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ORIGINAL PAPER

Enhanced decolourization of Direct Red-80 dye by the white rot fungus *Phanerochaete chrysosporium* employing sequential design of experiments

Sukhwinder Singh · Kannan Pakshirajan · Achlesh Daverey

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Abstract Decolourization of Direct Red 80 (DR-80) by the white rot fungus *Phanerochaete chrysosporium* MTCC 787 was investigated employing sequential design of experiments. Media components for growing the white rot fungus were first screened using Plackett-Burman design and then optimized using response surface methodology (RSM), which resulted in enhancement in the efficiency of dye removal by the fungus. For determining the effect of media constituents on the dye removal, both percent dye decolourization and specific dye removal due to maximum enzyme activity were chosen as the responses from the experiments, and the media constituents glucose, veratryl alcohol, KH_2PO_4, $CaCl_2$ and $MgSO_4$ were screened to be the most effective with P values less than 0.05. Central composite design (CCD) followed by RSM in the optimization study revealed the following optimum combinations of the screened media constituents: glucose, 11.9 g l^{-1}; veratryl alcohol, 12.03 mM; KH_2PO_4, 23.08 g l^{-1}; $CaCl_2$, 2.4 g l^{-1}; $MgSO_4$, 10.47 g l^{-1}. At the optimum settings of the media constituents, complete dye decolourization (100% removal efficiency) and a maximum specific dye removal due to lignin peroxidase enzyme of 0.24 mg U^{-1} by the white rot fungus were observed.

S. Singh · K. Pakshirajan (✉) · A. Daverey
Department of Biotechnology, Indian Institute of Technology, Guwahati, Assam 781039, India
e-mail: pakshi@iitg.ernet.in

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Introduction

Azo dyes such as Direct Red-80 (DR-80) are important colorants and constitute the largest class of dyes for application in textile, paper, leather, gasoline, foodstuffs and cosmetics industries (Dias et al. 2003). These dyes, generally characterized by the presence of one or more azo linkages (–N=N–) and aromatic rings (Nigam et al. 1996), are known to be toxic and mutagenic to living organisms (Moller and Wallin. 2000; Stolz. 2001; Mechichi et al. 2006). Discharge of wastewaters from dye manufacturing units and textile processing industries containing such azo dyes therefore results in pollution of the receiving aquatic environment (Keharia and Madamwar 2003). Hence, treatment of such dye containing wastewater is essential to prevent deterioration as well as to sustain the natural flora and fauna of the ecosystem. Currently, the major methods of treating dye containing wastewaters involve physical and/or chemical techniques such as photo catalytic degradation (Korbahti and Rauf 2008), electrocoagulation (Aleboyeh et al. 2008), ozonation (Hassan and Hawkyard 2002), treatment using Fenton's reagent, electrochemical destruction etc. (Lin and Chen 1997). However, these techniques are either inefficient in

complete removal of dyes or are economically non-viable (Chang and Lin 2000). On the other hand, microbial degradation and decolourization of azo dyes is considered more efficient and cost competitive compared to the physico-chemical techniques. The white-rot fungus *Phanerochaete chrysosporium,* which belongs to a group of lignin-degrading basidiomycetes, has received considerable attention in the past for their very good bioremediation potential (Bumpus and Brock 1988; Cripps et al. 1990; Bakshi et al. 1999) owing to its natural capability to degrade complex lignin by their extracellular non-specific and non-stereoselective enzyme system composed of lignin peroxidases (LiP, EC 1.11.1.14), and manganese peroxidases (MnP, EC 1.11.1.13) that function together with H_2O_2 producing oxidases and secondary metabolites (Hatakka 1994). The same unique non-specific mechanisms that give these fungi the ability to degrade lignin also facilitate degradation of a wide range of recalcitrant pollutants including polycyclic aromatic hydrocarbons, chlorinated phenols, polychlorinated biphenyls, dioxins, pesticides and explosives (Pointing 2001). The DR-80 is a water-soluble azo dye and is used for dyeing silk, wool, cellulose and cotton (Mahmoodi et al. 2005). Although important to decolorize or degrade this dye in aqueous systems, there is no report as such on its biodegradation. However, decolourization of DR-80 by UV oxidation in the presence of hydrogen peroxide employing TiO_2 as a photo catalyst or by electrochemical method using three different materials as anodes, iron, polypyrrole (PPy) and boron doped diamond (BDD), have been reported. As mentioned earlier, all these methods suffered from the main drawback in not being efficient to degrade the dye (Mahmoodi et al. 2005; Aleboyeh et al. 2008). Hence, biodecolorization of DR-80 dye by *P. chrysosporium* was investigated in the present study. Since media constituents play vital role in growth of *P. chrysosporium* and in production of the lignin degrading enzyme—lignin peroxidase, which is directly involved in dye decolourization process, proper understanding of the effects of media constituents and optimization of their levels are essential to significantly improve the dye decolourization efficiency by the fungus.

