Biodegradation of a Dye by Different White–rot Fungi on a Novel Agro Residue Based Medium

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The present study highlights a simple and novel method for the production of ligninolytic enzymes on wheat straw (a cheap agricultural waste) extract and employing cell free enzyme extracts of seven white-rot fungal cultures to decolourise Poly R– 478 (a standard dye). The ligninolytic enzyme activities were correlated with dye degradation ability. The study has also been consolidated using immobilized fungal bioreactor at laboratory scale. The affectivity of degradation was assessed by analyzing the dye decolourisation with US-visible spectroscopy, studying decrease in chemical oxygen demand and toxicity of treated samples. The production of three ligninolytic enzymes was independent of incubation conditions with exception of laccase which was in general, better produced under stationary conditions. The *Irpex flavus*, *Dichomitus squalens* and *Phlebia brevispora* were the better dye degraders at bioreactor level. The ligninolytic enzyme maxima coincided with the maximum dye degradation rate. The chemical oxygen demand of the dye sample was lowered significantly by the *D. squalens*, *P. brevispora* and *P. floridensis*.

Keywords: decolourisation; fungal bioreactor; ligninolytic enzymes; wastewater; white–rot fungi

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INTRODUCTION

Lignocellulosics are the major form of carbon present on earth. The fungi play an important role in their degradation. Lignin, a phenyl-propanoid polymer comprising 25 to 30% of plant biomass, is second only to cellulose as carbon repository. It is quiet resistant to microbial degradation under natural conditions still it acts as a potential substrate for different transformations (Arora and Sharma 2009). White–rot fungi (WRF) have got the potential for its complete mineralization to CO_2 (Coulibaly et al. 2003). The ligninolytic ability of such fungi has been used for delignifying wood chips, wheat straw and bamboo sticks (Arora et al. 2002; Reid 1989). The key ligninolytic enzymes (LE) are extracellular and thus obviate the need for intracellular uptake of the lignin and/or related xenobiotic compounds (Kandelbauer et al. 2004; Liu et al. 2004; Lopez et al. 2004; Tien and Kirk 1984). Ligninolytic fungal systems find applications in diverse fields such as, improving the digestibility and nutritive value of animal feeds, degradation of toxic pollutants, xenobiotics and industrial effluents thereby significantly reducing their toxicity, mutagenicity and BOD/COD loads (Chander et al. 2014; Fu and Viraraghvan 2001; Lucas et al. 2008; Papinutti and Forchiassin 2004).

The ligninolytic white–rot fungi have been considered as potential agents for treatment of variety of dyes like acidic, anthraquinonic, azo, nitro, triphenylmethane, xanthene etc (Gill et al. 2002; Nerud et al. 2004; Yang and Yu 1996). These synthetic/industrial dyes are being released as effluents of textile and other industries. The coloured contents of these wastes reduce the penetration of light in water bodies thus affecting the metabolic activities of photosynthetic aquatic flora. The production of extracellular ligninolytic enzymes by white– rot fungi can prove to be of potential significance for biocleaning of industrial dyes and related wastes (Santos et al. 2004; Unyayar et al. 2006; Chander et al. 2014). Most of the biodecolourisation studies have been carried out using enzyme extracts obtained by growing the fungi on synthetic media (Arora and Chander 2004; Palmieri et al. 2005; Yesilada and Ozcan 1998; Yesilada et al. 2003). However, a little work has been done using cheap and cost effective natural agroresidues for enzyme production (Wesenberg et al. 2003; Osma et al. 2007). Some of the studies have been extended to lab level reactor (Borchert and Libra 2001; Nerud et al. 2004; Yesilada et al. 2003), but have a limited scope due to their cost considerations. The use of dead or living mycelia/cells for dye absorption/adsorption in turn may create more severe pollution problems for their safe disposal (Coulibaly et al. 2003). The present study highlights the use of indigenous resources in the development of relatively less studied WRF based sustainable eco-friendly biocleaning technology. Further it may be exploited for future scale up also. The cell free enzyme extracts (CFEE) obtained from agroresidue based medium have been used in the present study. Potentially more efficient white–rot fungal cultures have also been tested in a semi–continuous immobilized reactor for ligninolytic enzyme production and simultaneous decolourisation of Poly R–478. An effort has also been made to correlate the ligninolytic enzyme production with the degradation of dye.

