ISSN:2319-2607 IISUniv.J.Sc.Tech. Vol.3(1),22-30 (2014)
RESEARCH ARTICLE

A Study on Immobilization of White Rot Fungal Cultures for Addressing the Toxicity Concerns of Paper Industry

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Abstract

The production and use of paper has a number of adverse effects on the environment which are known collectively as Paper pollution. The most important problem created by the pulp and paper industry is the release of waste bleach waters from conventional bleaching of chemical pulps into receiving waters. Fungi are robust organisms that have a high tolerance to toxic environments making them ideal to use for bio-remedial purposes. Especially, the white rot fungi have been shown to mineralize a wide variety of environmental pollutants. Immobilized viable cells, which serve as 'controlled catalytic biomass', have opened new avenues for continuous fermentation on heterogeneous catalysis basis by serving as self-proliferating biocatalysts. Keeping in view of this, the present study was chosen with an aim to evaluate various commonly available, cheap, solid support materials for their suitability to be used for immobilizing white rot fungal cultures for using them further in decolourization of effluents from the paper industry. For this, a total of eighteen support materials including beads/pearls, plastic stripes, springs, sand stone, buttons, tyre tube pieces, fiber glass, pieces of net, sponge etc were taken to develop a simple and cost-effective process of immobilization of white rot fungi for the paper industry. The white rot fungal cultures available in the Biotech lab of KNHPI was used in the present study. The extent of immobilization of the fungus studied varied from matrix to matrix used and maximum of it could be observed on the materials like tyre tube/carpet/sandstone pieces. On evaluating the potential of immobilized fungal cultures for decolourizing the black liquor effluent generated from pulping of banana fiber, it was observed that the addition of urea promoted removal of colour as compared to those treated without adding urea. The present study gives an insight into the vast possibilities of developing a cost-effective system of immobilization of white rot fungal cultures on easily available solid matrices so as to use them further for addressing the toxicity concerns especially the problem of colour associated with papermaking.

Keywords

Black liquor, Decolourization, Effluents, Handmade paper, Immobilization, Paper making, White rot fungi

Introduction

Pulp and paper industry is the fifth largest consumer of energy, accounting for four percent of the entire world's energy use. The pulp and paper industry uses more water to produce a ton of product than any other industry. Worldwide consumption of paper has risen by 400% in the past 40 years, with 35% of harvested trees being used for paper manufacture.

NO_2, SO_2 and CO_2 are all emitted during paper manufacturing. NO_2 and SO_2 are major contributors of acid rain whereas CO_2 is a green house gas. Waste water discharges from paper industries contain solids, nutrients (N and P), dissolved organic matter and a large amount of colour. Dioxins, produced mainly during pulp bleaching with chlorine and chlorine compounds are persistent organic pollutants that are generally recognized among the most toxic human released pollutants in existence. Their health effects on humans include reproductive, developmental, immune and hormonal problems. They are known to be carcinogenic. Over 90% of human exposure is through food, primarily meat, dairy, fish and shell fish as dioxins accumulate in the food chain in the fatty tissue of animals.

The most important problem created by the pulp and paper industry is the release of waste bleach waters from conventional bleaching of chemical pulps into receiving waters. An effective purification of bleach plants' effluents must involve elimination of both low and high molecular weight chlorinated compounds. One possibility for removal of these polymeric compounds is the use of white rot fungi which are the only micro-organisms able to completely degrade polymeric lignin. However, there are no microorganisms known that can grow on lignin alone

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and lignin degradation by white rot fungi seems to require energy. They need an extra, more easily degradable carbon source than lignin to provide energy and H_2O_2 for lignin degradation.

