

Biobleaching of pulp and paper mill effluent using mixed white rot fungi

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ABSTRACT

In the recent past, white rot fungi have received a considerable attention for the removal of color from pulp and paper mill effluents. Investigation was carried out to compare the biobleaching potential of reported four immobilized white-rot fungal strains (a) *Phanerocheate chrysosporium*, (b) *Trametes versicolor*, (c) *Daldelia flavida* and (d) *Pleurotus ostreatus*. Also, the most efficient decolorizer was co-immobilized with best Chemical Oxygen Demand (COD) reducer and the suitability of the same was studied. The treated and pre-treated combined effluent samples for the study was collected from Varindra-Agros Paper Mills Ltd., Barnala, Punjab, and stored at 4±1°C, until further use. Polyurethane Foam (PUF) pieces were used for adsorption and immobilization of white-rot fungi. The study revealed *T. versicolor* MTCC 138 as the best decoloriser with 87.7% color reduction, while *P. chrysosporium* BKMF 1767 was found to reduce COD up to a maximum of 42.3%, after 72 hours incubation time at 30±1°C. The mixed immobilized cultures of *P. chrysosporium* BKMF 1767 and *T. versicolor* MTCC 138 supported the maximum color reduction of 88.84% and a corresponding COD reduction of 41.60% after 120 hours of incubation at 30±1°C. The study suggests that co-immobilized system can be more efficient for decolorization and COD reduction of pulp and paper mill effluent and paves a good platform for further investigation of enzymatic remedial processes for the same.

Key words: Biobleaching, Co-immobilized, *P. chrysosporium*, *T. versicolor*, COD.

INTRODUCTION

There has been an ever increasing demand for a wide variety of paper products worldwide. The growth of pulp and paper industry is an index of social, cultural, technical, industrial and economical development of a nation. The history of this industry is very old in India and has an installed capacity of over 3 million tones of paper per annum¹. The pulp and paper industry is one of the sector which has generated concern about the hazardous pollutants continuously released into water bodies. The daily pollution load contributed by this industry is equivalent to that contributed by 7.12 million people². It is estimated that about 273-450 m³ of water is required per ton of paper produced [3] that consequently generates 300 m³ as waste water⁴.

This waste water is brown in colour and is associated with high biological oxygen demand (BOD), chemical oxygen demand (COD), total solids and organic carbon⁵⁻⁶. This colour is mainly due to presence of lignin and its derivatives⁷, which are produced mainly from the pulping, bleaching and chemical recovery process in pulp and paper mill.

Several physico-chemical process for colour removal have been developed⁸⁻⁹. These processes, however quite effective in decolorization of pulp and paper mill effluents, are unattractive for industrial applications because of high costs [10]. Biological treatment processes have attracted the attention of research workers worldwide. Although conventional biological treatment processes, like anaerobic digestion, are able to reduce BOD and

COD to much extent, the colour of the pulp and paper industry effluent is not removed even after the treatment, because the biological treatment systems lack micro-organisms, which can degrade lignin or its polymeric products.

Recent development of new technologies and/or improvement of existing technologies for the treatment of effluents of pulp and paper industries has included the use of white-rot fungi *Phanerochaete chrysosporium* and *Trametes versicolor* for the effluents of the above mentioned industries in the recent past¹¹⁻¹⁴.

The concept of immobilization of microbial cells on solid supports was introduced a number of years ago. Since then, it was developed into an important technique in biotechnology¹⁵. The ability of some white-rot fungi in decolorization of pulp and paper industry effluents have also been demonstrated^{12,16-17}. The immobilized fungal system have shown more potential than free cell system for the biobleaching of paper mill effluents, because it reduces the problem of viscosity, oxygen transfer and biomass recycling^{16,18}.

A number of microbial strains have been used for biobleaching of pulp and paper mill effluent. The literature survey reveals no report on use of mixed-immobilized or co-immobilized cultures for decolorization of pulp and paper mill effluent. Therefore, the present investigation was carried out to screen the comparative biobleaching potential of reported and immobilized white rot fungal strains and also the biobleaching potential of co-immobilized culture maximum colour reducing strain and best COD reducing strain.

