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Biodecolourisation of reactive red an industrial dye by *Phlebia* spp.

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Abstract

Four white rot fungi namely *Phanerochaete chrysosporium*, *Phlebia floridensis*, *P. radiata* and *P. brevispora* were selected for their ligninolytic enzymes *viz.*, lignin peroxidase, manganese peroxidase and laccase. Cell free enzyme extracts (as such and concentrated) obtained from these fungi were tested for their ability to decolourise reactive red 28 (Congo red), an industrial dye. The use of cell free enzyme extracts helped to overcome the problem of adsorption of dye to mycelia. Laccase production was best expressed in *P. brevispora*. Increase in decolourisation percentage by concentrated culture extract was comparable to increase in enzyme activity. *P. floridensis* proved to be a better dye decolouriser in comparison to *Pha. chrysosporium*, thus showing its potential for biocleaning of industrial wastes and wastewaters.

Key words

Congo red, Decolourisation, Enzyme extract, Fungi, Ligninolytic enzymes

Introduction

Lignin is the most abundant renewable natural material next to cellulose. It is an amorphous and optically inactive plant polymer. Despite its structural value to plants, it provides protection from oxidative stress (Wong, 2009). White rot fungi are the potential degraders of lignin, which produce extracellular lignin modifying enzymes, such as laccases , lignin peroxidases, manganese peroxidases and H_2O_2 generating oxidases (Arora and Sharma, 2010). Ligninolytic potential of such enzymes finds numerous applications in different areas of biotechnology. Delignification in pulp and paper industries is a foremost step in pulping (Baucher *et al*., 2003; Liew *et al*., 2011). White rot fungi are also capable of degrading a wide range of hazardous xenobiotic including various synthetic dyes (Coulibally *et al.,* 2003; Gomi *et al*., 2011). These dyes are widely used in industries viz. textile, leather, paper, food and cosmetics etc. Such extensive use of colour often leads to problem of colored waste waters, which need pre-treatment for colour removal prior to its disposal (Malaviya *et al*., 2012). Treatment of coloured effluents has not received much attention earlier as it was thought to be of aesthetic concern only. Release of azo dyes into the environment from the effluents of dye based industries has become major problem in waste water treatment (McMullen *et al*., 2001; Coulibaly *et al*., 2003). These dyes form the largest and important group followed by triphenylmethane dyes (Olikka *et al*., 1993; Gill *et al*., 2002; Liu *et al*., 2004). Acid azo dyes are characterized by the presence of a chromophoric azo group in addition to sulfonic acid group (Mautaoukkil *et al*., 2004).

Under aerobic conditions these dyes are non-degradable by bacteria (Paszczynski *et al*., 1991) though some strains of *Pseudomonas* have shown to degrade the sulphonated azo dyes aerobically (Zimmerman *et al*., 1982; Baeta *et al*., 2012). Anaerobic transformation of azo dyes results in the formation and accumulation of colorless aromatic amines which can be highly toxic and carcinogenic (Kulla *et al*., 1983, Coulibaly *et al*., 2003). The azo group of dyes includes Orange II, Azure B, Tropaeolin-O, Congo red, Amaranth, Orange G and other industrial dyes (Cripps *et al*., 1990; Arora and Chander, 2004). These ligninolytic fungi and their peroxidases have been reported to be biotransforming untreated pharmaceutical active compounds found in industrial effluents (Morales *et al*., 2012). These untreated pharma compounds such as estrogens, diclofenac, sulphamethoxazole etc. have ecotoxicological effects (Eibes *et al*., 2011; Morales *et al*., 2012).

