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Decolorization of different azo dyes by *Phanerochaete chrysosporium* RP78 under optimal condition

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ABSTRACT: Detoxification of synthetic dyes is one of the main challenges in clearing textile industry wastes. Biodegradation of azo-dyes using *Phanerochaete chrysosporium* is one the most environmentally friendly methods available. The main enzymes responsible for mycodecolorization process are lignin and manganese peroxidases. Here, optimization of expression conditions has been carried out with manipulating culture condition and nutrient sources. Therefore, the effects of buffer and temperature as well as nitrogen source on lignin peroxidase and manganese peroxidase production were investigated at two levels and four levels, respectively. For this purpose, *P. chrysosporium* RP78 based on Taguchi design of experiment has been applied. Maximum lignin and manganese peroxidase activities of 182 ± 2.5 U/L and 850 ± 41 U/L were obtained under predicted optimum conditions, respectively. Thereby, about 100 % decolorization was achieved after 24 h for two most widely used groups of azo dyes in textile industry consisting reactive and acidic. The physical adsorption of the azo dyes by mycelia was not significant which indicated that the enzymatic degradation of the dyes was occurred. Time profile of these enzymes showed that manganese peroxidase was peaked on 9th day while lignin peroxidase peaked on 13$^{th.}$ day and remained stable in the culture. The extracellular expression profiles of both were studied by 2 dimensional gel electrophoresis to partially characterize the enzymes.

Keywords: *Biodegradation; Lignolytic enzymes; Phanerochaete chrysosporium; Taguchi method*

INTRODUCTION

A great variety of synthetic dyes are used for textile dying and other industrial applications. The structural diversity of dyes derives from the use of different chromophoric groups (e.g., azo, anthraquinone, triarylmethan, and phthalocyanine groups) and different application technologies (e.g., reactive, direct, disperse and vat dying) (Heinfling *et al.*, 1998; Bandyopadhyay and Chattopadhyay, 2007). Approximately, 10,000 different dyes and pigments are used industrially and over 7×10^5 tons of these dyes are produced annually worldwide (Spadaro *et al.*, 1992). Physical and chemical technologies for decolorization of textile effluents are very expensive and commercially unattractive (Beydilli *et al.*, 1998). Biodegradation is an alternative to these technologies which is more cost-effective, environmentally friendly and do not produce large quantity of sludge (Azmi *et al.*, 1998; Gueu *et al.*, 2007). Azo dyes which are used extensively in many industries are the largest class of synthetic dyes with a wide variety of color and structure (Minussi *et al.*, 2001; Samarghandi *et al.*, 2007; Gharbani *et al.*, 2008). Most of these compounds are highly resistant to microbial attack and therefore they are hardly removed from effluents by conventional biological wastewater treatments, such as activated sludge (Cripps *et al.*, 1990; Heinfling *et al.*, 1998; Igbinosa and Okoh, 2009). The lignin-degrading white rot fungus *P. chrysosporium* mineralizes a wide variety of priority aromatic pollutants (Spadaro *et al.*, 1992; Heinfling *et al.*, 1998; Yadav *et al.*, 2006; Okafor and Opuene, 2007). This microorganism produces various isoforms of extracellular oxidases and peroxidases, which are involved in the degradation of lignin in their natural lignocellulosic substrates (Sato *et al.*, 2007). The first lignolytic peroxidases were isolated from *P. chrysosporium* and called as lignin peroxidase (LiP)

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and manganese peroxidase (Heinfling *et al.*, 1998). Many studies were performed on the production of lignolytic enzymes by *P. chrysosporium* because of the dependence of this enzymatic system on nutrient conditions (Dosoretz *et al.*, 1993; Naidu *et al.*, 2003; Ambrósio and Campos-Takaki, 2004; Sato *et al.*, 2007). Time profiles of these enzymes under different conditions were studied to find the time corresponding to the maximum enzyme activities which is different from one fungus to another (Dosoretz and Grethlein, 1991; Dosoretz *et al.*, 1993). The expression profile of extracellular proteins from *P. chrysosporium* growing on solid and liquid substrates has been recently analyzed by two-dimensional gel electrophoresis (Sato *et al.*, 2007).