MATERIAL AND METHODS

White Rot Fungal Strains

Three white rot fungal strains, namely, *Trametes versicolor* MTCC 138, *Pleurotus ostreatus* MTCC 142 and *Daldelia flavida* MTCC 145 were procured from Institute of Microbial Technology, Chandigarh, India, for the study. *Phanerochaete chrysosporium* BKMF 1767 was a courtesy gift from Dr. T.K. Kent, USA. All the strains were maintained by sub-culturing aseptically, at fortnight intervals. *Phanerochaete chrysosporium* BKMF 1767 was grown

on potato dextrose agar slants while other 3 strains, namely *Trametes versicolor* MTCC 138, *Pleurotus ostreatus* MTCC 142 and *Daldelia flavida* MTCC 145 were grown on Glucose Yeast Extract (GYE) slants. In all the cases, the pH of medium was adjusted to 5.5 and slants were incubated at $30\pm 1^\circ\text{C}$ for 7 days. The slants were stored at $4\pm 1^\circ\text{C}$ for further use.

Effluent Sample Collection

The pre-treated combined effluent was collected from Varindra-Agros paper Mills Ltd., Barnala, Punjab and stored at $4\pm 1^\circ\text{C}$ until further use.

Analysis of effluent sample

Physical Characteristics

The pH of the effluent sample was recorded using a pH meter (Jencons, 8521N, Singapore). The pH of the effluent sample was adjusted to 7.6 (using acetic acid/2M NaOH) and was filtered through Whatman's filter paper No.1 to remove the suspended solids and colour was estimated spectrophotometrically at 645 nm using Spectronic 20D spectrophotometer (Bausch and Lomb, USA).

The procedure of the Indian Standard Institution (1977) was employed to calculate the contents of Total Solids (TS), Total Dissolved Solids (TDS) and Total Suspended Solids (TSS) in the effluent sample.

Chemical Characteristics

The Biological Oxygen Demand (BOD_5) was determined as per the method prescribed by APHA¹⁹. The Chemical Oxygen Demand (COD), an indicator of pollution strength of waste water, was determined by closed reflux method [19] using Hach COD reactor and Hach DR/2000 spectrophotometer system (Hach company, Loveland, USA).

Biological Characteristics

The effluent sample was analyzed for its microflora following the serial dilution technique. The bacterial count was taken using Nutrient Agar (NA) medium while the fungal count was taken by plating the samples on Potato Dextrose Agar (PDA) medium. The presence of coliform bacteria in the samples was detected by presumptive test using single and double strength McConkey Broth. The samples displaying acid production in lactose solution were taken as coliform positive.

Immobilization of fungal cultures

Polyurethane foam (PUF) was used as a carrier material for immobilization in the present investigation. PUF pieces of size 2.0cm x 2.0cm x 1.0cm were cut, washed thoroughly with distilled water and used for adsorption of fungal culture.

To immobilize the fungal culture of *P.chrysosporium*, four PUF pieces were steam sterilized in the Potato Dextrose Broth (50ml in 250 ml conical flask). To immobilize the fungal cultures of *Trametes versicolor*, *Daldelia flavida* and *Pleurotus ostreatus*, four PUF pieces for each, were steam-sterilized in the Glucose Yeast Extract media (50 ml contained in 250 ml conical flask). Then the flasks containing the carrier material were inoculated with their respective cultures under aseptic conditions and incubated at $30\pm 1^\circ\text{C}$ for seven days, under stationary conditions. The growth of fungus took place throughout the carrier and the mycelia were easily attached to the supporting material. The PUF pieces were tilted upside down after 4 days of inoculation, aseptically, so that growth can be had throughout the carrier.

Growth measurement

The fungal growth was measured in the terms of dry weight of fungus using a moisture analyzer (Mettler LJ16, USA).

Bioleaching of pre-treated combined effluent using immobilized fungal strains

The effluent filtered through ordinary filter paper was supplemented with 1% (w/v) glucose and adjusted to pH 5.5. It was (100 ml in 250 ml capacity conical flasks) then inoculated with PUF pieces loaded to a known weight of mycelium of the four strains of white-rot fungi, separately. The flasks were incubated at $30\pm 1^\circ\text{C}$ for 72 hours under stationary conditions. The samples were analyzed for change in pH, colour and COD at 48 hours and 72 hours of incubation.