Screening and optimization of media constituents can be done by either varying one variable at a time or non-conventionally by using statistical methods. Conventional screening and optimization techniques involve varying factor and their levels by maintaining the other factors at an unspecified constant level; by doing so, the combined effect of the factors is, however, neglected. In addition, these techniques are time-consuming and require sufficiently large number of experimental runs. Such limitations of a classical method can be eliminated by screening and optimizing all the affecting factors collectively by employing statistical experimental design and empirical model building using regression analysis. The most successfully used technique for optimization based on statistical methods is known as response surface methodology (Chen 1994), which further helps in understanding the effects of individual variables and their interaction on a given response (Montgomery 1991). To reduce the number of factors to be used in an optimization study, screening of factors is normally performed by employing another statistical design such as Plackett-Burman. Although RSM has been effectively used in optimization of various biotechnological and industrial processes (Asgher et al. 2008; Mohana et al. 2008; Reddy et al. 2008; Purama and Goyal 2008; Sheth and Dave 2009; Tir and Moulai-Mostefa 2008), the use of such nonconventional statistical techniques in screening followed by optimization of media constituents for enhancing dye decolourization has not been reported so far in the literature. Therefore, in the present study, decolourization of DR-80 by *P. chrysosporium* was studied systematically by screening and optimization of media constituents using Plackett-Burman design and RSM, respectively, with an aim to enhance the efficiency of dye removal by the fungus.

Materials and methods

Chemicals and reagents

Azo dye (Direct Red-80) and veratryl alcohol (3, 4-dimethoxybenzyl alcohol, 96% pure) were purchased from Sigma (St. Louis, Mo, USA). All other chemicals and solvents, of Guaranteed Reagent (GR) grade, were purchased from either High Media Mumbai (India), SRL (India) or Merck (India).

Microorganism and its maintenance

The fungus *P. chrysosporium* MTCC 787 used in this study was procured from IMTECH, Chandigarh,

India, and was maintained at 25°C on potato dextrose agar (PDA) slants. For spore production, the slants were incubated at 39°C for 2–5 days in media containing glucose: 10 g l^{-1}, malt extract: 10 g l^{-1}; peptone: 2 g l^{-1}; yeast extract: 2 g l^{-1}; asparagine: 1 g l^{-1};KH_2PO_4: 2 g l^{-1}; $MgSO_4{\cdot}7H_2O$: 1 g l^{-1}; thaimin-HCl: 1 mg l^{-1} and agar: 20 g l^{-1} (Tien and Kirk 1983). The media used for studying decolourization of DR-80 dye composed of basal medium (KH_2PO_4, 20 g l^{-1}; $MgSO_4$: 5 g l^{-1}; $CaCl_2$: 1 g l^{-1}); trace elements ($MgSO_4$: 3 g l^{-1}; $MnSO_4$: 0.5 g l^{-1}; NaCl: 1 g l^{-1}; $FeSO_4{\cdot}7H_2O$: 0.1 g l^{-1}; $CoCl_2$: 0.1 g l^{-1}; $ZnSO_4{\cdot}7H_2O$: 0.1 g l^{-1}; $CuSO_4$: 0.1 g l^{-1}; $AlK(SO_4)_2{\cdot}12H_2O$:10 mg l^{-1}; H_3BO_3:10 mg l^{-1}; $Na_2MoO_4{\cdot}2H_2O$: 10 mg l^{-1}; nitrilotriacetate: 1.5 g l^{-1}) and other ingredients—glucose: 100 g l^{-1}; 2,2-dimethylsuccinate: 0.1 *M* (pH 4.2); thiamine:100 mg l^{-1} (filter sterilized); veratryl alcohol: 4 m*M* stock (filter sterilized) and NH_4Cl: 4.68 g l^{-1} (Radha et al. 2005).

Plackett-Burman design for screening of media constituents

A Plackett-Burman design of total 12 experimental runs was employed to screen the media constituents for their effects on DR-80 decolourization by *P. chrysosporium.* All the media constituents, except the trace elements, and inoculum size, which are known to influence both the fungus growth and its lignin peroxidase enzyme activity, were chosen as factors in the screening study. Since the trace elements, temperature, pH etc. are known to play a role in the fungal growth and not necessarily on the activity of its enzyme activity, these were not included in the study. Table 1 presents the experimental combinations of the factors and their levels adopted in the screening study, where −1 and +1 are the coded levels of each factor representing the respective minimum and maximum feasible concentrations of the factors in the study. In all the experimental runs that were performed in duplicates using an initial dye concentration of 50 mg l^{-1}, percentage dye decolourization and specific dye removal were recorded as the responses, and the results analyzed using the statistical software package Minitab®, 15, PA, USA. The response expressed as percentage dye decolourization was simply calculated from equation (1)

$$D = \left(\frac{C_0 - C}{C_0}\right) \times 100 \quad (1)$$

where D is percentage dye decolourization, C_0 and C are the respective initial and final dye concentrations (mg l^{-1}) in the media. The response on specific

Table 1 Plackett-Burman design matrix showing the experimental combinations adopted in the screening study along with the observed percentage dye decolourization and specific dye removal