EXPERIMENTAL

Materials

Dye

Poly R–478, a reference dye belonging to polyanthraquinoic group was used in the present work. It was procured from Sigma, USA while the remaining chemicals were from HiMedia, E–Merck and Qualigens India.

Microorganisms

Daedalea flavida MTCC 145, *Irpex flavus* MTCC 168, *Polyporus sanguineus* MTCC 137 and *Salmonella typhimurium* (TA98) MTCC 1251 were obtained from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. *Phanerochaete chrysosporium* BKM–F–1767 was received as a gift from Prof. T.W. Jeffries, Institute of Microbial and Biochemical Technology, United States Department of Agriculture, Madison, USA. *Dichomitus squalens* FP–105351–sp, *Phlebia brevispora* HHB–7030–sp and *P. floridensis* HHB–9905–sp were received as gift from Dr. Rita Rentmeester, Forest Products Laboratory, Madison, WI, USA.

Methods

Preadaptation of fungal cultures

The cultures were preadapted on yeast glucose agar (YGA) medium supplemented with (10mg l^{-1}) Poly R–478. The cultures were maintained at –80^{o}C in 10% glycerol.

Preparation of wheat straw extract

The wheat straw as agricultural waste was obtained from local market. It is very economical and is available throughout the year. The wheat straw extract (WSE) was prepared by steeping the washed, dried and 2–3mm grounded wheat straw in distilled water (pH 4.5) overnight for 10h. Activated charcoal (5g l^{-1}) was added to the dark coloured yellowish brown filtrate and the extract was heated under pressure in an autoclave at 10psi for 10min. The mixture was then filtered through Whatmann filter paper no. 1 to obtain clear WSE.

Dye decolourisation by CFEE

Two hundred ml of WSE taken in one litre flasks were sterilized by autoclaving at 15psi for 15 min. The autoclaved extracts were supplemented with 10ml filter sterilized trace elements (One litre stock containing Nitrilotriacetic acid 1.5g, $MnSO_4.H_2O$ 0.48g, NaCl 1.0g, $CoSO_4.7H_2O$ 10mg, $FeSO_4.7H_2O$ 10mg, $ZnSO_4.7H_2O$ 10mg, $CuSO_4.5H_2O$ 8mg, H_3BO_3 8mg, $Na_2MoO_4.2H_2O$ 8mg) and thiamine hydrochloride (1mg). Each flask was inoculated with twenty fungal discs (8mm diameter). Thirty ml of CFEE was removed at different periods of incubation i.e. 4, 6, 8, 10 and 12 days under stationary and shaking conditions to estimate ligninolytic enzymes (LE) and their dye decolourisation ability. To an aliquot of 20ml CFEE, filter sterilized dye stock was added to get its final concentration of 50 mg l^{-1}. The samples were analysed for decrease in their absorption peaks due to decolourisation using Labtronics UV-Visible spectrophotometer. The decrease in optical density of the treated samples was measured at absorption maxima (λ_{max}) of dye. The percentage of dye decolourisation was calculated as under

$$\text{Percent dye Decolourisation} = \frac{O.D._0 - O.D._t}{O.D._0} \times 100$$

$O.D._o$ = Absorbance of the dye recorded at 0 hour.
$O.D._t$ = Absorbance of the dye recorded at the given time interval.

Dye Decolourisation by Various White–Rot Fungi in U–Tube Reactor

The reactor having working volume of 100 ml was used in present. The polyurethane foam (PUF) was selected as support medium for packing the reactor for dye decolourisation studies under immobilized conditions (Figure 1). The PUF sheet was cut in equal cubes of 0.5x0.5x0.5mm size and used as immobilization support. The reactor was fed with 80 ml of 4% WSE and inoculated with 10ml of respective fungal biomass grown for 7–8 days on shaker in WSE at their optimum growth temperatures. The reactor was kept at stationary conditions for 8 days. For the next two days it was continuously fed with 4% WSE using BioAge Peristaltic Pump. From 10th day onward, 4% WSE

supplemented with 50 mg l^{-1} of Poly R–478 were fed to reactor. The flow rate was adjusted to 3 ml h^{-1}. The hydraulic retention time for the reactor was 33.3 h. During 12th to 20th day of reactor operation the samples were taken at an interval of 48 h for experimental studies.