In the present work, a synthetic azo dye, reactive red 28 (Congo red) was selected for biocleaning study. Though its use is

banned in few countries, it is widely used in dyeing wool and silk in Asian countries. It has been used for decades to identify amyloid deposits in post mortem tissue (Styren *et al*., 2000). It is highly toxic and may produce carbon monoxide on oxidation. It is a red colour dye having intense benzidine based chromophore, which is a known human carcinogen. Due to its mutagenic, carcinogenic and teratogenic nature, it causes harmful effects on various body systems viz. urino-genital system, central nervous system, eyes and ear (Patnaik *et al*., 1999; Purkait *et al*., 2004).

Biological decolourisation of Congo red is necessary as it is quite resistant to degradation under natural conditions. *Phlebia* spp. has been used to decolourise Congo red. Cell free enzyme extracts obtained from different *Phlebia* spp. helped to overcome the problem of fungal adsorption of dye. Further, effort were made to enhance the dye decolourisation using concentrated (lyophilized) cell free enzyme extracts. This paper for the first time reports the effectiveness and biocleaning application of lyophilized cell free enzyme extracts obtained from different white rot fungi.

Materials and Methods

Media composition (per liter) : Mineral salts broth (MSB) was prepared using 10g glucose; 2g KH_2PO_4, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2.2H_2O$, Thiamine HCl 1mg, Ammonium tartarate 0.2g, 10ml Trace element solution, 1l Distilled water and pH adjusted to 4.5.

Trace element solution : 1.5g Nitrilotriacetic acid, 0.48g $MnSO_4.H_2O$, 1.0g NaCl, 10mg $CoSO_4.7H_2O$, 10mg $FeSO_4.7H_2O$, 10mg $ZnSO_4.7H_2O$, 8mg $CuSO_4.5H_2O$, 8mg H_3BO_3, 8mg $Na_2MoO_4.2H_2O$, 1l Distilled water. Yeast extract glucose agar medium comprised of 10g glucose, 5g Peptone, 3g Yeast extract, 20g Agar, 1 l Distilled water and pH adjusted to 5.0.

Production of ligninolytic enzymes: For production of ligninolytic enzymes, triplicate set of 500 ml flasks containing 150 ml of MSB was sterilized and inoculated with 12 discs (8 mm diameter) per flask, taken from the periphery of 7-8 days grown fungal colony on yeast extract glucose agar medium plates. The flasks were incubated at 25°C for *Phlebia* spp. and 37°C for *Pha. chrysosporium* as shake cultures at 200 rpm on BOD incubator shaker. After 8 days, the contents of each flask were filtered through preweighed Whatmann filter paper No. 1 to obtain cell free enzyme extract.

Laccase assay: Laccase estimation was done according to Sandhu and Arora (1985). Five ml reaction mixture containing 3.8 ml of sodium acetate buffer (10 mM, pH 5), 1 ml guaiacol (100 mM), 0.2 ml of cell free enzyme extract was incubated at 25°C for 2 hr and the absorbance was read at 450 nm. In control, enzyme was replaced by equal volume of acetate buffer. The enzyme activity is expressed as colorimetric units per ml (CU ml^{-1}).

Lignin peroxidase assay : Lignin peroxidase activity was assayed according to Tien and Kirk (1988).The reaction mixture in a total volume of 2.5 ml, contained 1 ml sodium tartarate buffer (pH 3, 125 mM), 0.5 ml veratryl alcohol (10 mM), 0.5 ml of H_2O_2 (2 mM) and 0.5 ml of cell free enzyme extract. The reaction was initiated by adding H_2O_2 . The increase in absorbance was monitored at 310 nm for 10 min using UV-Visible spectrophotometer. The lignin peroxidase activity was calculated from the standard curve of veratraldehyde and expressed as moles of veratraldehyde formed per min per ml of the cell free enzyme extract (IU ml^{-1}).