White rot fungi (WRF), a group of basidiomycetous are the potential organisms capable of mineralizing the complex wood polymer and a wide variety of recalcitrant compounds like xenobiotics, lignin and dyestuff by their extracellular lignolytic enzyme system. WRF offer significant advantages over bacterial system since their extracellular lignolytic enzyme system consisting of lignin peroxidases, manganese dependent peroxidases, manganese independent versatile peroxidases, and laccases degrade a wide variety of complex aromatic dyestuffs (Boer *et al,* 2004; Kamistsuji *et al*, 2005). The degradation of persistent organic pollutants by white rot fungi *Phanerochaete chrysosporium* was reported in 1985 (John, 1985). Afterwards, Kirk and Farell (1987) found out the non specificity of the lignin peroxidase system produced by white rot fungi at the secondary metabolism stage. White rot fungi can degrade lignin and many organic pollutants, such as polycyclic aromatic hydrocarbons, chloro-aromatics, dyes and pesticides (Aitken *et al*, 1989; Bumpus, 1989; Hammel, 1989). White-rot fungi do not require preconditioning to particular pollutants, because enzyme secretion depends on nutrient limitation, nitrogen or carbon, rather than presence of pollutant. The extracellular enzyme system also enables white-rot fungus (WRF) to tolerate high concentration of pollutants (Knapp *et al,* 1997; Arulmani *et al*, 2008).

Generally, removal of dyes/colour by microorganisms takes place through three mechanisms: biosorption, bioaccumulation and biodegradation. Biosorption is defined as binding of solutes to the biomass by processes which do not involve metabolic energy or transport, although such processes may also occur simultaneously where live biomass is used. The process of biosorption can occur in either living or dead biomass (Tobin *et al*, 1994) whereas bioaccumulation is defined as the accumulation of pollutants by actively growing cells by metabolism- and temperature-independent and metabolism-dependent mechanism steps (Sadettin and Donmez, 2006). Biodegradation is an energy dependent process and involves the breakdown of dye into various by products through the action of various enzymes. When biodegradation is complete, the process is called mineralization (Bennett and Faison, 1997). Use of growing cultures in bioremoval has the advantage over the non-living and resting cells as the simultaneous removal of dye is obtained during growth of the organism and separate biomass production could be avoided (Charumathi and Das, 2010).

The use of freely suspended microbial cells for dye removal is limited owing to their inherent disadvantages such as small particle size, possible clogging and low mechanical strength of the biomass. Immobilized cells offer advantages over dispersed cells such as high cell density, strong endurance of toxicity, lower operating costs, simple maintenance management and lower residual sludge. In recent years, several studies have shown that the mechanisms for decolorization using immobilized microorganisms may include both biodegradation and adsorption (Chen *et al*, 2003; Pazarlioglu *et al*, 2005)

Compared with suspended microorganism technology, immobilized microorganism technology possesses many advantages, such as high biomass, high metabolic activity, and strong resistance to toxic chemicals, and so on (Zhou *et al*, 2008). An immobilized microorganism is defined as a microbe that is prevented from moving independently of its neighbors to all parts of the aqueous phase of the system by natural or artificial means (Tampion and Tampion, 1987). Zhou *et al* (2008) have reviewed the latest patents which emphasize the characteristic of the immobilization carriers as well as bioreactors. Several methods can be applied to immobilization microorganism on the carriers including adsorption, covalent bonding, cross-linking of microorganism and encapsulation into a polymer-gel and entrapment in a matrix, and so on (Casidy *et al*, 1996).

Romaskevic *et al* (2006) have reviewed the application of polyurethanes in biochemical and biotechnological fields, concerning its biocompatibility and stability. They have reviewed the synthesis of different kinds of polyurethanes, their application for the immobilization of enzymes and whole cells, and their employment in constructions of biosensors. PU foam is reported to be a perfect support for the cells growth. Cell growth kinetics of the immobilized *Catharanthus roseus* has been investigated by Hu and Yuan (1995).

Keeping in view of this, the present study was chosen with an aim to evaluate various commonly available, cheap, solid support materials for their suitability to be used for immobilizing white rot fungal cultures for using them further in decolourization of effluents from the paper industry. For this, a total of eighteen support materials including beads/pearls of different shapes, plastic stripes, springs, Regmal, buttons, glass beads, tyre tube pieces , fiber glass, pieces of net, sponge etc were taken to develop a simple and cost-effective process of immobilization of white rot fungi for the paper industry.