Fig 1. U–tube reactor showing immobilized *Phanerochaete chrysosporium* on PUF

Determination of chemical oxygen demand (COD) and toxicity studies

The COD was measured by open reflux calorimetric method as per APHA standard (APHA, 1998). The toxicity assay was carried out for untreated dye (Poly R–478 as standard; at a concentration 50 mg l^{-1}) and their treated samples using bacterial reverse mutation assay (Ames test) as modified by Cappuccino & Sherman (2004) using a fresh culture of *Salmonella typhimurium* TA98 (MTCC 1251), a tryptophan auxotroph.

UV-visible spectroscopy

The decolourised samples were scanned for decrease in their absorption peaks in range of 280-750nm wavelength using a double beam Labtronics UV-Visible LT-2900 spectrophotometer. The decrease in the peaks qualitatively indicates the dye degradation.

Enzyme assays

Lignin peroxidase (EC 1.11.1.14; Diarylpropane: Oxygen, Hydrogen Peroxidase Oxidoreductase) assay was done according to Tien and Kirk (1984). The enzyme activity has been expressed as μmoles of veratraldehyde formed $min^{-1}ml^{-1}$ of the enzyme extract.

Manganese peroxidase (E.C. 1.11.1.13; Mn (II): H_2O_2 Oxidoreductase) assay is based on the oxidation of phenol red (Orth et al. 1991). One unit of enzyme activity is equivalent to an absorbance increase of 0.1 unit ml^{-1} of enzyme extract.

Laccase (EC 1.10.3.2; phenol oxidase, benzenediol: oxygen oxidoreductase) activity was measured according to Arora and Sandhu (1985). Laccase activity has been expressed as colorimetric unit ml^{-1} (CU ml^{-1}).

RESULTS & DISCUSSION

Ligninolytic Enzyme Production and Poly R–478 Decolourisation at Flask Level

The cell free enzyme extracts obtained from various fungi grown in WSE under stationary as well as shaking conditions, efficiently decolourised Poly R–478 (Table 1). In general, maximum decolourisation in first 5h of reaction was achieved using CFEE obtained from 8 day grown cultures. In case of *D. squalens*, *Pha. chrysosporium*, *P. brevispora* and *P. floridensis* an appreciable decolourisation was also observed using extracts obtained on day 12 as well.

Table 1. Maximal Production of Different Enzymes and Maxima for Decolourisation of Poly R–478

Fungus	LiP		MnP		Laccase		decolourisation %	
	Static	Shaker	Static	Shaker	Static	Shaker	Static	Shaker
D. flavida	2.10 (12)	1.90 (10)	–	–	1.10 (10)	0.04 (12)	22 (8)	30 (8)
D. squalens	–	–	0.0025 (12)	0.003 (12)	0.06 (8)	0.10 (4)	57 (8)	57(8)
I. flavus	4.00 (10)	1.00 (10)	0.0020 (10)	0.001 (10)	–	–	38 (8)	37 (8)
Pha.chrysosporium	2.00 (4,10)	2.60 (12)	0.0100 (10)	0.004 (10)	–	–	29 (8)	57 (12)
P. brevispora	0.76 (10)	0.50 (10)	0.0030 (10)	0.002 (6)	0.22 (10)	0.63 (10)	57 (8)	57 (8)
P. floridensis	1.70 (10)	2.00 (10,12)	0.0230 (12)	0.016 (10)	0.04 (4)	0.08 (10)	60 (8)	60 (8)
P. sanguineus	0.62 (12)	1.20 (10)	0.0010 (12)	0.001 (10)	0.05 (10)	0.03 (10)	28 (8)	41 (8)

(–) No activity detected; Figures in the parenthesis indicate days of incubation

Daedalea flavida

Daedalea flavida produced LiP and laccase to a maximum level during 10–12 days stationary cultures, respectively (Figures 4, 5, 8, 9). While another peak for LiP was recorded on day 4 at stationary conditions (Figure 4). However, the cell free enzyme extract obtained from 8 day grown static as well as shake cultures of *D. flavida* caused maximum dye decolourisation of 22 and 30%, respectively (Figures 2, 3).

