area

Isolation and culture of bacteria from Paper mill effluent disposal site

Serial dilution was done for each of the samples of paper mill effluent collected from different sites, followed by pour plating in MPA agar medium at 37°C and colony counting was done at 48 hrs, 72 hrs and 96hrs (Kojima et. al., 1961). Microscopic examination and staining tests were done for identification of the microbes predominantly growing in effluent of NPM, at different time of survey. Pure culture of all total nine bacterial isolates was developed, and named serially as S1, S2...to S9.

Table 3 : Composition of modified MPA medium

Contents	Quantity g/L
$Na_2HPO_4.12H_2O$	3.78
KH_2PO_4	0.5
NH_4Cl	5.0
$MgSO_4.7H_2O$	0.2
Yeast Agar	0.99
Mineral Medium	

(Kojima et. al., 1961) Mineral medium of following composition in 1L distilled water was tested.

K_2HPO_4	4.35
KH_2PO_4	1.7
$MgSO_4$	0.2
NH_4Cl	2.1
$MnSO_4$	0.05
$FeSO_4.H_2O$	0.01
$CaCl_2.H_2O$	0.03
Agar	20

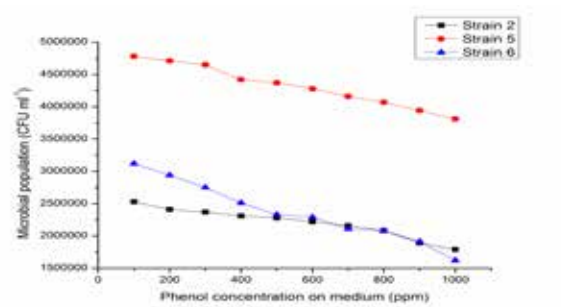
Isolation and culture of phenol degrading bacteria

A defined mineral salts medium was used for isolation and cultivation of the phenol-degrading strains (Taylor et. al., 1970) from different disposal sites of paper mill effluent. Enrichment cultures were prepared in the defined 100ml of mineral salts medium in 250-ml of Erlenmeyer flask. Phenol was added at a starting concentration of 1000ppm. Cultures were incubated in shaking rotor at 30°C at 120rpm and were monitored for phenol loss and turbidity due to bacterial growth. Periodically, when phenol was depleted, 500ppm was added after an interval of 72hours, and nitrate (10 mM) was added when phenol loss halted. The cultures were diluted 10-fold twice, and after each dilution 500ppm phenol were added three times. After the last addition of phenol 10-fold serial dilutions were prepared. Colonies of phenol-degrading organisms were obtained from the highest dilutions by using MPA medium plates containing 500ppm phenol and 1 mM nitrate and were transferred to 100ml of mineral salts medium in 250-ml of Erlenmeyer flask containing 500ppm phenol, and 5 mM nitrate under Laminar air flow cabinet. Out of nine strains, only three bacterial strains were isolated by enrichment techniques, which showed growth at 1000ppm, were selected as the efficient phenol degrading strains. The cultures were checked for purity by plating on tryptic soy agar (TSA), as well as by microscopic observation (Paula & Young, 1966). Cell density was measured spectrophotometrically at 600nm using UV-Vis spectrophotometer at 12 hours intervals (Table 4).

The selected cultures were purified by repeated streaking on Mineral Salt Medium containing 100ppm of phenol, and the working culture was maintained by culturing in Mineral Salt Medium or broth containing 1000ppm concentration of phenol at two weeks intervals (Ali et. al. 1998).

The three strains were inoculated in 100ml of MPA Broth Medium containing different concentrations of phenol viz., 100ppm, 200ppm, 500ppm up to 1000ppm at pH 7.0. The flasks were incubated at 30°C±2°C in a shaking incubator at 120rpm. Microbial population counting was done using a Digital colony counter, after 48hours of incubation. Graphical plot of phenol concentration verses microbial population of strains S2, S5 and S6 has been shown in Fig. 1.

Fig. 1: Effect of phenol concentrations on the growth of microorganisms in mineral medium (after 48 hrs of incubation)



Chemical precipitation experiments

For chemical precipitation experiments, 100 mL of raw paper mill effluent was taken in separate 200 mL glass bottles and the metal salt (dose 0-5.0 gL^{-1}) was added to it. The contents in the bottle were thoroughly mixed and the pH was adjusted to the required value. The bottles were kept in unshaken conditions for 24 hours to facilitate the formation and sedimentation of precipitates. The paper mill effluent samples collected from different sites of the HPCL Paper mill, Jagiroad, at different time were analyzed after 24 hours drawing aliquots of effluent from the bottles, for the analysis of phenol content.

Analysis of phenol (Spectrophotometric analysis)

As the phenolic material react with 4-Amino Antipyrine in the presence of ferricyanide at pH 10 to form stable antipyrine dye, which can be measured by taking the absorbance at 510nm by direct photometric method (Gales & Booth,1976). Absorbance of antipyrine was measured and compared with, the absorbance of standard phenol solution (Behera & Jena, 2009). In the present work, the concentration for phenol degradation by

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microbes was selected in a wide range based on review literature. In order to estimate phenol concentration, the microbial cultures were centrifuged at 5000rpm for 20 minutes at 4°C, to separate the biomass and simultaneously bacterial growth was measured spectrophotometrically at 510 nm with a double beam UV-Vis spectrophotometer (model SICAN-2301) and population was determined at regular interval of 12hours, up to 72hours of incubation (Martin, 1949).