Materials and Method

Immobilization Studies

For the present study, white rot fungal culture available in the Biotech lab of KNHPI was used. Fungus was cultivated in Potato Dextrose Agar plates at 37 ˜C. A total of eighteen support materials including beads/pearls of different shapes, plastic stripes, springs, Regmal, buttons, glass beads, tyre tube pieces, fiber glass, pieces of net, sponge etc were chosen to explore the possibilities of fungal immobilization. These support materials were added into the Erlenmeyer flasks containing potato dextrose broth and autoclaved at 121 psi before aseptically inoculating them with the constant number of fungal disc from a 5-7 day old freshly grown fungal culture on PDA plates using the special tool of cork borer. Cork borer (Fig.1.) is a metal tool for cutting a circle of a solid media containing fungal culture in the petri dish for inoculation. The cork borer available in a set of nested size along with a solid pin for pushing the cutted media circle out of the borer was used. Two flasks were thus inoculated for each of the solid matrix chosen. The flasks were then incubated at 37 ˜ C under static (in bacteriological incubator) and shaking conditions (at 75 rpm in orbital shaking incubator) for all the respective matrices. The extent of immobilization of fungus was observed under static and shaking conditions after an interval of 3-6 days.

Decolourization Studies

The solid matrices having good amount of immobilized fungus were then selected for further studies on decolourization. To study the potential of immobilized fungal culture for decolourization, black liquor generated from banana fiber pulping was chosen. The black liquor was first characterized for the parameters of interest viz. total solids, colour and Residual Active Alkali (RAA).

The original liquor was diluted with distilled water in the ratio of 70:30 for using it further to inoculate with fungus. The diluted black liquors were then added with sucrose (0.5% and 1.0%) and urea (0.5% and 1.0%) as a carbon source for the fungus. Thus the four flasks were then autoclaved at 15 psi for each of the matrix immobilized with fungus chosen for decolourization studies. One flask containing the diluted black liquor was also autoclaved to be used as a control. All the autoclaved flasks were then inoculated at room temperature with the equal number of chosen matrices under aseptic conditions except the control flask. The flasks were then incubated in the incubator shaker at 37 ˜ C and 50-100 rpm for a period of 2-3 weeks. 200 µl of the samples were collected from each of the flasks in microcentrifuge tubes on the 4th and 8th day of incubation under aseptic conditions. These sample aliquots were then analysed for colour at 465 nm with the help of UV/Vis Spectrophotometer.

Fig.1. Cork Borer

Results and Discussion

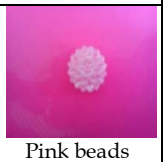
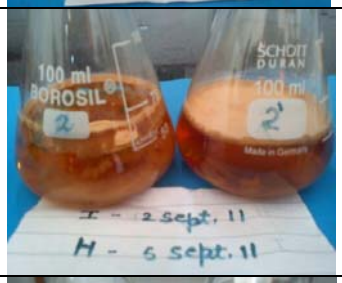
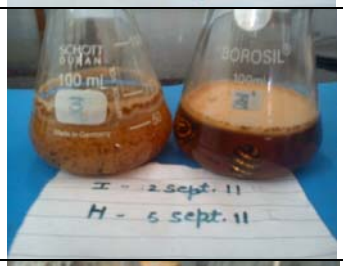
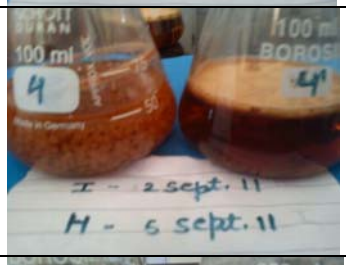
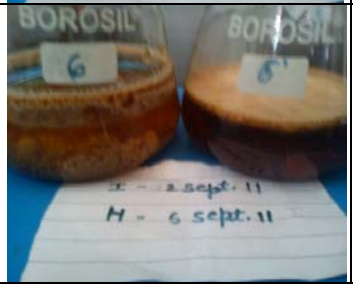
Immobilization of Fungus on Cost Effective Solid Matrices