Dichomitus squalens

A reasonable laccase production was observed under static as well as shake conditions. The peak values were obtained on day 8 and 4, respectively (Figures 8, 9). MnP was produced to a moderate level only, which gave its peak on 12th day under static as well as shake conditions (Figures 6, 7). No LiP activity was detected in this fungus. Poly R–478 was decolourised appreciably (34 to 57%) by the CFEE obtained from *D. squalens* grown for different incubation periods. However, maximum dye decolourisation was caused by extracts obtained from 8 day grown cultures (Figure 2).

Irpex flavus

Irpex flavus was capable of producing LiP and MnP, with maximum activity observed on day 10 under both static as well as shake flask conditions (Figures 4-7). No laccase activity was detected throughout the incubation period. Maximum dye decolourisation (38%) was caused by CFEE obtained from 8 day grown culture of *I. flavus* (Figures 2, 3).

Phanerochaete chrysosporium

The static culture of *Pha. chrysosporium* gave maximum LiP and MnP activity on day 4 and 10, respectively (Figures 4-7). However, a second peak for LiP was also observed on 10th day (Figure 4). The CFEE harvested from shake flask cultures revealed the maxima for LiP and MnP on day 12 and 10, respectively (Figures 5, 7). No laccase activity was observed in both static as well as shake cultures. The CFEE obtained from its static and shake cultures on day 8 caused 29 and 54% colour loss, though the maximum colour loss (57%) was caused by 12 day extracts obtained from shake cultures (Figure 2,3).