Manganese peroxidase assay: The manganese peroxidase activity was assayed according to Orth *et al*. (1991). The reaction mixture in a total volume of 10 ml, contained 2 ml sodium succinate buffer (pH 4.5, 500mM), 2 ml of sodium lactate (pH 4.5, 500 mM), 1 ml of gelatin (1000 mg/l), 1.4 ml of phenol red (2.5 mM), 0.8 ml of $MnSO_4$ (2.5 mM), 0.8 ml of H_2O_2 (1.25 mM) and 1 ml of cell free enzyme extract. The reaction was started by adding H_2O_2. One ml of reaction mixture was taken every min upto 10 min and 40 µl of 5N NaOH was added to it to stop the reaction. Manganese peroxidase activity was expressed as an absorbance increase of 0.1 unit ml U ml^{-1} of enzyme extract at 610 nm.

Dye decolourisation study in cell free enzyme extract: Twenty ml of cell free enzyme extract was taken from different fungal cultures and 200 µl of dye from stock solution (30 mg l^{-1}) was added to obtain a final concentration of 30 µg ml^{-1}. It was incubated at shaking conditions at 25°C for *Phlebia* spp. and 37°C for *Phanerochaete chrysosporium*. Absorbance of dye at 530 nm was recorded at hourly intervals from 0, 1, 2, 3, 5 to 24 hrs. The decolourisation percent of the dye was calculated by dividing the difference of absorbance of dye at zero hour and absorbance of the dye recorded at the given time by absorbance of dye at zero hour.

Ligninolytic enzymes in lyophilized CFEE and their dye decolourisation potential: One hundred twenty ml of cell free enzyme extract was lyophilized to obtain 200 mg of powder. It was dissolved in distilled water so as to get 6 times concentrated extract which was then used for assaying enzyme activities. Dye Congo red from stock solution was added to 15 ml of lyophilized cell free enzyme extract to get a final concentration of 50 µg ml^{-1}. The reaction mixture was incubated and percentage of dye decolourisation was calculated as above.

Data analysis: The data in subsequent sections represent standard deviation values of two experimental repetitions wherein triplicate samples were processed for every study.

Results and Discussion

The release of dye-based effluents into water bodies is only a small proportion of water pollution, but as a result of the chromophoric or colouring properties, these dyes are visible even in their trace amounts. The conventional physico-chemical

Table 1 : Comparative ligninolytic enzymes activity of cell free enzyme extract of some white–rot fungi

Organisms	Enzyme Activity					
	Manganese peroxidase (Unit ml^{-1})		Lignin peroxidase (IU ml^{-1})		Laccase (CU ml^{-1})	
	A	B	A	B	A	B
Pha. chrysosporium	0.05±0.007	0.07±0.001	0.06±0.005	0.08±0.005	x	x
P. brevispora	0.03±0.001	0.05±0.002	0.05±0.014	0.07±0.003	0.065±0.002	0.115±0.001
P. radiata	0.04±0.006	0.06±0.015	0.05±0.006	0.06±0.013	0.035±0.004	0.075±0.004
P. floridensis	0.06±0.014	0.08±0.001	0.06±0.013	0.07±0.002	x	x

Values of mean of three replicates ± SD (n=3); A- Enzyme activity in unlyophilized cell free enzyme extract; B - Enzyme activity in lyophilized cell free enzyme extract; x - No activity detected

Table 2 : Comparative decolourisation of Congo red by cell free enzyme extract of some white–rot fungi

Hours of reaction	Percentage decolourisation							
	Pha. chrysosporium		*P. floridensis*		*P. radiata*		*P. brevispora*	
	A	B	A	B	A	B	A	B
0	2±0.449	5±0.064	3±0.209	7±0.089	3±0.314	5±0.082	3±0.182	4±0.216
1	6±0.171	31±2.082	16±0.946	83±3.604	7±0.142	11±0.256	11±0.198	6±0.683
2	17±0.842	69±1.856	67±2.024	86±3.962	10±0.684	62±1.641	17±0.942	52±2.946
3	35±1.642	77±3.584	79±2.942	90±3.462	20±1.024	78±2.861	24±1.004	63±1.846
5	74±2.886	90±3.268	87±3.483	94±2.476	41±2.046	87±3.875	34±1.269	71±2.165
24	92±3.972	98±2.441	93±2.546	96±4.102	83±3.182	94±2.925	79±2.021	91±2.9833