Exp. run no.	Experimental variables								Percentage dye decolourization (%)	Sp. dye removal (mg U^{-1})
	Glucose (g l^{-1})	NH_4Cl (g l^{-1})	KH_2PO_4 (g l^{-1})	$MgSO_4$ (g l^{-1})	$CaCl_2$ (g l^{-1})	Veratryl alcohol (mM)	Tween 20 (%)	Inoculum size (OD)		
1	15 (+1)	0.68 (−1)	30 (+1)	1 (−1)	0.2 (−1)	1 (−1)	0.2 (+1)	1.2 (+1)	97	0.4529
2	15 (+1)	8.68 (+1)	10 (−1)	10 (+1)	0.2 (−1)	1 (−1)	0.05 (−1)	1.2 (+1)	100	0.5549
3	5 (−1)	8.68 (+1)	30 (+1)	1 (−1)	1.8 (+1)	1 (−1)	0.05 (−1)	0.4 (−1)	38	0.6784
4	15 (+1)	0.68 (−1)	30 (+1)	10 (+1)	0.2 (−1)	7 (+1)	0.05 (−1)	0.4 (−1)	62	0.7643
5	15 (+1)	8.68 (+1)	10 (−1)	10 (+1)	1.8 (+1)	1 (−1)	0.2 (+1)	0.4 (−1)	100	0.5863
6	15 (+1)	8.68 (+1)	30 (+1)	1 (−1)	1.8 (+1)	7 (+1)	0.05 (−1)	1.2 (+1)	47	0.7580
7	5 (−1)	8.68 (+1)	30 (+1)	10 (+1)	0.2 (−1)	7 (+1)	0.2 (+1)	0.4 (−1)	32	0.5130
8	5 (−1)	0.68 (−1)	30 (+1)	10 (+1)	1.8 (+1)	1 (−1)	0.2 (+1)	1.2 (+1)	55	0.5333
9	5 (−1)	0.68 (−1)	10 (−1)	10 (+1)	1.8 (+1)	7 (+1)	0.05 (−1)	1.2 (+1)	83	0.8635
10	15 (+1)	0.68 (−1)	10 (−1)	1 (−1)	1.8 (+1)	7 (+1)	0.2 (+1)	0.4 (−1)	99	0.6104
11	5 (−1)	8.68 (+1)	10 (−1)	1 (−1)	0.2 (−1)	7 (+1)	0.2 (+1)	1.2 (+1)	59	0.5774
12	5 (−1)	0.68 (−1)	10 (−1)	1 (−1)	0.2 (−1)	1 (−1)	0.05 (−1)	0.4 (−1)	76	0.1955

Coded levels of the factors are indicated in parentheses

DR-80 removal (mg U^{-1}) due to maximum enzyme (LiP) activity was calculated using equation (2)

$$SDR = \left(\frac{C_0 - C_t}{E_{max}}\right) \quad (2)$$

where SDR is specific DR-80 removal due to maximum enzyme activity (mg U^{-1}), E_{max} is the maximum enzyme activity (U l^{-1}) and C_t is the dye concentration remaining in the media corresponding to the time at E_{max}.

Optimization of media constituents using response surface methodology

Based on the results obtained from Plackett-Burmann design for screening the media components, central composite design (CCD), containing the five factors glucose, $MgSO_4 \cdot 7H_2O$, KH_2PO_4, $CaCl_2$ and veratryl alcohol, was employed in optimizing the levels of the media constituents for enhancing both the responses on percentage dye decolourization and specific dye removal due to maximum enzyme activity by *P. chrysosporium*. Table 2 shows the 2^5 full factorial CCD and 10 center point replicates along with the observed and predicted responses in the study. All the experiments were carried out in 250 ml Erlenmeyer flasks containing 100 ml of the media containing DR-80 (50 mg l^{-1}) and the media constituents, as per the design shown in Table 2. After inoculation with five day old spores ($OD_{650\ nm} = 0.5$) produced on PDA slants, the flasks were incubated at 39°C and 180 rpm for 6 days.

Analytical methods

Estimation of lignin peroxidase enzyme and DR-80 dye concentration

Samples taken during the experiments were centrifuged at 10,000×*g* for 10 min at 4°C to remove fungal biomass. Biomass free samples so obtained were divided into two portions: one portion containing lignin peroxidase enzyme was assayed by spectrophotometric method (Kirk et al. 1990), which was based on the oxidation of veratryl alcohol to veratraldehyde. One unit (U) of lignin peroxidase enzyme activity was defined as the amount that converted 1 mol of veratryl alcohol to veratraldehyde per min per ml of the culture filtrate; the enzyme activities were expressed as U l^{-1} (Linko and Haapala 1993). The other portion was used for determining residual DR-80 concentration by measuring its absorbance (λ_{max} DR- 80) at 528 nm using a UV–visible spectrophotometer (Carry 100, Varian, USA).

Results and discussion

Lignin peroxidase activity and dye decolourization profiles

In the present study both percentage dye decolourization and specific dye removal due to maximum LiP activity by the fungus were simultaneously investigated by screening and optimization of the media constituents with an aim to enhance the dye decolourization efficiency. Typical profiles of both LiP activity and DR-80 decolourization obtained from experimental run 1 of the Plackett-Burman screening design are presented in Fig. 1. It could be seen that the enzyme activity reached a maximum value of 85.14 U l^{-1} at 48 h of culture, and after which the activity decreased considerably. Similar profiles of lignin peroxidase enzyme by the fungus have been reported in the literature by other authors, as well (Sayadi and Ellouz 1995). Lignin peroxidase is one such important enzyme produced by *P. chrysosporium* that it is mainly responsible for decolourization of the azo dye. This aspect is clearly revealed in Fig. 1 where the concentration of DR-80 in the media was found to decrease with an increase in the enzyme activity that resulted in a significant decrease ($\sim$80%) of the dye within the first day itself (Fig. 1). Similar profiles of lignin peroxidase and decolourization of DR-80 were obtained in all the other experimental runs, and these are depicted in Table 1. However, variations in these two responses were obtained depending upon the factor and combinations thereof in each of the runs strongly suggesting the role of media constituents and their levels on the dye decolourization efficiency by the fungus.