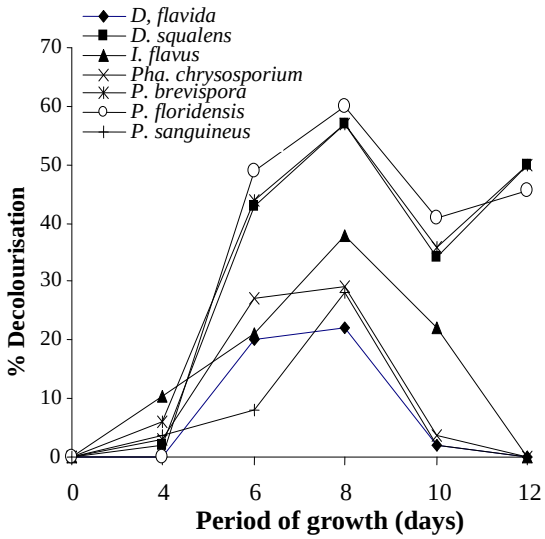

Fig. 2. Decolourisation of Poly R-478 by WRF under static conditions

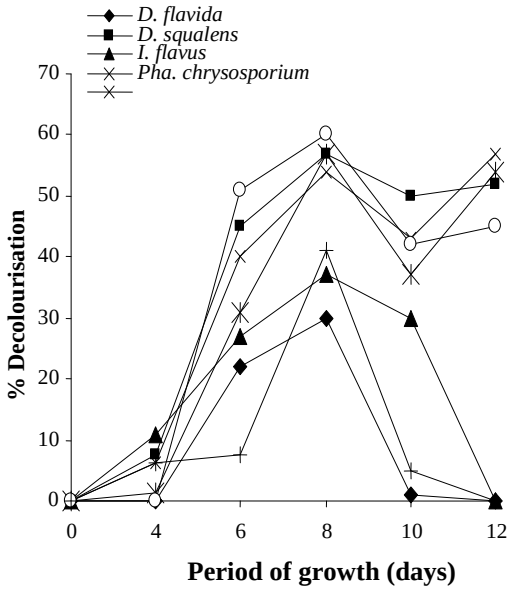

Fig. 3. Decolourisation of Poly R 478 by WRF under shaking conditions

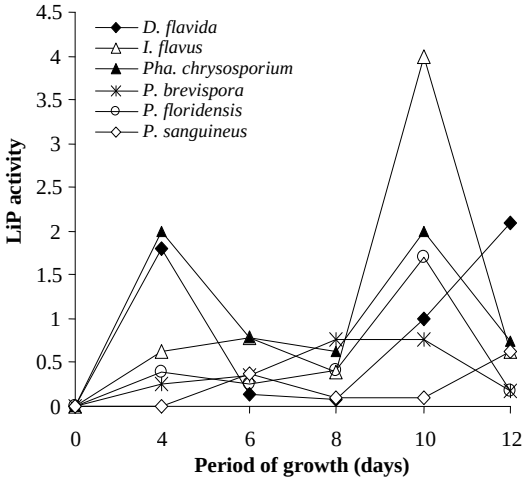

Fig. 4. Production of lignin peroxidase by Different WRF under static conditions

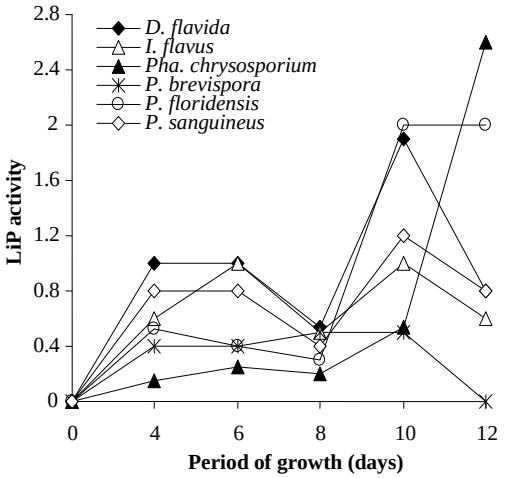

Fig.5. Production of lignin peroxidase by different WRF under shaking conditions

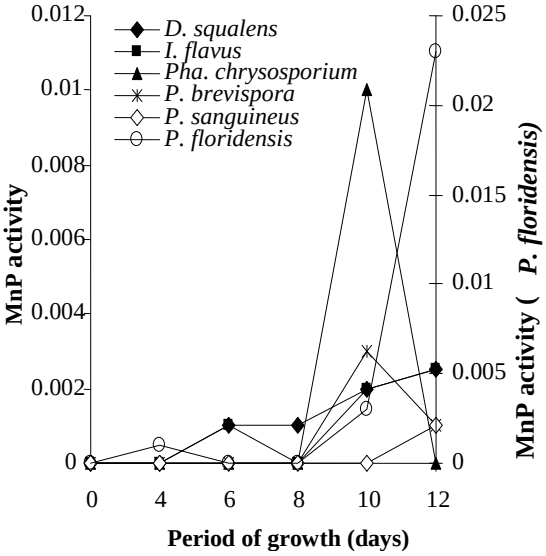

Fig. 6. Production of manganese peroxidase By different WRF under static condition

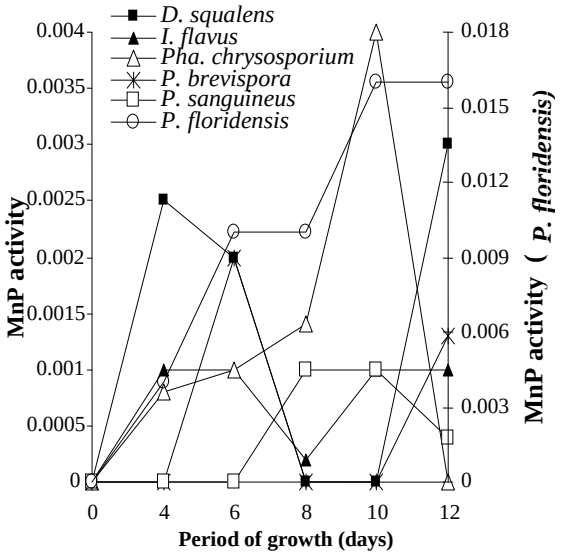

Fig. 7. Production of manganese peroxidase By different WRF under shaking condition