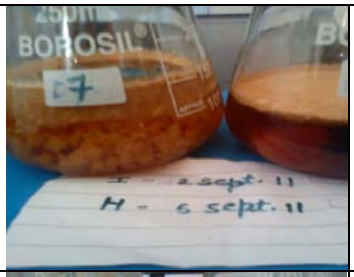
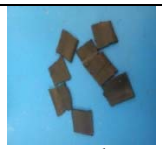
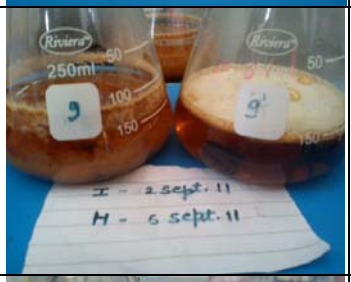
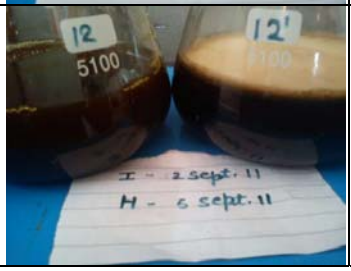
All the flasks incubated under stationery and shaking conditions at 37 ° C were observed after an interval of 3 days and six days each. It was observed that almost all the flasks incubated under stationery conditions showed white coloured growth on the broth-surface and those under shaking conditions resulted into the formation of fungal beads of varying sizes in most of the substrate matrices (pink beads, golden beads, plastic strips, springs, sandstone, flower beads, kundan beads, glass beads, tyre tube pieces, carpet pieces, net pieces, fiber glass, glass pieces, clay pieces) while fungal clumps could be seen in a few of them viz. silver circles, sponge white, sponge 40D) and no growth at all could be seen in the broth containing iron pieces as substrate matrix under shaking condition (Table 1).

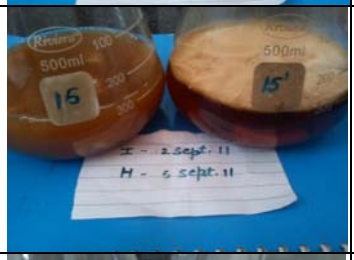
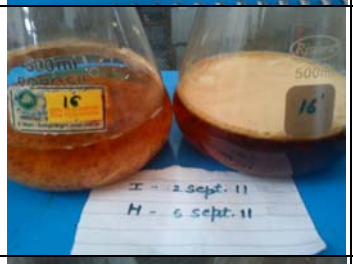
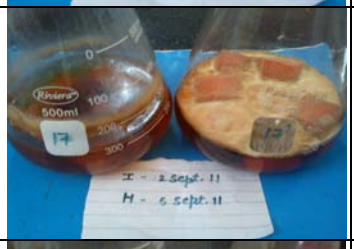
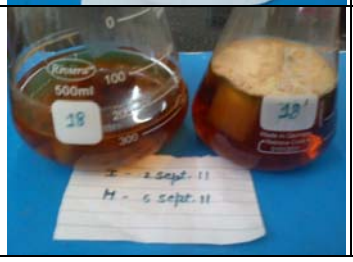
In addition to this, the extent of substrate matrix' surface covered was also different with different matrix used. About 80-90% of the surface was covered in the case of clay pieces, glass pieces, fiber glass, carpet pieces, tyre tube pieces and sandstone. The surface of kundan beads, plastic strips, flower beads, glass beads and net pieces was covered with fungal growth to the range of 51-80% while there was only 30-50% coverage of the surface in the case of sponge white, sponge 40D, springs, golden beads, pink beads and 10-30% on silver circles, glass beads and no growth at all on iron pieces (Table 2).

