INTERNATIONAL JOURNAL OF PLANT, ANIMAL AND ENVIRONMENTAL SCIENCES
IJPAES
VOLUME-2, ISSUE-1, JAN-MAR-2012 ISSN 2231-4490
Accepted: Jan-2012 *Research Article*

DECOLOURIZATION OF PAPER MILL EFFLUENT BY IMMOBILIZED CELLS OF *PHANEROCHAETE CHRYSOSPORIUM*

[1]Gomathi, V Cibichakravarthy, B [2]Ramanathan, A [3]Sivaramaiah Nallapeta, [4]Ramanjaneya V, R Mula, [5]Jayasimha Rayalu, D

[1]Centre for Plant Molecular Biology, Tamil Nadu Agricultural University, Coimbatore 641003, India
[2]Centre for plant protection studies, Tamil nadu Agricultural University, Coimbatore 641003, India
[3]Birla Institute of Scientific Research, Jaipur-302001, India.
[4]Torrey Pines Institute of Molecular Studies, Florida 3497, USA
[5]Department of Biotechnology, Global Institute of Biotechnology, Himayat nagar, Hyderabad. India.

ABSTRACT: Paper and pulp industrial waste waters are a major environmental concern. As the treatment of these effluents is a major problem, exploring novel and feasible biotechnological interventions is very important. Utilization of white rot fungi as a means of biological treatment seems to be a viable option**.** The study was undertaken to use immobilization technique and *Phanerochaete chrysosporium*, a commonly used white rot fungus for decolourization. The optimum conditions under which significant decolourization observed were temperature (30-35^{o} C), agitation (125rpm), sucrose (0.25 – 1.0%), ammonium chloride (0.25 – 1.0%), pH and duration 24-96 hrs. The addition of 1g/ L sucrose and 1g/L ammonium chloride has significant effect on the degradation of lignin by the white-rot fungi as immobilized cells**.** Reduction of lignin, phenol, chemical oxygen demand (COD) and biological oxygen demand (BOD) from paper mill effluents using different concentration of carbon and nitrogen (0.25, 0.50, 0.75, and 1.0 %) was investigated. Maximum decolourization of 83% was achieved when 1% sucrose and 1% ammonium chloride was added the effluent containing immobilized cells of *P. chrysosporium*. Significant reduction in BOD, and COD of the effluent was observed in the presence of *P. chrysosporium respectively*. A pH shift of 7.0 to 6.3 had the significant effect (83%) on biological decolourization process.
Key words: Waste water treatment, immobilization, ligninolytic fungi

INTRODUCTION

Industrial effluents represent a significant environmental and economic problem. The pulp and paper industry typically generates large quantities of wastewater whose correct treatment prior to discharge into the environment is critical. Paper factory effluent is one of the major pollutants on the earth. The pulp and paper mill effluent is highly coloured. The persistent dark brown colour in the released paper and pulp industrial effluent from wastewater treatment facilities is brown or black in colour due to dissolved lignin based synthetic, aromatic and chlorinated compounds derived from the blow heat condensate, pulp decker washing, chlorine and alkali bleach waste, black liquor spillage and foul evaporator condensate. These compounds being non-degradable, by chemical and conventional biological methods, pose problems in the removal of colour (Bajpai and Bajpai 1994). The brown color of the effluent may increase water temperature and decrease photosynthesis, both of which may lead to decreased concentration of dissolved oxygen (Kingstad and Lindstrom 1984). Lignin and its derivatives are difficult to degrade because of the linkages within the molecule, especially the biphenyl type carbon-to-carbon linkages. Because chlorolignins are not easily biodegradable, conventional biological treatment methods cannot efficiently decolorize these effluents (Eaton et al. 1982). The growing awareness of environmental issues and their potential health hazards caused by industrial waste water has prompted many countries to impose limits on the discharge of certain improperly /untreated effluents. In India 34 large scale paper mills account for 51% of total capacity and 271 small paper mills account for the remaining 49%. Chemical recovery is not carried out in small paper mills due to economic reasons.