Bioleaching of pre-treated combined effluent using mixed cultures of immobilized *P.chrysosporium* & *T. versicolor* and *D. flavida* & *P.ostreatus*

The effluent filtered through ordinary filter paper was supplemented with 1% (w/v) glucose and adjusted to pH 5.5. In one set, it was (100ml in 250

ml capacity conical flask) inoculated with PUF pieces loaded to a known equal weight of mycelia of both *P.chrysosporium* and *T. versicolor*. The other set (100 ml in 250 ml capacity conical flask) inoculated with PUF pieces loaded to a known equal weight of mycelia of both *D. flavida* & *P.ostreatus*. Then the flasks were incubated at 30°C for 120 hours under stationary conditions. The samples were analyzed for change in pH, colour and COD at 24 hours intervals from 48-120 hours of incubation.

RESULTS

Characteristics of pulp and paper combined effluent

The comprehensive result of physico-chemical and biological analysis of the pulp and paper combined effluent are presented in Table 1.

Physico-chemical analysis

The colour of the combined effluent was estimated as O.D. units at wavelength of 465 nm and it was measured 0.278 units. The pH was found to be 7.02.

The amount of total solids (1,800 mg/l), total dissolved solids (1,400 mg/l) and total suspended solids (400 mg/l), chemical oxygen demand (73,000 mg/l), biological oxygen demand (24,500 mg/l) of the tested sample was found to be much higher than the federal permissible limits governing discharge of effluents in India. However, pH of the effluent was within the permissible limits if Indian Standards.

Biological analysis

The qualitative analysis of the effluent samples for their microflora revealed that the bacterial density was in the range of 4.75×10^4 cells/ml, while the fungal density was in the range of 3.01×10^4 cells/ml.

The sample was found coliform positive. The most probable number (MPN) of coliform in the combined effluent was found in the range of 7-11 100ml^{-1} .

The characteristics of pulp and paper mill effluent showed more or less the same trend as observed by other Indian workers [2,4,5,6,13,17].

Thus it is an imperative need to develop an alternative low cost system for reducing not only the colour but also bringing the TSS, BOD and COD within the BIS specification before its discharge into main streams of water, land etc.

Immobilization of white-rot fungi

The polyurethane foam (PUF) pieces were used for the adsorption of white-rot fungi *i.e.*, *P. chrysosporium* BKMF 1767, *T. versicolor* MTCC 138, *P. ostreatus* MTCC 142 and *D. flavidia* MTCC 145.

Table 1: Characteristics of pulp and paper effluent

pH	Physico-chemical characteristics					Biological characteristics		
	TS (mg/l)	TSS (mg/l)	TDS (mg/l)	COD (mg/l)	BOD ₅ (mg/l)	Bacterial Count (Cells/ml)	Fungal Count (Cells/ml)	MPN (/100ml)
7.02	1800	400	1400	73,000	24,500	4.75x10 ⁴	3.01x10 ⁴	7-11

Table 2(a): Decolorization and COD reduction of pulp and paper mill effluent using immobilized white rot fungi

Fungal Culture	pH		% Reduction	
	Initial	After Treatment	Colour	COD
<i>Phanerochaete chrysosporium</i> BKMF 1767	5.5	4.37	80.5	41.70
<i>Trametes versicolor</i> MTCC 138	5.5	4.67	82.0	37.70
<i>Pleurotus ostreatus</i> MTCC 142	5.5	4.74	74.1	36.10
<i>Daldelia flavidia</i> MTCC 145	5.5	5.40	71.9	35.08

Treatment conditions

Age of inoculum	:	7 days
Temperature	:	30±1°C
Condition	:	Stationary
Mycelial load	:	646 mg dry weight/100ml
Treatment time	:	48 hours.

Table 2(b): Decolorization and COD reduction of pulp and paper mill effluent using immobilized white rot fungi

Fungal Culture	pH		% Reduction	
	Initial	After Treatment	Colour	COD
<i>Phanerochaete chrysosporium</i> BKMF 1767	5.5	4.28	81.6	42.30
<i>Trametes versicolor</i> MTCC 138	5.5	4.58	87.7	38.60
<i>Pleurotus ostreatus</i> MTCC 142	5.5	4.69	76.9	36.30
<i>Daldelia flavidia</i> MTCC 145	5.5	5.33	74.4	35.50

Treatment conditions:

Same as Table 2a except treatment time of 72 hours.

The growth of the culture took place on the PUF pieces and the mycelia were easily attached to the carrier. Seven days old culture, in general, has been recommended for decolorization and COD reduction of pulp and paper mill effluents (Singh, 1993). The white rot fungi in immobilized form have been reported as efficient producer of extra-cellular lignin peroxidase¹⁸.