Plackett-Burman design for screening of media constituents

The results obtained in the screening study (Table 1) were analyzed in the form of analysis of variance

Table 2 Central composite design matrix showing the factors and their levels in the optimization study along with the measured and predicted responses

Exp. run no.	Experimental variables (mg l^{-1})					Percentage dye decolourization (%)		Sp. dye removal (mg U^{-1})	
	X_1	X_2	X_3	X_4	X_5	Exp.	Pred.	Exp.	Pred.
1	6.9 (−1)	4.2 (−1)	13.8 (−1)	5.6 (−1)	1.6 (−1)	66	63	1.3689	0.9881
2	16.9 (+1)	4.2 (−1)	13.8 (−1)	5.6 (−1)	1.6 (−1)	75	70	0.8841	0.7829
3	6.9 (−1)	10.2 (+1)	13.8 (−1)	5.6 (−1)	1.6 (−1)	64	79	0.4969	0.4997
4	16.9 (+1)	10.2 (+1)	13.8 (−1)	5.6 (−1)	1.6 (−1)	86	88	0.3370	0.3718
5	6.9 (−1)	4.2 (−1)	33.8 (+1)	5.6 (−1)	1.6 (−1)	65	66	0.6327	0.6262
6	16.9 (+1)	4.2 (−1)	33.8 (+1)	5.6 (−1)	1.6 (−1)	65	69	0.4083	0.4605
7	6.9 (−1)	10.2 (+1)	33.8 (+1)	5.6 (−1)	1.6 (−1)	98	94	0.2881	0.3958
8	16.9 (+1)	10.2 (+1)	33.8 (+1)	5.6 (−1)	1.6 (−1)	100	99	0.3135	0.3074
9	6.9 (−1)	4.2 (−1)	13.8 (−1)	13.6 (+1)	1.6 (−1)	76	74	0.4948	0.6415
10	16.9 (+1)	4.2 (−1)	13.8 (−1)	13.6 (+1)	1.6 (−1)	75	77	0.4376	0.5197
11	6.9 (−1)	10.2 (+1)	13.8 (−1)	13.6 (+1)	1.6 (−1)	94	91	0.3426	0.2915
12	16.9 (+1)	10.2 (+1)	13.8 (−1)	13.6 (+1)	1.6 (−1)	95	95	0.3384	0.2470
13	6.9 (−1)	4.2 (−1)	33.8 (+1)	13.6 (+1)	1.6 (−1)	78	75	0.6929	0.5329
14	16.9 (+1)	4.2 (−1)	33.8 (+1)	13.6 (+1)	1.6 (−1)	77	74	0.6466	0.4506
15	6.9 (−1)	10.2 (+1)	33.8 (+1)	13.6 (+1)	1.6 (−1)	100	104	0.4274	0.4408
16	16.9 (+1)	10.2 (+1)	33.8 (+1)	13.6 (+1)	1.6 (−1)	100	104	0.4124	0.4359
17	6.9 (−1)	4.2 (−1)	13.8 (−1)	5.6 (−1)	3.2 (+1)	69	68	0.5296	0.6170
18	16.9 (+1)	4.2 (−1)	13.8 (−1)	5.6 (−1)	3.2 (+1)	69	71	0.5872	0.5361
19	6.9 (−1)	10.2 (+1)	13.8 (−1)	5.6 (−1)	3.2 (+1)	96	94	0.2688	0.3302
20	16.9 (+1)	10.2 (+1)	13.8 (−1)	5.6 (−1)	3.2 (+1)	95	98	0.2734	0.3266
21	6.9 (−1)	4.2 (−1)	33.8 (+1)	5.6 (−1)	3.2 (+1)	65	65	0.3278	0.2968
22	16.9 (+1)	4.2 (−1)	33.8 (+1)	5.6 (−1)	3.2 (+1)	66	64	0.3232	0.2555
23	6.9 (−1)	10.2 (+1)	33.8 (+1)	5.6 (−1)	3.2 (+1)	98	102	0.3720	0.2680
24	16.9 (+1)	10.2 (+1)	33.8 (+1)	5.6 (−1)	3.2 (+1)	98	103	0.3555	0.3039
25	6.9 (−1)	4.2 (−1)	13.8 (−1)	13.6 (+1)	3.2 (+1)	69	70	0.6354	0.4653
26	16.9 (+1)	4.2 (−1)	13.8 (−1)	13.6 (+1)	3.2 (+1)	70	69	0.6406	0.4678
27	6.9 (−1)	10.2 (+1)	13.8 (−1)	13.6 (+1)	3.2 (+1)	95	97	0.3371	0.3168
28	16.9 (+1)	10.2 (+1)	13.8 (−1)	13.6 (+1)	3.2 (+1)	95	97	0.3488	0.3967
29	6.9 (−1)	4.2 (−1)	33.8 (+1)	13.6 (+1)	3.2 (+1)	66	66	0.4135	0.3984
30	16.9 (+1)	4.2 (−1)	33.8 (+1)	13.6 (+1)	3.2 (+1)	67	60	0.3898	0.4405
31	6.9 (−1)	10.2 (+1)	33.8 (+1)	13.6 (+1)	3.2 (+1)	100	103	0.4501	0.5079
32	16.9 (+1)	10.2 (+1)	33.8 (+1)	13.6 (+1)	3.2 (+1)	100	100	0.4445	0.6273
33	0.008 (−α)	7.2 (0)	23.8 (0)	9.6 (0)	2.4 (0)	100	96	0.3665	0.4786
34	23.79 (+α)	7.2 (0)	23.8 (0)	9.6 (0)	2.4 (0)	100	100	0.3697	0.3764
35	11.9 (0)	0.065 (−α)	23.8 (0)	9.6 (0)	2.4 (0)	14	22	0.2868	0.5972
36	11.9 (0)	14.34 (+α)	23.8 (0)	9.6 (0)	2.4 (0)	100	88	0.4300	0.2385
37	11.9 (0)	7.2 (0)	0.016 (−α)	9.6 (0)	2.4 (0)	75	72	0.5144	0.6522
38	11.9 (0)	7.2 (0)	47.58 (+α)	9.6 (0)	2.4 (0)	80	80	0.5149	0.4960
39	11.9 (0)	7.2 (0)	23.8 (0)	0.086 (−α)	2.4 (0)	88	81	0.4012	0.4877
40	11.9 (0)	7.2 (0)	23.8 (0)	19.11 (+α)	2.4 (0)	88	91	0.4277	0.4601
41	11.9 (0)	7.2 (0)	23.8 (0)	9.6 (0)	0.497 (−α)	91	89	0.4622	0.6030
42	11.9 (0)	7.2 (0)	23.8 (0)	9.6 (0)	4.305 (+α)	93	90	0.4114	0.3894