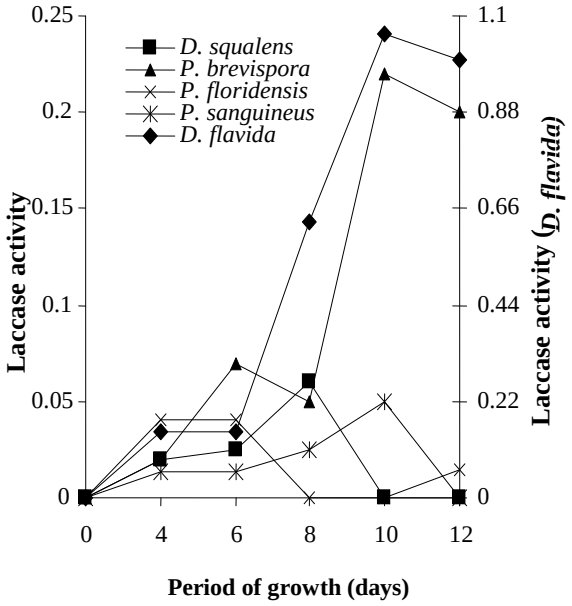

Fig. 8. Production of laccase by different WRF under static

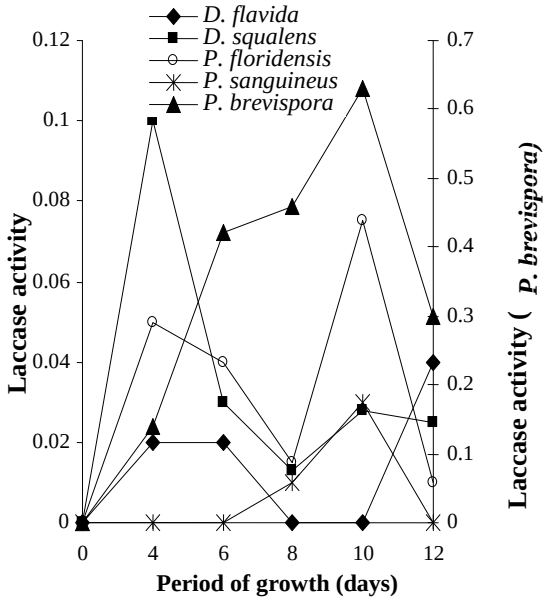

Fig.9. Laccase production by different WRF under shaking condition

Phlebia brevispora

Phlebia brevispora was capable of producing all of the three LE under static as well as shake conditions (Figures 4-9) and their maximum production was observed on day 10, except for MnP which peaked on day 6 under shaking condition (Figure 7). The CFEE obtained on 8th day from static and shake cultures of *P. brevispora* decolourised 57% of Poly R–478 (Figures 2, 3). It was the second best dye decolourising culture.

Phlebia floridensis

All the three LE were produced by *P. floridensis* where LiP and MnP were best produced during day 10–12 days under both the culture conditions (Figures 4-9). Laccase activity was maximum on day 4 and 10 in static and shake cultures of *P. floridensis*, respectively, while giving second maxima on day 6 in former growth conditions (Figures 8, 9). The CFEE obtained from 8 day grown cultures caused the highest dye decolourisation (60% in 5h) (Figures 2, 3).

Polyporus sanguineus

The shake culture of *P. sanguineus* gave maxima of three LE on day 10. The static cultures produced maximum laccase and LiP/MnP on day 10, 12 respectively (Figures 4-9). The CFEE obtained from 8 day grown static as well as shake cultures caused maximum dye decolourisation (Figures 2, 3).

Dye Decolourisation in U–Tube Immobilized Reactor

The above studies on dye decolourisation at flask level revealed *D. squalens*, *P. brevispora* and *P. floridensis* to be better biocleaning agents. These WRF were further

evaluated for their dye decolourisation potential on semi–continuous immobilized reactor using 4% WSE medium. Three of the tested fungi (including both the *Phlebia* spp.), which were capable of producing three LE caused an equal or relatively high dye decolourisation in comparison to *Pha. chrysosporium* during 20 days of reactor operation (Table 2). These results are in consonance with earlier studies. It ranged from 50–56% and 47–61% in case of *P. brevispora* and *P. floridensis*, respectively. While *D. squalens* and *Pha. chrysosporium*, which were unable to produce LiP and laccase, respectively gave relatively low level of dye decolourisation (Table 2). The maximum titre of three LE in general, coincided with their maximum dye decolourisation rate. The enzyme production maxima almost peaked with dye decolourisation. In comparison to *Pha. chrysosporium*, all the selected fungi caused higher reduction in chemical oxygen demand (COD) of dye. The lowering of COD may be attributed to enzymatic breakage of chemical structure of dye or biodegradation. On 16th day of reactor operation, *P. floridensis* removed 79% of total COD load followed by *P. brevispora* and *D. squalens* which lowered the oxygen demand by 74 and 62%, respectively (Table 2). *Pha. chrysosporium* lowered 60% COD in similar period of reactor operation (Table 2). The tested fungi caused a significant loss in toxicity of dye 35.5 to 89.4%. *P. floridensis* was the best in lowering the toxicity in 20 days of reactor operation (Table 2).