Values are mean± SD (n=3); A- Percentage decolourisation by unlyophilized cell free enzyme extract; **B-** Percentage decolourisation by lyophilized cell free enzyme extract

methods are of little utility because these are either costly or are only partially competent in treating these wastes (Namasivayan and Kavitha, 2002). The treatment system based on fungi, especially the white-rot, has not been applied extensively due to many factors such as high cost, longer fungal growth periods (Wesenberg *et al.,* 2003) and an early inactivation of enzymes (Cing and Yesilada, 2004). The use of fungal and algal biosorption systems itself, faces disposal problem (Coulibaly *et al*., 2003). Hence, severe problem in terms of safe disposal of biomagnified biomass is created. In present study, use of cell free enzyme extracts obtained from the tested fungi have solved these problems. Different white rot fungi were grown for 8 days to study the production of ligninolytic enzymes. All the three ligninolytic enzymes were produced by the tested fungi except *Pha. chrysosporium* and *P. floridensis* which did not produce laccase under the given nutritional conditions, while *P. brevispora* was the best laccase producer. *P. floridensis* was the best producer of manganese peroxidase, followed by *Pha. chrysosporium*, *P. radiata* and *P. brevispora*. Lignin peroxidase was maximally produced by *P. floridensis* and *Pha. chrysosporium* (Table 1).

An increase in the activity of different enzymes was observed, though no laccase activity could be detected even in the concentrated cell free enzyme extract of *P. floridensis* and *Pha. chrysosporium*. Two fold increase was observed in *P. radiata,* followed by *P. brevispora.* An average increase of 1.5 and 1.2 times was observed in manganese peroxidase and lignin peroxidase activity respectively in concentrated cell free enzyme extract obtained from different fungi (Table 1,2).

Cell free enzyme extract obtained from different fungi caused extensive decolourisation of Congo red in 24 hrs of reaction. *Pha. chrysosporium* decolourised 92% (Fig. 1) of the dye, closely followed by *P. floridensis* (93%) in 24 hrs (Fig. 2). It was followed by *P. radiata* and *P. brevispora* which caused 83% and 79% decolourisation of Congo red (Fig. 3, 4). On comparing the initial rate of decolourisation, *P. floridensis* was found to be a faster decolouriser in comparison to *Pha. chrysosporium* and caused 90% decolourisation within three hours of the reaction (Table 2). The dye removal rate of *P. radiata* was however slow, followed closely by *P. brevispora* (Fig. 3,4).

The concentrated cell free enzyme extract significantly decolorized Congo red. *Pha. chrysosporium* caused maximum decolourisation followed by *P. floridensis*, *P. radiata* and *P. brevispora* in 24 hrs (Table 2). However, significant decolourisation was recorded in first 5 hrs with no further decolourisation during extended period of reaction (Fig. 1-4).