Comparative decolorization of pulp and paper mill effluent by immobilized white-rot fungi

The results obtained during the course of experiment are comprehensively presented in Table 2a and 2b. Among the four white-rot fungal cultures, *T.versicolor* showed the highest degree of decolorization (87.7%). The corresponding COD reduction by this culture was 36.6%. The order of decolorization by these fungal cultures was *T. versicolor* > *P.chrysosporium* > *P.ostreatus* > *D. flavida*. *P.chrysosporium* supported the maximum COD reduction (42.3%). However, minimum reduction in COD (35.5%) was recorded by *D. flavida*.

The highest decolorization strains, *i.e.*, *T. versicolor* and highest COD reducing culture, *i.e.*, *P. chrysosporium*, were used in mixed immobilized form for further experimentation. The ability of *P. ostreatus* and *D. flavida* in immobilized mixed system was also tested for colour and COD reduction. The per-treated combined effluent samples (100 ml in 250ml conical flasks, supplemented with 1% w/v dextrose and adjusted pH 5.5) were inoculated with known amount (646 mg dry weight) of mycelia, immobilized on PUF pieces. The samples were incubated at 30±1°C for 72 hours and were analyzed for change in pH, colour and COD. The results obtained during the course of investigation are presented in Table 3a and 3b. The combination of *P.chrysosporium* and *T. versicolor* in mixed immobilized form supported decolorization by 88.84% after a treatment time of 120 hours, under stationary conditions. The corresponding COD reduction by these two fungal cultures in combination was 41.60%. The maximum reduction in colour by *P. ostreatus* and *D. flavida* in combination

Table 3a: Decolorization potential of immobilized mixed culture of *P. chrysosporium* & *T. versicolor*

Incubation time (hours)	pH		% Reduction	
	Initial	After Treatment	Colour	COD
48	5.5	4.97	79.85	36.66
72	5.5	4.65	83.09	37.72
96	5.5	4.21	86.33	40.80
120	5.5	4.20	88.84	41.60

Treatment conditions: Same as Table 1a except different incubation time

Table 3(b): Decolorization potential of immobilized mixed culture of *P. ostreatus* & *D. flavida*

Incubation time (hours)	pH		% Reduction	
	Initial	After Treatment	Colour	COD
48	5.5	4.89	70.86	35.50
72	5.5	4.72	72.66	37.75
96	5.5	4.67	75.18	35.91
120	5.5	4.61	74.10	37.91

Treatment conditions: Same as Table 1a except different incubation time

was 75.18% after 96 hours treatment period. However, maximum COD reduction by this mixed culture was 37.91% after 120 hours of incubation.

DISCUSSION

Since the discovery of lignin peroxidase (LiP) in the white rot fungus *Phanerochaete chrysosporium*²⁰, the production of this enzyme or the corresponding activity has been reported in a number of other white rot²¹⁻²⁹. LiP is believed to be one of the key enzymes in lignin degradation. Further studies in lignin degradation have also resulted in enzyme and gene localization, isolation and cloning of extracellular LiP enzyme in *P. chrysosporium* and *T. versicolor*^{23,30-31}. *D. flavida* and *P. ostreatus* have also been studied and reported to have lignolytic activity²⁹. All the studies reported till date for biobleaching of pulp and paper mill effluent have been either free cell or immobilized culture of single white rot fungal strain. The present investigation attempted to look into the biobleaching studies in conjunction with COD reduction of pulp and paper

mill effluent using mixed immobilized cultures of white rot fungi. The study reflects that the mixed immobilized culture of *P. chrysosporium* and *T. versicolor* is more efficient in colour and COD reduction than *D. flavida* and *P. ostreatus* combination. Also, the fungal culture in mixed immobilized form has been found to be more efficient than the cultures immobilized individually. The screening of the four white rot fungal strains studied reflects that *T. versicolor* MTCC 138 is the best decolorizing culture of pre-treated pulp and paper mill effluent while *P. chrysosporium* BKMF 1767 is the best COD reducer of the same, which is in good accordance with the previously reported findings^{11-13,17}. Thus, the present investigation suggests that the co-immobilized system can be more efficient for decolorization and COD reduction for pulp and paper mill effluent. Also, the experimentation carried out in flask culture at bench scale would provide useful guidelines for further investigations for the development of treatment system for pulp and paper mill effluents.

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