Using working solution of phenol at a concentration of 100ppm per ml, standard curve was prepared by measuring the absorbance at 510nm. The pH of the samples and standards was adjusted to pH 10±0.2., and the estimation of phenol was done by modified spectrophotometric technique based on standard method of phenol estimation (Mordocco et. al., 1999) adding 2ml of buffer solution (16.9g of NH_4Cl in 145ml of conc. NH_4OH and diluted to 250ml) to 100ml of diluted sample and mixed well, followed by addition of 2ml of potassium ferri-cyanide solution and mixed thoroughly. After 15 minutes, absorbance reading was taken at 510nm, for each sample at regular interval of 12 hours of incubation. Phenol degradation was quantified by comparing the absorbance with the standard curve of phenol along with the control, using UV-Vis Spectrophotometer. Percentage of phenol degradation for selected bacterial strains showing growth at 1000ppm of phenol concentration was calculated from O.D. readings at 510nm, at 12 hours interval up to 72hours.

Percent phenol degradation was computed using the formula,

$$\% \text{ Phenol degradation} = \frac{\text{Initial phenol conc.} - \text{Residual phenol conc.}}{\text{Initial Phenol conc.}} \times 100$$

Table 4: Cell density and percentage of phenol degradation for selected bacterial strains showing growth at 1000ppm concentration as per calculation from O.D. readings.

S. No.	Strain Code	Cell density as OD at 600nm*						Percent of Phenol degradation as calculated from OD at 510nm**					
		12hrs	24hrs	36hrs	48hrs	60hrs	72hrs	12hrs	24hrs	36hrs	48hrs	60hrs	72hrs
1	Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	Strain2	0.14	0.20	0.24	0.27	0.32	0.35	21	26	31	38	41	45
3	Strain5	0.21	0.26	0.32	0.38	0.44	0.48	34	38	43	49	52	57
4	Strain6	0.19	0.24	0.29	0.33	0.38	0.42	27	31	36	40	44	48

***Absorbance at 510nm in UV-Vis spectrophotometer (double beam) & for control blank medium was taken and value was nullified to zero.**
** Formula used for calculation: [(Initial phenol - Residual phenol) ÷ Initial phenol] x100

Characterization of the isolates
Identification of the efficient phenol degrading bacteria was done based on morphological and biochemical tests according to Bergey's manual of determinative bacteriology (Bergey & Hendricks, 1957). Bacterial strains isolated were examined for colony morphology, pigmentation, cell shape and Gram reaction as per the standard procedures as per (Anon, 1957; Barthalomew & Mittewer, 1950). Cell morphology was determined by microscopic observation under Digital Research Microscope (model Motic BA-210). Motility was assessed by direct microscopic observations during growth, and by testing the ability of the strains to migrate from the point of inoculation through semisolid (0.3%) agar plates containing 20 mM succinate (Adler, 1966).

The pH range and optimum pH for growth for each strain were determined by monitoring the residual phenol concentrations in cultures inoculated into medium adjusting the pH at 7.0; a 5% (v/v) inoculums and a starting phenol concentration of 100ppm were used in these experiments. The highest concentration of phenol at which each isolate initiated growth (i.e., the phenol tolerance) was determined by monitoring the optical densities of cultures growing on phenol at initial concentrations ranging from 100ppm to 1000ppm. (Beaulieu et. al.,2000)

RESULT
Paper mill effluent collected from Nagaon Paper Mill, Jagiroad, was chosen as the material for studying its effect on the soil, its physico-chemical characteristics The phenol content of the untreated paper mill effluent (71.64+12.2mg/ml) is found to be higher than the current OSHA (1994), permissible exposure limit of 19 mg/ml as an 8-hour time-weighted average (TWA) concentration. Percent of soil moisture content in treated effluent irrigated soil and the raw effluent irrigated soil was observed to be higher than the control soil. The soil pH was found to be more in REIF soil than in TEIF soil. Moisture percent was recorded to be higher in distillery paper mill effluent affected soil than in paper mill effluent contaminated soil. However, percent of moisture was observed to be highest in REIF soil *in silico,* among all other soil samples from treated paper mill effluent irrigated and control fields.

The collected samples of paper mill effluents were enriched in MSM enrichment medium with 1000ppm of phenol concentration for five times, each after 72 hours interval. All total nine strains were isolated, out of which three efficient strains were screened for phenol degradation by growing in MSM medium containing 1000ppm of phenol concentration. The efficient strains were identified by morphological characteristics according to Bergey's Manual of Determinative Bacteriology (Bergey et.al., 1957), and identified as Pseudomonas sp., Azatobacter and Streptococcus sp. Among the three isolated strains for phenol degrading ability, strain 5 of Pseudomonas sp. was found to be most efficient among them, with respect to its growing ability at phenol concentration of 1000 ppm at the time of active growth viz., between 12-72 hrs of incubation in MSM medium followed by Strain 6 of Azatobacter sp. and Strain 2 of Streptococcus sp. For strain 5 percent of phenol degradation at regular time-interval of incubation in MSM medium containing 1000ppm of phenol concentration, was found to be the highest among the three strains, followed by strain 6 and 2 respectively. With the advancement of incubation-time, all the three strains showed increasing trend in percent of phenol degradation.

CONCLUSION
The present study leads to the conclusion that the paper mill effluent disposed to the soil affect its physico-chemical parameters, which ultimately leads to different levels of pollution. Moreover, paper mill effluent contains high amount of phenol content, having deleterious effect on soil, can be degraded efficiently with microorganisms. Development of different technologies, for efficient use of microbial sources can solve the problems to a considerable extent in an eco-friendly and economic way.

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