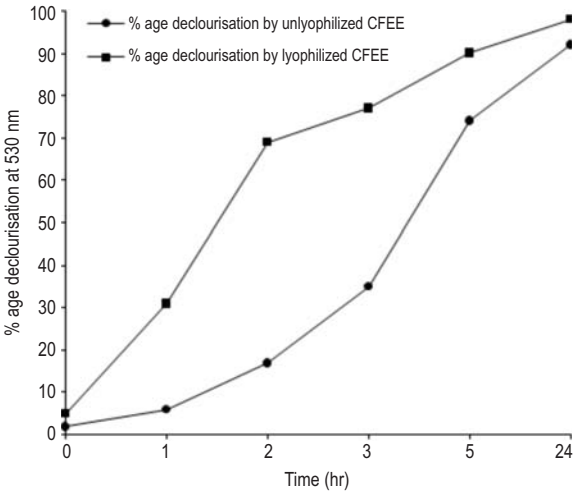

Fig. 1 : Decolourisation of Congo red by *Phanerochaete chrysosporium*

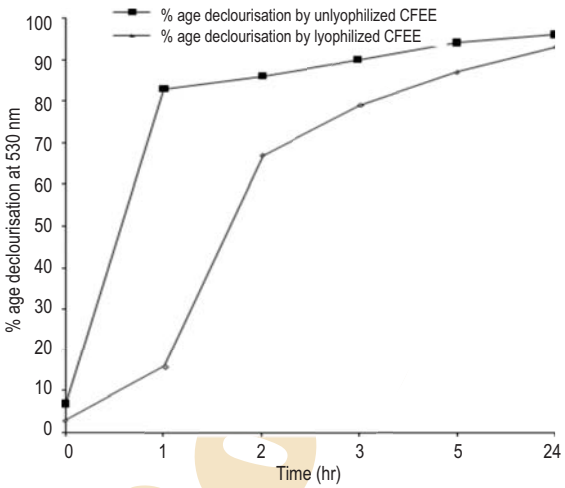

Fig. 2 : Decolourisation of Congo red by *Phlebia floridensis*

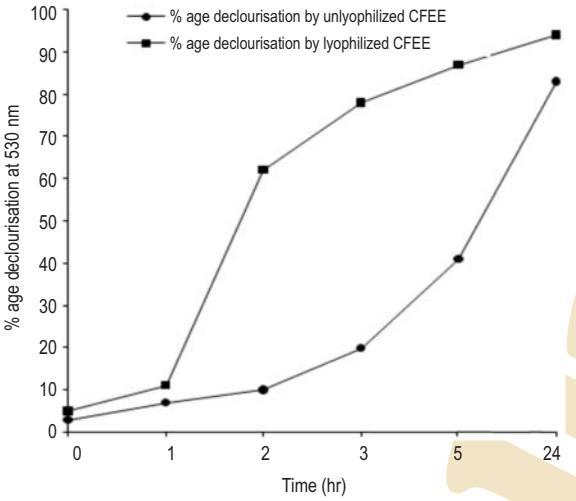

Fig. 3 : Decolourisation of Congo red by *Phlebia radiata*

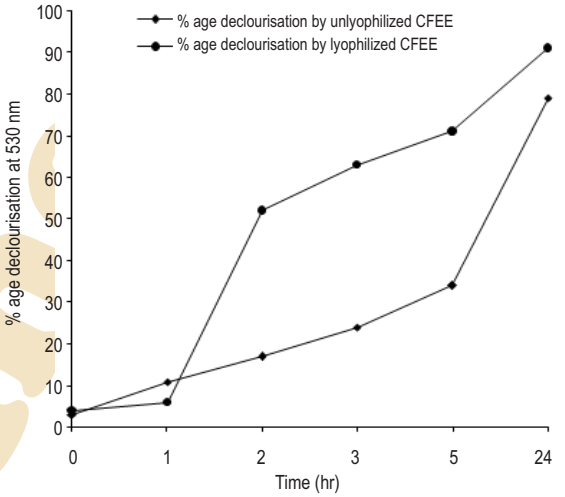

Fig. 4 : Decolourisation of Congo red by *Phlebia brevispora*

The concentrated lyophilized cell free enzyme extract increased the initial rate of dye decolourisation. The initial rate of *P. floridensis* was 90% in 3 hrs of reaction as comparised to *Pha. chrysosporium* (77%) (Fig. 1 and 3).

Most of the white rot fungi produce ligninolytic enzymes in relatively small amounts, thus limiting their commercial use. Moreover, whatever information has been generated is centered on *Pha. chrysosporium* (Conneely *et al*., 1999; Pointing, 2001), while *Phlebia* spp. are more recent to attract scientific attention (Kirby *et al*., 2000; Chander and Arora, 2004; Sharma and Arora 2011; Shrari *et al.,* 2011). Most of the previous studies confined were to agar plate and broth culture method for decolourisation of azo dyes (Cripps *et al*., 1990; Gill *et al*., 2002) but the present study make use of cell free enzyme extract of different fungi so as to avoid the adsorption of dye to the mycelium.