The pollution load in terms of BOD from small paper mills is 2.5 times higher than that of large paper mills, which employ the soda recovery process (Sastry 1986). These produce nearly a million tonnes of all varieties of paper per annum. White-rot fungi are primarily responsible for the initial decomposition of lignin in wood, which occurs via an oxidative and relatively nonspecific process (Hatakka 1994; Hammel et al. 2002). *Phanerochaete chrysosporium*, a white-rot-wood decaying basidiomycete, produces a potent lignin degrading system that oxidizes lignin completely to CO_2 (Breen and Singleton 1999). The utilization of *P. chrysosporium* in waste water treatment is gaining importance in paper industries, because of their ability to degrade lignin in wood (Eaton *et al.* 1980). Attempts have been made to remove the colour of the effluent from a Kraft mill by using *P. chrysosporium* by Thomas *et al*.(1981) and from the pulp waste by *Tinctoria* sp (Fukuzumi 1980) and *Aspergillus* sp (Dutta *et al.* 1985). Belsare and Prasad (1988) demonstrated that for degrading lignin lignolytic fungus like *P. chrysosporium requires* exogenous carbon and nitrogen nutrients. In the present study, an attempt was made to find out the efficacy of white rot fungus *P. chrysosporium* for decolourization of pulp industry effluents .White rot fungi have been receiving attention due to their efficiency and presence of diverse ligninolytic enzyme systems involving lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac). These fungi have been reported as having promising capabilities of decolorizing wastewater from a wide range of industries including those producing olive oil, alcohol, textile, and pulp and paper mill effluent.

MATERIALS AND METHODS

Microorganism, Inoculum development, culture medium and conditions

The paper mill effluent samples were collected from M/S Seshasayee Paper and Boards Ltd., Pallipalayam, Tamil Nadu, India. The physico-chemical and biological characteristics of the combined effluent were analyzed using standard methods (APHA 1975).The white rot fungi *P. chrysosporium,* obtained from the Department of Environmental Sciences, Tamil Nadu Agricultural University at Coimbatore in India was used for decolourization experiments. Pure mycelia of all fungal isolates were maintained at 4 °C in distilled water. *P. chrysosporium* were cultured on PDA plates and incubated at 32°C. The growth of white rot fungi measured as colony diameter was monitored on daily basis. All observations and data were collected from the experiments performed in triplicate.

Immobilization of white rot fungi

The fungal isolate was grown in 100 ml malt extract media (pH 6.5). After 5 days, the mycelial pellets were washed with sterile phosphate buffer (pH 7.0) and then the mycelial mat was homogenized by grinding with acid washed sand using pestle and mortar for enzymatic analyses and immobilization. The 2 ml of homogenized fungal mat was added aseptically using a sterile 5 ml glass syringe fitted with a 16-T gauge needle to sodium alginate (2% w/v) and entrapped in 0.1M calcium chloride as calcium alginate. A 50 immobilized beads (4 mm approximately) of mycelial mat trapped in alginate ware added to 250 ml Erlenmeyer flask containing 100 ml effluent. A control without immobilized *P.chrysosporium* beads was used for comparison purposes. At specified time intervals 3- 4 ml of the samples were drawn from the inoculated treatment and centrifuged at 7000 rpm for 10 min. The supernatant was collected and colour removal (CU values) was measured for seven days at 465 nm in the spectrophotometer. The same experiment was carried out by different treatments with different concentration (0.25-1.0%) of carbon source viz., sucrose and nitrogen source viz., ammonium chloride. The flasks were incubated under at room temperature (32°C) in an environmental rotary shaker.

Decolourization of effluent using white rot fungi:

Paper and pulp mill effluent was scanned in a spectrophotometer to ascertain the specific wavelength where maximum absorbance occurs. A maximum absorbance at 465 nm was observed. The rate of decolourization was monitored at this wavelength for change of colour determination. The effluent was centrifuged at 10000 rpm for 30 min to remove all the suspended particulate material.

Absorbance values were transformed in to colour units (CU) using the following equation: CU= 500 A_2/A_1. Where A_1 is the absorbance of 500 CU platinum – cobalt standard solution (A_{465}= 0.132) and A_2 is the absorbance of the effluent sample Since the white rot fungus can not grow on lignin alone, basic nutrients such as carbon and nitrogen were added to the growth media to stimulate the growth of the fungus and the breakdown of lignin in the effluent. The carbon sources for the fungus included sucrose and effluent, while ammonium chloride was used as nitrogen source. All the nutrients were added in % concentration. The fungal culture was inoculated for 5 days at room temperature. The untreated and treated effluent was analyzed for pH, BOD, and COD as described in APHA (1975) and the change in the colour was determined using a spectrophotometer (465 nm).