Statistical methods of design of experiments are known by DOE as useful tools help to gain more information about the optimum conditions in a few trials (Montgomery, 2001). Among all DOE methods, Taguchi method, a kind of fractional factorial design, involves establishment of large number of experimental situations described as orthogonal arrays to reduce experimental errors and to enhance their efficiency and reproducibility of the laboratory experiments (Krishna Prasad *et al.*, 2005). The effect of the culture conditions consisting O_2 and nitrogen and carbon source limitations on the regulation of LiP and MnP was studied (Dosoretz and Grethlein, 1991). However, there are no reports available on the application of Taguchi method to investigate the effect of culture parameters on lignolytic enzymes in the field of biodecolorization.

In the present study, an attempt has been made to obtain the culture conditions in which the *P. chrysosporium* showed the highest LiP activity in order to degrade seven azo dyes from four different groups. The experiments were designed based on Taguchi method. Furthermore the expression profile of two main peroxidases, LiP and MnP during the decolorization of reactive orange 16 has also been investigated.

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical grade and purchased from Sigma and Merck Company and dyes were obtained from Sigma-Aldrich Company.

Microorganism and culture maintenance

The microorganism *Phanerochaete chrysosporium* RP78 was obtained from Forest Products Laboratory, Madison, WI, USA through Center for Biotechnology, Acharya Nagarjuna University, India and was maintained on potato dextrose agar at 30 °C. Subcultures were routinely made every 30 days.

Preparation of spore inoculum

Spore production required 6 days growth on the PDA medium. Spores were prepared by suspension in sterile water. The spores were separated from mycelia by centrifuging at 5000g for 5 min. Spore concentration was determined by measuring absorbance at 650 nm (an absorbance of 1.0/cm is approximately 5×10^6 spores/mL) by spectrophotometer (Beckman DU530, USA).

Medium and optimization methodology

According to Taguchi's orthogonal array L8, eight experiments were used to evaluate the effect of three variables on the production of the lignolytic enzymes (Table 1). The decision on the levels of these components was based on literature data. The factors and their levels were as follows: temperature (30°C, 37°C), buffer (acetate and sodium succinate, 20 mM), and ammonium tartrate as nitrogen source (1.2, 12, 24 and 48 mM). The composition of the medium was varied according to the experimental plan. The constant constituents of the medium consisted of 56 mM glucose, 1.5 mM veratryl alcohol and mineral salts and 1 mg/L thiamin at pH 4.5 as described previously (Tien and Kirk, 1988). Stationary liquid cultures were established by inoculating the 20 mL media with 0.1 mL fungal spores with absorbance of 0.5 at 650 nm. The cultures were prepared in 250-mL flask equipped with inlet and outlet and flushed with pure oxygen at the time of inoculation and then every three days (Tien and Kirk, 1988).

Biodegradation tests

The optimized cultures of *P. chrysosporium* were allowed to grow for 8 days at 30 °C. On day 8, one of the dyes was added to each culture. The dye stock solutions were prepared in distilled water at 0.5 mg/mL and were added to give final concentration of 40.483, 25.206, 34.736, 62.4, 30.09, 100.5 and 54.182 µM for Reactive Orange 16, Reactive Black 5, Direct Violet 51, Acid Red 88, Acid Red 114, Basic Orange II and Bismarck Brown R, respectively. The final dye concentration of 24 mg/L was considered for mycodecolorization studies based on the previous data (Naidu *et al.*, 2003; Gao *et al.*, 2006). Dye

disappearance was detected by spectrophotometer (Beckman DU530, USA) at or near the absorption maximum for each dye at 24 h intervals for 5 days. Results were reported as the mean values of decolorization percent for three replicates.

Enzymatic assays

Lignin peroxidase activity was measured according to the method of Tein and Kirk (1988). One unit of activity (U) represents 1 µM veratryl alcohol oxidized to the aldehyde per min at room temperature. Manganese peroxidase (MnP) activity was measured by spectrophotometery (Beckman DU530, USA) in presence of Mn^{2+} as a substrate (Paszczynski *et al.*, 1988)

Electrophoresis

Equal volumes of extracellular fluid were precipitated with trichloroacetic acid (TCA) and subjected to SDS-PAGE (Laemmli, 1970) and also 2D gel electrophoresis as previously described (O'Farrells, 1975). Then the gels were stained with silver nitrate.