Table 2 continued

Exp. run no.	Experimental variables (mg l^{-1})					Percentage dye decolourization (%)		Sp. dye removal (mg U^{-1})	
	X_1	X_2	X_3	X_4	X_5	Exp.	Pred.	Exp.	Pred.
43	11.9 (0)	7.2 (0)	23.8 (0)	9.6 (0)	2.4 (0)	96	96	0.2671	0.2772
44	11.9 (0)	7.2 (0)	23.8 (0)	9.6 (0)	2.4 (0)	97	96	0.2704	0.2772
45	11.9 (0)	7.2 (0)	23.8 (0)	9.6 (0)	2.4 (0)	98	96	0.2708	0.2772
46	11.9 (0)	7.2 (0)	23.8 (0)	9.6 (0)	2.4 (0)	97	96	0.2646	0.2772
47	11.9 (0)	7.2 (0)	23.8 (0)	9.6 (0)	2.4 (0)	97	96	0.2702	0.2772
48	11.9 (0)	7.2 (0)	23.8 (0)	9.6 (0)	2.4 (0)	97	96	0.2702	0.2772
49	11.9 (0)	7.2 (0)	23.8 (0)	9.6 (0)	2.4 (0)	97	96	0.2702	0.2772
50	11.9 (0)	7.2 (0)	23.8 (0)	9.6 (0)	2.4 (0)	97	96	0.2702	0.2772
51	11.9 (0)	7.2 (0)	23.8 (0)	9.6 (0)	2.4 (0)	97	96	0.2702	0.2772
52	11.9 (0)	7.2 (0)	23.8 (0)	9.6 (0)	2.4 (0)	97	96	0.2702	0.2772

X_1 = Glucose (g l^{-1}), X_2 = Veratryl alcohol (mM), X_3 = KH_2PO_4 (g l^{-1}), X_4 = $MgSO_4$ (g l^{-1}), X_5 = $CaCl_2$ (g l^{-1})

Coded levels of the factors are indicated in parentheses

(ANOVA), which is a statistical technique that subdivides the total variation in a data set into component parts associated with specific sources of variation for the purpose of testing null hypotheses on the parameters in a model (Montgomery 1991). Table 3 presents the ANOVA of percentage dye decolourization and specific dye removal at maximum LiP enzyme activity, in which the mean sum of squares (MS) of the model terms was obtained from the ratio of sum of squares (SS) and degrees of freedom (df); the Fisher's F value was calculated by dividing the MS owing to the model by the MS owing to error. Generally, a large F value and a corresponding low value of P for a term in the model indicate high significance of the term (Montgomery 1991). Therefore, from the ANOVA table, the term for main effects of media constituents in both the models for percentage dye decolourization and specific dye removal were highly significant at P value less than 0.01. Table 3 also shows a term for error in the models, the MS value of which indicates that the amount of variation in the response data that was left unexplained by the models is low. To assess the significance of each individual medium constituent on percentage dye decolourization and specific dye removal, Student's t-test was performed and the results are presented in Table 4. From this table, glucose was found to have a maximum effect on percentage dye decolourization followed by KH_2PO_4, NH_4Cl and veratryl alcohol; however, only $CaCl_2$ and $MgSO_4{\cdot}7H_2O$ were found to significantly effect specific removal of the dye. Hence, out of the seven media constituents, the five media constituents of glucose, KH_2PO_4, veratryl alcohol, $CaCl_2$ and $MgSO_4{\cdot}7H_2O$ were further chosen for optimizing their levels. In a similar study by Mohana et al. (2008), glucose and such media components were studied to be important in decolourization of Direct Black 32 dye by a bacterial consortium. Moreover, the observation that NH_4Cl did not show any significant effect on both the responses was found to be in conformity with the literature where it is reported that under nitrogen excess conditions enzyme activity shown by the fungus is low, which