RESULTS AND DISCUSSION

The pulp and paper mill effluent imparts dark black/brown color to the water body. The color is mainly due to lignin and its derivatives released during various stages in the paper-making process. The complex nature of such lignin compounds and their phenolic content make them extremely resistant to biological degradation. Conventional treatment methods such as aerated lagoons and activated sludge process are ineffective in removing color. The physical and chemical treatment methods including ultra filtration, ion-exchange, and lime precipitation, are expensive and are also less efficient. Therefore, alternate low-cost biological treatment processes are now being considered as viable options. Most of these biological processes are based on lignin-degrading fungi. Depending on the treatment process, the fungal inoculum for decolourization could be used in the form of mycelium, cell free system or the immobilized state. The decomposition of lignin is an enzymatic process employing various ligninases being produced by the fungal species. Soluble and immobilized ligninolytic enzymes have also been employed for effluent decolourization.

The physical, chemical and biological characteristics of the paper mill effluent used in the study are presented in the Table1. Growth on glycerol leads to carbon limitation which affects the onset of secondary metabolism (Buswell et al., 1984). Therefore, glucose (10 g/L) was selected as carbon source. The maximum colour removal by the growth of *P.chrysosporium* was observed with the addition of 1.0% each of sucrose and ammonium chloride to 50 ml of the effluent (Table 2). Chang *et al*. (1987) showed that the enzyme catalyzed process by whole cells of *P. chrysosporium* could effectively remove the colour from paper mill effluent. Addition of metabolizable nutrients such as glucose, sucrose or cellulose favored fungal decolourization of the effluent (Eaton *et al*. 1982). The nutrients added were able to induce sugar oxidizing enzyme activity which oxidized the sugar and produced hydrogen peroxide necessary for the degradation of lignin (Erikson et al. 1986). Sugar is also a substrate for biosynthesis of veratryl alcohol, an inducer of ligninases. Ligninase activity in *P. chrysosporium* is stimulated by incubating cultures with various substrates for the enzyme, including veratryl (3, 4-dimethoxybenzyl) alcohol, which is a secondary metabolite of this fungus. Ligninase activity increased 2 to 4 h after the addition of exogenous veratryl alcohol to ligninolytic cultures (Faison et al. 1986). The data revealed that by adding 1.0g sucrose and 1.0g ammonium chloride per liter to the initial effluent increased the BOD and COD. The effluent itself had native microflora which caused a decline in BOD (19%) and COD (7.2%) of untreated effluent. *P. chrysosporium* reduced the BOD and COD levels to 40% on the first day treatment (Eaton *et al*. 1982).

There was a reduction in the pH of the effluent supplemented with sucrose and ammonium chloride presumably due to the release of organic acid in the fermentation process. Prabhu *et al.* (2005) reported that 84% effluent decolourization along with 79% COD reduction by *P. chrysosporium*. The effluent chlorinated phenol degradation was 91% by the fungus when added with 1% glucose as co-substrate. Sehanat et al. (2009) reported that *Daedaleopsis* sp. and *P. chrysosporium* exhibited the highest ability to decolorize wastewater (52%) and (86%), respectively. *P. chrysosporium* also greatly reduced the pH level.