RESULTS AND DISCUSSION

Biodegradation of widely used synthetic dyes in textile industry especially azo ones by *P. chrysosporium* has been investigated previously (Cripps *et al.*, 1990; Goszczynski *et al.*, 1994; Beydilli *et al.*, 1998; Naidu *et al.*, 2003). The lignin degrading system of white-rot fungi is consisted of various extracellular enzymes such as laccases, peroxidases and oxidases (Shah and Nerud, 2002). Therefore, they are able to degrade a wide range of organic pollutants e.g. phenolic compounds and synthetic dyes (Leatham *et al.*, 1983; Cripps *et al.*, 1990; Minussi *et al.*, 2001). The ability of *P. chrysosporium* to degrade a lot of synthetic dyes has been reported (Shah and Nerud, 2002). This is because, this white-rot fungus is able to produce a family of extracellular glycosylated heme-containing lignin peroxidases. These ligninase isoenzymes are highly homologous, with molecular weight ranging from 38-46 KDa and Pi ranging from 3.3-4.9 (Chaudhry, 1994). Expression of lignolytic enzymes by *P. chrysosporium* is idiophathic, occurs under nitrogen, carbon or sulfur limitation and is particularly active at high O_2 tension (Dosoretz *et al.*, 1993). LiP was considered as the main component among these enzymes and the effect of nitrogen limitation on its production was investigated. Furthermore, LiP synthesis under different temperatures with two buffers was simultaneously studied using Taguchi experimantal design. The obtained data from eight Taguchi trials were analyzed using Design Expert software (version 6.0.10, Stat-Ease Inc., USA) for LiP activity as response (Table 1). From the calculated ratios (*F*), it can be referred that the factors considered in the experimental design are statistically significant at 95 % confidence limit. The results showed that all three factors (ammonium tartrate concentration, temperature and type of buffer) and two interactions were significant (Table 2). The ANOVA of LiP activity has the model *F*-value of 450.17 and Prob > F lower than 0.05 that implies it is significant and can be used to navigate in the design space. The model also indicated that the multiple correlation coefficient of R^2 is 0.999 i.e. it can explain 99 % variation in the response. Furthermore, the predicted R^2 of 0.975 is in reasonable agreement with the adjusted R^2 of 0.997. The model shows standard deviation, mean, C.V. and predicted residual sum of square values of 2.85, 52.75, 5.40 and 463.93, respectively. The software proposed the following equations for prediction and description of the responses according to the actual values of the factors.

Eq. 1 while succinate was as buffer:

$$\text{LiP Activity} = -287.974 - 4.906 \times A + 14.179 \times B \quad (1)$$

Eq. 2 while acetate was as buffer:

Table 1: The factors and their levels according to L8 Taguchi orthogonal array

Trial No.	A: nitrogen source (mM)	B: Temp (°C)	C: buffer	LiP activity U/L
1	1.2	30	succinate	3.9±129.0
2	1.2	37	acetate	1.9±27.0
3	12.0	30	succinate	21.0±81.0
4	12.0	37	acetate	11.0±40.0
5	24.0	30	acetate	0.0
6	24.0	37	succinate	21.0±120.0
7	48.0	30	acetate	13.5±25.0
8	48.0	37	succinate	0.0

Table 2: ANOVA results for Taguchi orthogonal array L_8

Source	Sum of squares	DF	Mean Square	F value	Prob. > F
Model	18279.26	5	3655.85	450.17	0.0022
A	2550.01	1	2550.01	314.00	0.0032
B	3290.46	1	3290.46	405.17	0.0025
C	3469.19	1	3469.19	427.18	0.0023
A×C	6182.74	1	6182.74	761.32	0.0013
B×C	313.56	1	313.56	38.61	0.0249
Residual	16.24	2	8.12		
Cor Total	18295.50	7			

$$\text{LiP activity} = -250.670 + 1.068 \times A + 7.489 \times B \quad (2)$$

Where, A is ammonium concentration in the culture medium and B is temperature.

According to Taguchi analysis the maximum LiP activity of 230 ± 39 U/L at the 99 % interval was predicted at 37 ºC in the presence of succinate buffer and 1.2 mM ammonium tartrate (Fig. 1). Therefore, the validation test was carried out in triplicates and LiP activity of 182 ± 2.5 U/L was achieved.