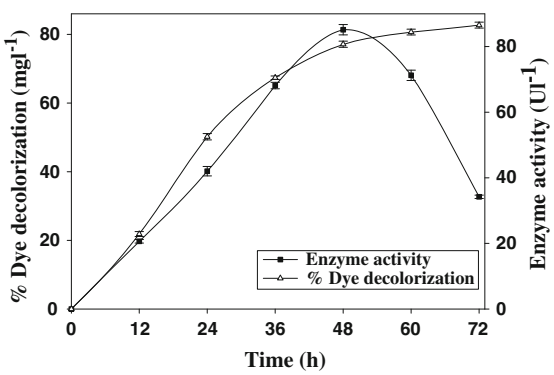

Fig. 1 LiP activity and DR-80 decolourization profiles obtained in experimental run no. 1 of the Plackett-Burman screening design

Table 3 ANOVA of percentage dye decolourization and specific dye removal in the screening study

Source	Percentage dye decolourization (%)					Specific dye removal (mg U^{-1})				
	SS	df	MS	*F*	*P*	SS	df	MS	*F*	*P*
Main effects	11252.7	8	1406.58	18.92	0.000	0.45784	8	0.06789	3.03	0.031
Curvature	751.9	1	751.89	10.11	0.005	0.1070	3	0.0356	1.59	0.233
Residual error	1264.0	17	74.35			0.33628	15	0.02241		
Pure error	1148.2	14	82.01			0.04028	14	0.00287		
Total	13268.5	26					26			

df degrees of freedom, *SS* sum of squares, *MS* mean sum of squares

Table 4 Student *t* test for screening of media constituents by the Plackett-Burman method

Media constituents	Sp. dye removal due to maximum LiP activity (mg U^{-1})		Percentage dye decolourization (%)	
	T	*P*- value	*T*	*P*- value
Constant	21.81	0.000	42.35	0.000
Glucose	1.24	0.236*	5.33	0.000
Ammonium chloride	1.25	0.230*	−3.91	0.001
Potassium di hydrogen phosphate	−0.48	0.635*	−9.45	0.000
Calcium chloride	3.01	0.009	−0.40	0.692*
Magnesium sulphate	1.91	0.075*	0.07	0.944*
Tween 20	−1.72	0.106*	1.68	0.111*
Veratryl alcohol	0.26	0.801*	−3.39	0.004
Inoculum Size	0.95	0.357*	2.01	0.060*

* Indicates insignificant values of the factors at $P > 0.050$)

therefore results in poor degradation of xenobiotics (Tien and Kirk 1983; Nagarajan and Annadurai 1999).

Optimization using response surface methodology

Table 2, which was earlier referred to present the experimental combinations of the levels of screened media constituents in the optimization study, also presents the results of percentage dye decolourization and specific dye removal. Based on the results obtained, regression model equations were developed for depicting relationship between the various media constituents and the responses on percentage dye decolourization and specific dye removal. These two models are presented in equations 3 and 4 respectively.

$$\begin{aligned} f(X) = {} & -13.9 + 11.56X_2 + 1.29X_3 + 4.63X_4 \\ & + 8.61X_5 - 0.81X_2^2 - 0.03X_3^2 - 0.12X_4^2 \\ & - 1.88X_5^2 + 0.097X_2X_3 + 1.01X_2X_5 \\ & - 0.01X_3X_4 - 0.68X_4X_5 \end{aligned} \quad (3)$$

$$\begin{aligned} f(X) = {} & 3.08 - 0.16X_2 - 0.578X_3 - 0.11X_4 \\ & - 0.62X_5 + 0.002X_2^2 + 0.005X_3^2 + 0.002X_4^2 \\ & + 0.056X_5^2 + 0.002X_2X_3 + 0.021X_2X_5 \\ & + 0.002X_3X_4 + 0.015X_4X_5 \end{aligned} \quad (4)$$

ANOVA results of the models for percentage dye decolourization and specific dye removal (mg l^{-1}) were obtained as before in the screening study, and are given in Table 5. The Fisher's *F* values of both the models owing to regression were found to be high, which indicates that most of the variation in the responses can be explained by the regression model equations. The associated *P* value, which is used to judge whether F is large enough to indicate statistical significance or not, suggest that all the interaction terms in both the regression models were found to be highly significant ($P < 0.03$). Overall, the regression models for percentage dye decolourization and specific dye removal was found to be highly accurate with *P* value less than 0.003. Also, from Table 2,

both the experimental and predicted values of the two responses were found to match well with each other. These findings indicate that the second-order polynomial models for percentage dye decolourization and specific dye removal were adequate in representing the actual relationship between the media constituents and the responses. To determine significance of the regression coefficient of the factors in these models, the results were further subjected to Student *t*-test which is presented in Table 6. This particular result reveals that regression coefficients for the linear terms due to veratryl alcohol (X_2) and $MgSO_4$ (X_4) in the model for percentage dye decolourization were found to be highly significant ($P < 0.02$); also, the coefficient of KH_2PO_4 (X_3) was significant at $P < 0.05$ compared to those of other media constituents. However, the term for glucose (X_1) was found to be only slightly significant. Compared to the findings on the significance of coefficients of linear terms in the models, all the coefficients of the quadratic terms in the model were found to be highly significant ($P < 0.05$). On the other hand, most of the regression coefficient terms for two-way interaction between factors, i.e. $X_1 \times X_2$, $X_1 \times X_3$, $X_1 \times X_4$, $X_1 \times X_5$, $X_2 \times X_4$ and $X_3 \times X_5$, were found to be statistically insignificant ($P > 0.05$) on both the responses.