P. floridensis was found to be the best producer of manganese peroxidase in comparison to *Pha. chrysosporium* (Table 1) and caused almost same percentage of decolourisation (93%) as observed for *Pha. chrysosporium* (95%) with in 24 hrs of reaction (Fig. 1) though *P. floridensis* did not produce any detectable laccase activity. Absence of laccase activity is in agreement with the study conducted by Dhaliwal *et al*. (1992). It was found to drastically decrease in shaking cultures, as shaking had a mixed effect on laccase production. Laccase activity was

not detected in *Pha. chrysosporium* even in wider experimental conditions (Thurston, 1994; Baucher *et al*., 2003), though it has been reported in defined culture medium containing cellulose (Srinivasan *et al*., 1995). *P. radiata* and *P. brevispora* produced all the three enzyme (Table 1) but could remove only 83% and 79% of dye colour in 24 hrs (Table 2).

The use of concentrated lyophilized cell free enzyme extract led to an increase in enzyme activity and in dye decolourisation potential. The concentrated cell free enzyme extract could decolourise relatively higher concentration of (50 µg ml^{-1}) dye in comparison to unlyophilized extracts. The maximum decolourisation caused by *P. floridensis* was almost similar to *Pha. chrysosporium* in 24 hrs though the initial decolourisation rate was faster in *P. floridensis* (Fig. 1, 2). Laccase was not detected even in culture broth of *P. floridensis* (Table 1) when concentrated six times.

A significant increase in colour removal was also observed in *P. radiata* and in *P. brevispora* at 5 and 24 hrs of incubation (Fig. 3 and 4).

The results obtained show the differential susceptibility of the dye to fungal decolourisation. In contrast to previous study, (Caplash and Sharma, 1992) where the dyes were found to be inhibiting the decolourising ability of *P. chrysosporium*, appreciable amount of dye was decolourised by its cell free enzyme extracts in the present study (Table 2). Congo red was highly decolourised by all the fungi tested in the present study. Further, decolourisation was carried out by cell free enzyme extract, obtained from fungi wherein, much of the colour removal invariably occurred during first 5 hr which is in agreement with the previous studies (Arora and Chander, 2004). The decolourisation percentage rose to their maxima on 24 hr of incubation, with cell free enzyme extract obtained from all of the tested fungi. These results are in contrast to Yesilada and Ozcan (1998) wherein, the residual groups released by partial decolourisation of dye tested inhibited the activity of cell free enzyme extract. In agreement with earlier studies, cell free enzyme extract obtained from different fungi could cause an effective decolourisation of all the dyes tested without help of any inducer and mediator (Unyayar *et al.,* 2005). Keeping in view, the maximum production of manganese peroxidase, lignin peroxidase and higher percent decolourisation in *P. floridensis* alongwith *Pha. chrysosporium*, despite the absence of laccase activity, these two fungi may be considered as promising agents for the treatment of waste water emanating from textile and other dye based industries.

The above discussion reveals that concentrating the culture broth as a source of enzymes may prove to be more useful method for industrial applications. This will help in reducing the volume to be treated to avoid the complexities of different conditions used in waste water treatment. On correlating the dye decolourisation ability of different fungi with that of their potential to produce ligninolytic enzyme reveals that *Phlebia spp*. may prove better dye decolourising agents in comparison to much studied *Pha. chrysosporium*. The use of cell free enzyme extract and concentrating it by lyophilization improved the decolourisation efficiency further. *P. floridensis* apparently seems to be the better strain as compared to *Pha. chrysosporium* and can be exploited for the biocleaning of industrial effluents.

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