Shim and Kawamoto (2002) have also reported the immobilization of a white rot fungus namely P. *chrysosporium* in bioreactor to degrade PCP. The immobilized *P.chrysosporium* on polyurethane foam and rotating biological contactor (RBC) with polyethylene discs have been reported to degrade more than 90% 4-

Table 1. Extent of Fungal Immobilization on the Used Solid Matrices and Mycelial Growth Pattern in the Flasks Incubated Under Shaking and Static Condition

S. No	Sample detail	Status of Fungal Immobilization Under Shaking (1′-18′) and Static Incubation (1-18)	Growth Pattern	
			Shaking	Static
1.	 Pink beads (0.5 cm)		Numerous fungal beads of mustard size were formed.	White growth covered the whole surface of broth media on 3rd day. Fruiting bodies protruding out were observed on 6th day of incubation.
2.	 Kundan beads (0.5cm)		White coloured fungal clumps were formed which covered the matrix surface.	White coloured bulky growth covered the whole surface of broth media on 3rd day.Fruiting bodies were observed on 6th day of incubation.
3	 Golden beads (0.5cm)		White coloured fungal beads like mustard seeds were formed in the whole broth and covered the matrix surface approx.30%.	White coloured dense growth covered the whole surface of broth.
4	 Plastic strips (1x1cm)		White coloured fungal beads like mustard seeds were formed. 70% matrix surface was covered till 3rd day.Clumps started appearing by 6th day of incubation.	White bulky growth covered the whole surface of broth on 3rd day. Light yellow coloured fruiting bodies start appearing by 6th day of incubation.
5	 Springs (1cm)		White coloured fungal beads of pin head size were observed. Approx. 30% matrix surface was covered.	White bulky growth covered the whole surface of broth on 3rd day. ruiting bodies start appearing by 6th day of incubation.
6	 Regmal/ Sandstone (1x1cm)		White coloured fungal beads like mustard seeds were formed in whole broth. Approx. 90% of matrix surface was covered.	White bulky growth covered the whole surface of broth on 3rd day. ruiting bodies start appearing by 6th day of incubation.

7	 Flower buttons (1.2 cm)		White fungal beads of oat seed's size were formed. Matrix surface was covered upto 70%.	White bulky growth covered the whole surface of broth on 3rd day. ruiting bodies start appearing by 6th day of incubation.
8	Glass beads (0.5 cm)		White fungal beads of pin head size were formed throughout the broth. Matrix surface was covered by approx.60%.	White bulky growth covered the whole surface of broth on 3rd day. ruiting bodies start appearing by 6th day of incubation.
9	 Tyre tube pieces (1x1cm)		White coloured fungal beads of oat seed size were formed. Approx. 90% Matrix surface was covered.	White bulky growth covered the whole surface of broth on 3rd day. ruiting bodies start appearing by 6th day of incubation.
10	 Carpet pieces		White fungal beads like oat seeds were formed throughout the broth.Approx90% matrix surface was covered.	White bulky growth covered the whole surface of broth.
11	 Net peices		White fungal beads like pin head were formed in large number. The matrix surface was covered to the extent of 70%.	White bulky growth covered the whole surface of broth.
12	 Iron pieces		No growth could be observed.	White coloured growth appeared on the surface of broth.

13	 Fiber glass pieces		White fungal beads of oat seed's size were formed. Approx. 80% of matrix surface was covered.	White thick, bulky growth covered the whole surface of broth.
14	 Glass pieces		White fungal beads of different sizes were formed. Approx. 80% matrix surface was covered.	White bulky growth covered the whole surface of broth.
15	 Clay pieces		White fungal culture suspended in whole broth was observed. Approx. 80% matrix surface was covered.	White bulky growth covered the whole surface of broth.
16	 Silver circles		White coloured fungal clumps were formed. Approx. 10% of matrix surface was covered.	White bulky growth covered the whole surface of broth.
17	 Sponge 40D		White coloured fungal clumps were formed in broth.Approx50% matrix surface was covered.	White bulky growth covered the whole surface of broth .Matrix came to the surface.
18	Sponge white		Fungal clumps were formed. Approx.40% matrix surface was covered.	White bulky growth covered the whole surface of broth.

Name Of The Solid Matrices That Were Covered With Fungal Mycelium To an Extent				
0-10%	**10-30%**	**31-50%**	**51-80%**	**81-90%**
Iron Pieces.	Silver Circles	Sponge White	Kundan Beads	Clay Pieces
		Sponge 40D	Plastic Strips	Glass Pieces
		Springs	Flower Beads	Fiber Glass
		Golden Beads	Glass Beads	Carpet Pieces
	Glass Beads	Pink Beads	Net Pieces	Tyre Tube Pieces
				Regmal/Sandstone.