In general, 2D response surface contour plots are graphical representation of relationship between response and experimental variables that can be used for determining the optimum conditions (Haider and Pakshirajan 2007; Tanyildizi et al. 2005). These contour plots show relative effects of any two variables when the concentration of the remaining variable is held constant at its center-point value; from the nature of the response surface contours whether elliptical, circular or saddle point, interaction between the variables may be predicted. Figures 2, 3, 4 and 5 depict response surface contour plots with significant interaction effects between the media constituents obtained in the study. The response surface contour plots between the variables $CaCl_2$ and veratryl alcohol and that between KH_2PO_4 and veratryl alcohol (Figs. 2 and 3, respectively) were found to be elliptical and, hence, indicated significant interactions on percentage dye decolourization. Similarly, contour plot of mutual interaction between the variables KH_2PO_4 and veratryl alcohol (Fig. 4) on specific dye removal due to maximum enzyme activity indicated good significance. The interaction between glucose and KH_2PO_4 also showed good importance on the response (Fig. 5) which was, however, not revealed from the Student *t* test results presented earlier in Table 6. Ravikumar et al. (2005) also demonstrated such interaction effects between independent variables using contour plots on the removal of Astrazone Blue FRR (Basic Blue 69) and Teflon Blue ANL (Acid Blue 125) by a novel adsorbent based on 1:1 ratio of carbon and fly ash.

The model equations for percentage dye decolourization and specific dye removal due to maximum enzyme activity were solved by partially differentiating the equation and then equating it to zero. Corresponding local maxima were further checked by second-order sufficient condition using a Hessian matrix, and the optimized levels of the screened media constituents were thus obtained to be glucose: 11.9 g l^{-1}, veratryl alcohol: 12.03 mM, KH_2PO_4: 23.08 g l^{-1}, $CaCl_2$: 2.4 g l^{-1}, $MgSO_4$: 10.47 g l^{-1}. The optimum values of the constituents were also in

Table 5 ANOVA of percentage dye decolourization and specific dye removal in the optimization study

Source	Percentage dye decolourization (%)					Specific dye removal (mg U^{-1})				
	SS	df	MS	*F*	*P*	SS	df	MS	*F*	*P*
Regression	13182.7	20	659.13	25.84	0.000	1.22805	20	0.06140	3.14	0.002
Linear	8614.9	5	1722.99	67.55	0.000	0.40173	5	0.08035	4.11	0.006
Square	3772.4	5	754.47	29.58	0.000	0.30597	5	0.06119	3.13	0.021
Interaction	795.4	10	79.54	3.12	0.007	0.5202	10	0.05204	2.66	0.018
Residual error	790.7	31	25.51			0.60619	31	0.01955		
Pure error		9	0.12			0.00004	9	0.00001		
Total	13973.4	51					51			

df degrees of freedom, *SS*: sum of squares, *MS* mean sum of squares

Table 6 Estimated regression coefficients and their significance in the models for percentage dye decolourization and specific dye removal in the optimization study

Term	Percentage dye decolourization (%)				Specific dye removal (mg U^{-1})			
	Coef	SE Coef	*T*	*P*	Coef	SE Coef	*T*	*P*
Constant (*C*)	96.38	1.59	60.716	0.000	0.27720	0.0440	6.307	0.000
Glucose (X_1)	2.00	1.825	1.099	0.280	−0.0510	−0.0505	1.011	0.320
Veratryl alcohol (X_2)	32.95	1.825	18.053	0.000	−0.18	0.0505	−3.549	0.001
KH_2PO_4 (X_3)	3.73	1.825	2.042	0.050	−0.078	0.0505	−1.545	0.132
$MgSO_4$ (X_4)	4.60	1.825	2.522	0.017	−0.014	0.0505	−0.273	0.786
$CaCl_2$ (X_5)	0.51	1.825	0.281	0.781	−0.107	0.0505	−2.114	0.043
X_1^2	1.85	3.735	0.494	0.625	0.150	0.1034	1.454	0.156
X_2^2	−41.15	3.735	−11.02	0.000	0.141	0.1034	1.360	0.184
X_3^2	−20.30	3.735	−5.436	0.000	0.297	0.1034	2.871	0.007
X_4^2	−10.33	3.735	−2.766	0.009	0.197	0.1034	1.902	0.067
X_5^2	−6.64	3.735	−1.779	0.085	0.220	0.1034	2.118	0.042
X_1 X_2	2.6	5.050	0.515	0.611	0.109	0.1398	0.782	0.440
X_1 X_3	−5.21	5.050	−1.032	0.310	0.056	0.1398	0.400	0.692
X_1 X_4	−5.78	5.050	−1.144	0.261	0.118	0.1398	0.844	0.405
X_1 X_5	−5.60	5.050	−1.109	0.276	0.176	0.1398	1.258	0.218
X_2 X_3	16.45	5.050	3.267	0.003	0.364	0.1398	2.609	0.014
X_2 X_4	0.96	5.050	0.190	0.851	0.195	0.1398	1.399	0.172
X_2 X_5	13.71	5.050	2.715	0.011	0.285	0.1398	2.039	0.050
X_3 X_4	−2.61	5.050	−0.518	0.608	0.358	0.1398	2.562	0.015
X_3 X_5	−8.58	5.050	−1.699	0.099	0.059	0.1398	0.422	0.676
X_4 X_5	−12.45	5.050	−2.465	0.019	0.276	0.1398	1.971	0.058