Table1 Physico-chemical characteristics of the paper mill effluent

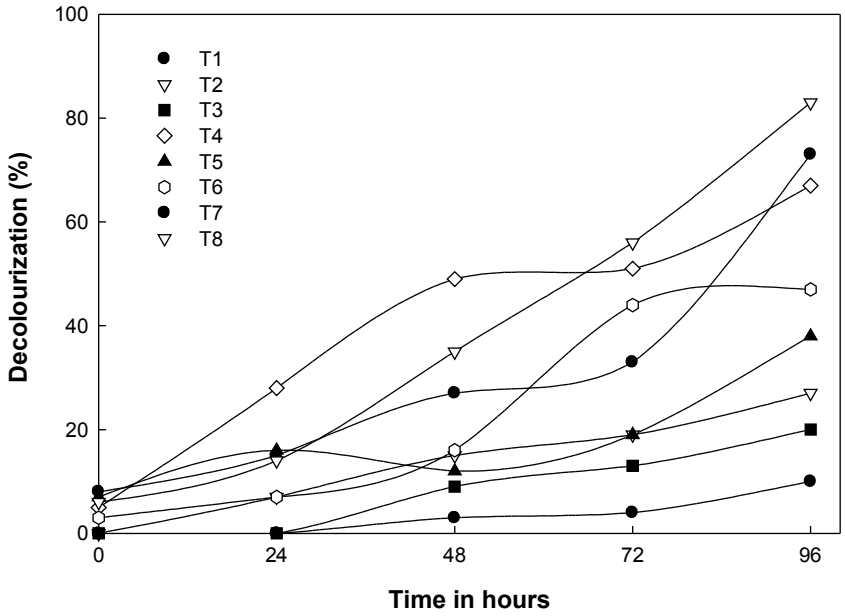
Parameters	Observed values
Color	Dark brown
Odor	Phenolic
PH	7.8-8.0
EC mmhos/ cm^{-1}	1.6
Suspended solids (mg l^{-1})	1120
Dissolved solids (mg l^{-1})	1870
Total solids (mg l^{-1})	2870
BOD (mg l^{-1})	162
COD (mg l^{-1})	392
Total N (mg l^{-1})	18.12
Available P mg l^{-1})	16.80
Available K (mg l^{-1})	10.62
Calcium (mg l^{-1})	132.78
Magnesium (mg l^{-1})	71.96
Sodium (mg l^{-1})	482.40
Sulphate (mg l^{-1})	440.60
Chloride (mg l^{-1})	143
Lignin (mg l^{-1})	290.75
Cellulose (mg l^{-1})	628
Reducing Sugars (mg l^{-1})	186.75
Polyphenol (mg l^{-1})	43.2

Table 2 Characteristics of the effluent after inoculation with *P.chrysosporium*

Treatment	**pH**	**EC mmhos/ cm^{-1}**	**BOD mg l^{-1}**	**COD mg l^{-1}**	**Bacteria 10 $^{6}ml^{-1}$**	**Fungi $10^{4}ml^{-1}$**	***Actino-mycetes*** **10^{4}**
Control	7.10	1.03	123	286	98	13	7
P.chrysosporium	6.72	1.17	118	281	121	7	5
Sucrose (1%)	6.58	1.25	96	275	114	5	4
Ammonium chloride (1%)	6.36	1.39	89	279	127	10	5
P. chrysosporium + Sucrose (0.25) + Ammonium chloride (0.25%)	6.29	1.42	78	266	131	21	5
P.chrysosporium + Sucrose (0.50) + Ammonium chloride (0.50%)	6.38	1.33	103	273	135	26	3
P.chrysosporium + Sucrose (0.75) + Ammonium chloride (0.75%)	6.96	1.28	112	276	140	30	4
P. chrysosporium + Sucrose (1.0%) + Ammonium chloride (1.0 %)	6.30	1.42	116	280	143	37	3

The present study further revealed that *P.chrysosporium* inoculation helped colour removal of the pulp and paper mill effluent by 83% within 96 hours (Fig.1). High oxygen levels stimulated decolourization. Aeration without inoculation of the fungus did not reduce the colour; however, there was reduction in BOD and COD. With proper aeration and exogenous addition of nutrients, *P.chrysosporium* can effectively reduce the colour of the effluent

Fig 1. Decolourization of the effluent by *P chrysosporioum*



T1 -Control
T2 - P.*chrysosporium*
T3 - Sucrose (1%)
T4 - Ammonium chloride (1%)
T5 - *P. chrysosporium* + Sucrose (0.25) + Ammonium chloride (0.25%)
T6 - *P.chrysosporium* + Sucrose (0.50) + Ammonium chloride (0.50%)
T7 - *P.chrysosporium* + Sucrose (0.75) + Ammonium chloride (0.75%)
T8 - P chrysosporium + Sucrose (1%) + Ammonium Sulphate (1%)

Decolourization of the effluent by *P.chrysosporium* (Color removal expressed in percentage)

Treatment	0 hr	24hr	48 hr	72 hr	96 hr
T1	0	0	3	4	10
T2	0	7	15	19	27
T3	0	0	9	13	20
T4	5	28	49	51	67
T5	7	16	12	19	38
T6	3	7	16	44	47
T7	8	15	27	33	73
T8	6	14	35	56	83

CONCLUSION

The isolate of white rot fungi obtained from the dept of environmental science were capable of secreting active ligninolytic enzymes and decolorizing the pulp and paper mill waste water. While glucose may be directly added to the wastewater source, it has been found to be more economical to reduce or substantially remove color from the wastewater by additionally adding white rot fungi in the presence of cellulose and hemi-cellulose.