Table 2. Decolourisation of Poly R–478 by Different WRF Immobilized on PUF in Semi–Continuous U–Tube Reactor

Fungus	Total days of reactor operation	Enzyme activity			% decol^{n}	Ames assay		% reduction in *COD
		LiP	MnP	Laccase		No of #revertant formed	% reduction in toxicity	
D. squalens	12	–	0.0024	0.06	50.0	38	50.0	40
	14	–	0.0024	0.03	52.0	24	69.2	38
	16	–	0.0001	0.03	53.5	20	73.6	62
	18	–	0.0010	0.03	47.0	25	67.1	40
	20	–	0.0020	0.02	50.0	36	52.6	48
Pha.chrysosporium	12	0.42	0.0010	–	41.0	49	35.5	35
	14	0.40	0.0008	–	52.0	36	52.6	42
	16	0.54	0.0020	–	55.0	19	75.0	60
	18	0.51	0.0010	–	46.0	25	67.1	39
	20	0.30	0.0010	–	46.4	29	61.8	40
P. brevispora	12	0.60	0.0028	0.15	50.0	42	44.7	42
	14	0.52	0.0026	0.05	54.0	18	76.3	58
	16	0.52	0.0010	0.30	56.0	16	78.9	74
	18	0.50	0.0010	0.50	56.0	13	81.5	74
	20	0.67	0.0016	0.30	56.0	14	81.6	76
P. floridensis	12	0.40	0.0016	0.01	47.0	23	69.7	45
	14	0.41	0.0026	0.02	59.0	16	78.9	60
	16	0.18	0.0020	0.04	61.0	10	86.8	79
	18	0.46	0.0024	0.05	56.0	13	82.8	74
	20	0.50	0.0020	0.06	60.0	8	89.4	77

#Untreated sample formed lawn of *S. typhi* (76 colonies in positive control), * COD of untreated sample was 26000 mg l^{-1}, – : No activity

The present study employs the WRF which have been earlier known to be producing various LE in different combinations such as LiP+MnP+laccase, MnP+laccase or LiP+Laccase (Arora et al. 2002; Chander and Arora 2007; Heinzkill et al. 1997; Vares et al. 1995). *Phlebia* spp. and *P. sanguineus* were capable of producing all the three LE while rest of the tested fungi were unable to produce either one or other LE. However, the production of three LE was independent of incubation conditions (static or shake) with exception of laccase which was, in general, better produced under stationary conditions. All the cultures under static growth conditions gave parallel production maxima for MnP and laccase except for *D. squalens* and *P. sanguineus*. In general, LiP activity peaked either on day 10 or 12. The shake flask cultures produced maximum MnP and laccase invariably on day 10, except *D. flavida*, *D. squalens* and *P. brevispora*. In the earlier studies, highest decolourisation of industrial dyes was achieved by the CFEE obtained from wild cultures grown for 8 days on mineral salts broth MSB (Chander et al. 2004) and this period could be reduced to 6 days when using preadapted cultures (Arora and Chander 2004; Chander et al. 2014). In MSB, the easily metabolizable substrate availability might have led to early enzyme production (Cing and Yesilada 2004) which could have been delayed due to complex nutritional status of WSE as used in present study. In consonance with earlier studies (Arora and Gill 2005), three enzymes showed two activity peaks which correlate well with the dye decolourisation on day 8 and 12 by *D. squalens*, *P. brevispora* and *P. floridensis*.