Coef coefficient, *SE Coef* coefficient of standard error

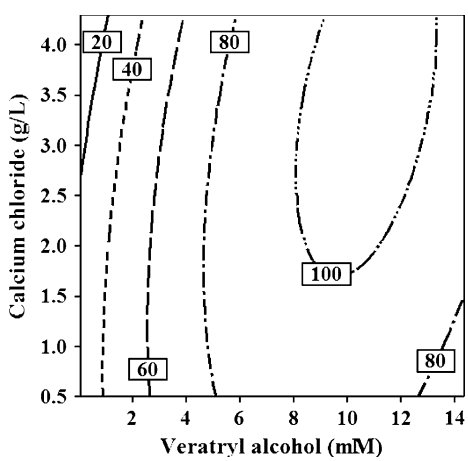

Fig. 2 2D contour plot showing interaction between calcium chloride and veratryl alcohol on the dye decolourization efficiency by *P. chrysosporium*

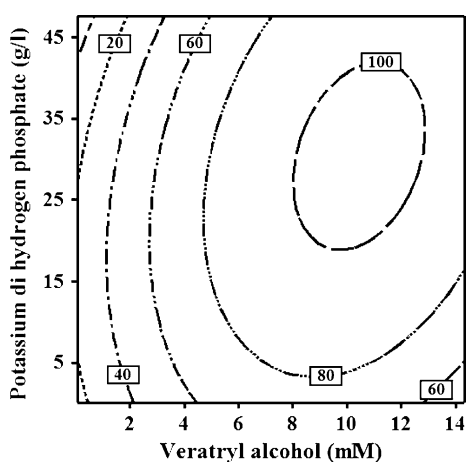

Fig. 3 2D contour plot showing interaction between KH_2PO_4 and veratryl alcohol on the dye decolourization efficiency by *P. chrysosporium*

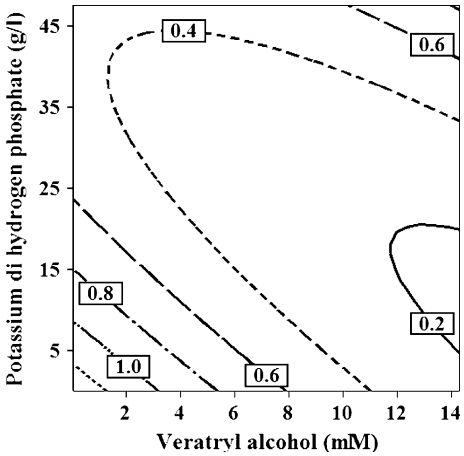

Fig. 4 2D contour plot showing interaction between KH_2PO_4 and veratryl alcohol on specific dye removal by *P. chrysosporium*

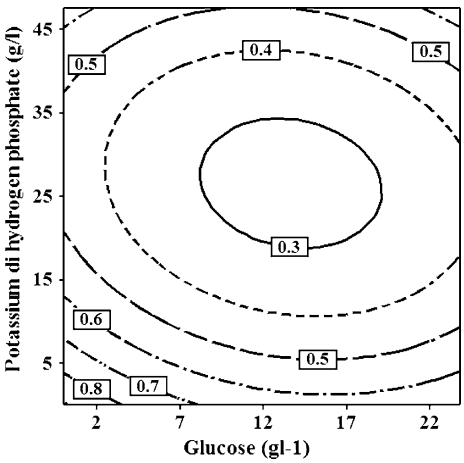

Fig. 5 2D contour plot showing interaction between KH_2PO_4 and glucose on specific dye removal by *P. chrysosporium*

close agreement with those found from the response surface contour plots (Figs. 2, 3, 4 and 5). Further, maximum predicted percentage dye decolourization and specific dye removal were found from the surface confined in the smallest curve of the contour diagrams. Experimental validation of the results were performed at the optimized settings of the media constituents in batch shake-flasks, and the corresponding percentage dye decolourization and specific dye removal were found to be 99.98% and 0.24 mg U^{-1} respectively, which perfectively matched with the predicted values of 99.99% of dye decolourization and 0.23 mg U^{-1} of specific dye removal.

Overall, the study not only gave good insight into the effect of various media constituents on DR-80 dye decolourization and LiP activity by the fungus, but also proved helpful in significant enhancement in dye decolourization by the fungus.

Conclusion

The present study clearly demonstrated enhancement in DR-80 decolourization efficiency by *P. chrysosporium* following screening and optimization of media constituents. Based on the Plackett-Burman design for screening the media constituents, glucose, $MgSO_4$, KH_2PO_4, veratryl alcohol and $CaCl_2$ were found to be the most influential factors affecting the percentage dye decolourization and specific dye removal due to maximum enzyme activity by the fungus. At the RSM optimized levels of the media constituents, *P. chrysosporium* showed complete (100%) dye decolourization efficiency with a specific DR-80 removal 0.24 mg U^{-1} due to its maximum LiP activity.

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