Table 3. Characterization of Black Liquor

S.No.	Parameter	Value
1.	pH	9.09
2.	Total Solids (%)	5.525%
3.	Residual Active Alkali (gpl)	0.84
4.	Colour (PCU)-original sample	12317

Table 4. Analysis of Colour in Black Liquor Treated With Immobilized Fungus

S.No.	Black Liquor Treated With Fungus Immobilized on the Solid Matrices	Carbon/Nitrogen Source Added			
		Sucrose		Urea	
		0.5%	1.0%	0.5%	1.0%
		Colour in PCU	Colour in PCU	Colour in PCU	Colour in PCU
1.	**Sandstone/Regmal**	8170	6768	14817	11951
2.	**Tyre Tube Pieces**	10854	8658	11707	9268
3.	**Plastic Strips**	7499.9	6829	12439	9878
4.	**Kundan Beads**	8719	8354	8902	7805
5.	**Carpet Pieces**	7134	7025	10548	8171
6.	**Fiber Glass**	5915	5719	8415	8963

chlorophenol in three repeated batches (Gao *et al*, 2008 ; Shim and Kawamato,2002). A white-rot fungus *Trametes versicolor* immobilized on PU foam has been used for biodegradation of pentachlorophenols in batch and continuous bioreactors. The PU foam-immobilized fungal culture yielded more than 99% of pentachlorophenols reduction with a residence time of 12 hours for the inlet pentachlorophenols concentrations from 20 to 25 mg/l (Pallerla and Chambers, 1998).

Decolourization Studies

***Characterization of Black liquor*:** Black liquor obtained from banana pulp after squeezing the cooked fiber was used for fungal decolourization studies. Its analysis for various parameters of interest including pH, total solids, colour and Residual Active Alkali is given in Table 3.

Solid Matrices with Immobilized fungus Used for Decolourization: Based on the extent of immobilization of fungus on different solid matrices, the matrices covered with more than 80% fungus were chosen to be used further for decolourization studies. Thus, the fungal cultures immobilized on the matrices namely sandstone/regmal, tyre tube pieces, plastic strips, carpet pieces, kundan beads and fiber glass were used for their decolourization potential.

In nature, most of the fungi grow in solid-state conditions, in the near absence of free water (Raghukumar *et al*, 2008). Such cultures when immobilized on a variety of solid supports have been shown to decolorize various effluents (Ohmomo *et al*, 1987; Shin *et al*, 2002; Šušla *et al*, 2007). Zhang *et al* (1999) immobilized a white-rot fungus strain F29 to decolorize Orange II, a toxic dye. Wu *et al* (2005)

have used biofilm of white-rot fungi grown on a porous plastic rings for decolorization of paper mill effluent.

Colour values evaluated in the black liquor samples treated with fungal cultures immobilized on different solid matrices are given in table-4. There was found to be more reduction in colour in the case of black liquors supplemented with 1% sucrose as compared to 0.5% sucrose. Similarly, lesser colour values were observed in the liquors added with 1% urea than those with 0.5% urea. Gumarces *et al* (2005) have reported use of *Phanerochaete chrysosporium* immobilized on PU foam modified by a rotating biological contractor reactor for the decolouration of sugar refinery wastewater. They have shown decolouration efficiency of 62% during 40 days of a repeated batch test. The PU foam-immobilized cells not only removed the colour of the effluent by 55%, but also reduced total phenols and chemical oxygen demand by 63 and 48%, respectively. Thus the present study showed the possibility of using immobilized fungal cultures for treating black liquor effluents of the paper industry.

Conclusion

In the present study, a range of solid matrices/support materials which are simple, easily available as waste or at the cheap prices were used to explore the possibilities of immobilizing white rot fungal culture with an aim to use them further for decolourizing black liquors generated during banana fiber pulping. The results have been encouraging and have shown potential involved in using immobilized fungal cultures for decolourization. However more detailed and systematic studies are required to be conducted for actually realizing the potential involved.

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