A relatively higher dye decolourisation was observed by the CFEE obtained from shake flask cultures. In general, the maximum decolourisation of Poly R–478 was caused by CFEE obtained from 8 day grown cultures. The *Phlebia* spp. producing the three LE was better decolourisers than *Pha. chrysosporium* under static conditions. *I. flavus* and *Pha. chrysosporium* which though produced sufficiently high levels of LiP decolourised Poly R–478 only to a moderate level under static conditions. On the contrary moderate MnP and laccase activities in *Phlebia* spp. in static as well as shake cultures caused maximum dye decolourisation. *D. flavida* and *I. flavus* which lacked one of the LE, caused relatively lower dye decolourisation under both conditions i.e. static and shaking except *D. squalens* and *Pha. chrysosporium*, which showed high decolourisation under both and shaking condition, respectively. In comparison to static conditions, during the shaking conditions the reaction mixtures may have uniform mixing of enzyme extracts with the dye hence causing higher dye decolourisation in case of *D. flavida* and *I. flavus* (Table 1). Similar observations have been made earlier where enzymatic combinations have been shown to play an important role in ligninolysis using wheat straw as substrate (Scholosser et al. 1997; Velaquez et al. 2004; Chander 2014).

Apparently, only a scant literature is available on the use of WRF based reactors in waste water treatment. In a study carried out by Blanquez et al. (2004) using bioreactor filled with pellets of *T. versicolor* removed 90% of dye Grey Lanaset G (150mg l^{-1}) in batch as well as continuous mode, while actively removing dye colour upto 40 days in the latter mode. The study advocated the use of rotating biological contactors allowing intermittent contact of the mycelium with the effluent, thus avoiding overgrowth and the problems arising in packed–bed reactors. To overcome this, Lopez et al. (2004), developed enzymatic membrane bioreactors for the oxidation of azo dyes by MnP. The study by Selvem et al. (2003) evaluated the potential of two WRF namely *Thelephora* sp.

and *Fomes lividus* to decolourise the dye based effluents. In comparison to the continuous system, reactors operated in batch mode decolourised the effluents to a greater extent (Chander et al., 2014). It was proposed that immobilized cultures produces higher LME and cause greater dye decolourisation. Our studies are in consonance with their results. As the WRF were grown and immobilized as batch cultures during first 8 day of reactor operation and onwards dye decolourisation studies was done in continuous mode, *P. floridensis* and *Pha. chrysosporium* gave a little higher dye decolourisation than that in flask level studies (Figure 2,3; Table 1,2). The Poly R–478 decolourisation potential of *D. squalens* and *P. brevispora* was equally expressed in three growth conditions viz. static, shake and reactor system. The present study also supports the concept of concerted action of LE in biocleaning of dyes.

The present study showed the continuous production of enzymes in bioreactor up to 20 days of operation causing significant colour loss of Poly R–478 (Table 1). Four of the fungi tested for their enzyme production and dye decolourisation on PUF immobilized reactor gave reasonable enzyme production and caused 40–60% decolourisation of Poly R–478. *Phlebia* spp. again proved to be better decolourisers than the much studied *Pha. chrysosporium* (Table 2). Under the reactor conditions the enzyme production by the four WRF showed only slight fluctuations from 10th day onward. There were no drastic changes in pattern of enzyme activity from 12–20 days and it did not require any change in reaction media or addition of inoculants as required in batch systems. The toxicity of the treated sample was reduced markedly by all the tested fungi. *P. floridensis* causing the decrease in COD and mutagenicity of Poly R–478 is the organism of choice (Table 2).

CONCLUSIONS

1. The present study reveals *Phlebia* spp. and *D. squalens* to be more efficient decolourisers of Poly R–478 in flask as well as immobilized reactor levels.
2. The Poly R–478 decolourisation potential of *D. squalens* and *P. brevispora* was equally expressed in three growth conditions viz. static, shake and reactor system.
3. No single enzyme could be held responsible for the biodecolourisation; however, their collective action plays an important role in decolourisation. The future studies on dye biodegradation potential of individual ligninolytic enzymes under selective production conditions or in purified forms may reveal their precise role.

ACKNOWLEDGEMENT

Dr. Mukesh Chander is grateful to the University Grants Commission, New Delhi, India for conferring a Major Research Project upon him.

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Article submitted: January 10, 2014; Peer review completed: March 22, 2014; revised version received and accepted: May 25, 2014; Published: June 13, 2014.