The immobilized cell of white rot fungi has also been found helpful to improve the reduction in color, biological oxygen demand, as well as chemical oxygen demand in paper mill waste water reduction. It was found that the combination of calcium alginate immobilized *P. chrysosporium* with the addition of C and N source helped to achieve high treatment efficiency in colour removal. The low concentration of carbon source in the effluent is essential for decolourization activity whereas sugar is a substrate for biosynthesis of veratryl alcohol, an inducer for ligninase production. When the immobilized culture was used under aerobic conditions, it was found that BOD and COD and other characteristics of the effluent were reduced and high transparency of the effluent was obtained. In particular, their ability to decolorize wastewater at higher temperatures makes them more suitable for tropical environments.

Acknowledgements: The financial support from the Seshasayee Paper Board ltd, Pallipalayam, Tamil Nadu, India and Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India are gratefully acknowledged.

REFERENCES

1) APHA, AWWA & WPCF (1975) *Standard Methods for the Examination of Water and Waste Water*, 14th edn. Washington, DC: American Public Health Association.
2) Bajpai P, Bajpai PK (1994) Biological colour removal of pulp and paper mill wastewaters. *J Biotechnol* 33:211-220
3) Belsare DK, Prasad DY (1988) Decolourization of effluent from the bagasse based pulp mills by white rot fungus. *Schizophyllum commune*. *Appl Microbiol Biotechnol* 301-304
4) Breen A, Singleton FL (1999) Fungi in lignocellulose breakdown and biopulping. *Curr Opin Biotechno* 10: 252–258
5) Buswell JA, Mollet B, Odier E (1984) Ligninolytic enzyme production on by *Phanerochaete chrysosporium* under conditions of nitrogen sufficiency. *FEMS Microbiol Let* 25: 295-299
6) Chang HM, Joyce TW, Kirk TK (1987) Process of treating effluent from a pulp or paper making operation. US patent No 4: 655
7) Dutta SA, Parhad NM, Joshi SR (1985) Decolourization of lignin bearing waste by *Aspergillus* sp. IAWPC *Technol Annual* 12: 32-37
8) Eaton D, Chang HM, Kirk TK (1980) Decolourization of kraft bleach effluent. *TAPPI* 63: 103
9) Eriksson K, Ander E, Patterson P (1986) Regulation of lignin degradation in P*hanerochate chrysosporium*. Proceedings of the Third International Symposium on Biotechnology in the Pulp and Paper Industry Stockholm Sweden P 24-27
10) Fukuzumi T (1980) Microbial decolourization and defoaming of pulping waste liquors. In: Kirk TK, Higuchi T, Chang HM (eds) Lignin Biodegradation Microbiology, Chemistry and Potential Applications CRC Press Boca Raton Florida.1: 215-230
11) Hammel KE, Kapich AN, Jensen KA, Ryan ZC (2002) Reactive oxygen species as agents of wood decay by fungi. *Enzyme Microb Technol 30***:** 445–453
12) Hatakka A (1994) Lignin modifying enzymes from selected white rot fungi- production and role in lignin degradation. *FEMS Microbiol Rev* **13**, 125-135
13) Prabhu PC, Udayasooriyan C (2005) Decolorization and degradation of phenolic paper mill effluent by native white rot fungus *Phanerochaete chrysosporium*. *Asian J Plant Sci* 4: 60-63
14) Sehanat P, Pongtharin T, Hunsa P (2009) Decolourization of pulp mill wastewater using thermo tolerant white rot fungi. *Science Asia* 35: 37–41
15) Homas W, Chang HM, Alton G, Kirk D, Kirk K (1981) Removal of kraft bleach plant colour by a ligninolytic fungus. Proceedings of the Applied Environmental conferences USA Pp 225-228
16) Kingstad KP, Lindstrom PK (1984) Spent liquors from pulp bleaching. *Environ Sci and Technol 18*: 236A– 248A
17) Eaton DC, Chang HM, Kirk TK (1980) Decolorization of kraft mill bleach plant effluent. *TAPPI Journal* 63: 103–106
18) Sastry CA (1986) Color removal from pulp and paper mill wastes. *Ind J Environ Protect* 6: 105–113.
19) Faison BD, Kirk TK, Farrell R L (1986) Role of Veratryl Alcohol in Regulating Ligninase Activity in *Phanerochaete chrysosporium. Appl Environ Microbiol* 52: 251–254.