The effect of initial nitrogen concentration on substrate consumption, LiP and MnP synthesis by *P. chrysosporium* has been studied (Dosoretz *et al.*, 1993). The result indicated that MnP and then LiP were appeared in direct response to nitrogen starvation under nitrogen limitation condition, but they were synthesized simultaneously after complete utilization of carbon source under sufficient and excess nitrogen conditions. However the LiP activity was seven times more than the activity observed under nitrogen limitation conditions (Dosoretz *et al.*, 1993). On the other hand, it has been reported that the gradual increase of nitrogen concentration resulted in the decrease of both enzymes (Cripps *et al.*, 1990; Chaudhry, 1994). MnP was active just under the nitrogen limitation condition (trials 1 and 2) and by increasing the nitrogen concentration, its activity completely disappeared. In these two nitrogen limitation conditions (trial 1 and 2); the maximum MnP activities of 850 ± 41 UL and 600 ± 37 U/L were obtained, respectively. The regression models represent the ammonium concentration has a negative effect on LiP activity while succinate is as buffer. It is in agreement with the previous finding (Cripps *et al.*, 1990; Chaudhry, 1994). As shown in Fig. 1, the higher activity was obtained at higher temperature (from 30 to 37 °C) in the presence of each buffer. But the maximum activity was achieved while succinare applied. In literature, both buffers have been applied in lignolytic media (Dosoretz and Grethlein, 1991; Sato *et al.*, 2007). Succinate has two carboxylic groups while acetate has one. The carboxylic group appears to be directly affected by lowering the pH of the solution. With decreasing pH, redox potential increased and LiP is able to oxidize its substrate (Oyadomari *et al.*, 2003). The optimum pH for LiP activity is 4.2. LiP is stable at 34ÚC and pH 4.2 for 100 h (Couto *et al.*, 2006). Therefore, it seems that succinate buffer is able to supply the optimum pH for Lip activity and stability rather than acetate buffer.

Daily assay for LiP and MnP under optimal conditions showed that LiP activity was first detected on day 6 and peaked on day 13 as 182 ± 2.5 U/L. MnP activity was also detected on day 6 and peaked on day 9 up to 850 ± 41 U/L. This pattern of the synthesis of the lignolytic enzymes is in agreement with previous reports under limiting nitrogen conditions (Dosoretz *et al.*, 1993). However, this culture remained lignolytic at least through day 15 (Fig. 2). Therefore, the azo dyes were added on day 8 in which both peroxidases were active in order to investigate their decolorization (Fig. 2). The effective mycodecolorization of 5 azo dyes (acid red 114, acid red 88, direct violet 51, reactive orange 16 and reactive black 5) related to three groups of azo dyes (acidic, direct and reactive) was observed. In this condition, more than 99 % of acid red 88, reactive black 5 and reactive orange 16 were degraded in 24 h while, acid red 114 and direct violet 55 were decolorized after 5 days up to 90 %. The mycelia adsorption was measured for the azo dyes of bismarck brown R, acid red 114 and direct violet 51. It was observed that bismarck brown R was not degraded at all, but adsorbed direct violet 51 and acid red 114 steadily released from mycelia and finally decolorized completely from the culture medium. Some dyes like basic orange II were not decolorized by the lignolytic system of *P. chrysosporium*. The results showed that this strain has a high potential to degrade three groups of azo dyes with one or two azo bonds. It has been reported that disperse and solvent azo dyes are also mineralized by *P. chrysosporium* (Spadaro *et al.*, 1992). The effectiveness of decolorization depends on the

structure and complexity of synthetic dyes (Minussi *et al.*, 2001; Ambrósio and Campos-Takaki, 2004), e. g. a nitro function may retard the mineralization process (Spadaro *et al.*, 1992). However, decolorization occurs when just the chromophore bond is degraded. The situation and availability of this bond in the dye structure affect the efficiency of decolorization. Generally, the larger molecules with weaker bonds are more frigid. Results also showed that the small molecules with strong bonds (e.g. basic orange II) were not affected efficiently by fungal enzymes.

In the presence of different dyes, the extracellular expression profile was studied by SDS-PAGE gel electrophoresis. Fig. 3 showed that there was no significant difference between the patterns of secreted proteins to the culture medium. Three bands were observed in presence and also absence of azo dyes in the medium. It seems that the medium compositions and operational conditions (i.e. no shaking, high aeration) directly affected the expression of lignolytic enzymes.

Two-dimensional gel electrophoresis was employed to characterize the extracellular proteins partially. Three protein spots with MW of 35-45 kDa and pI of 4.2-4.9 were observed in 2D gel electrophoresis (Fig. 4). LiP and MnP activities were also determined and depicted on Fig. 5. Since LiP and

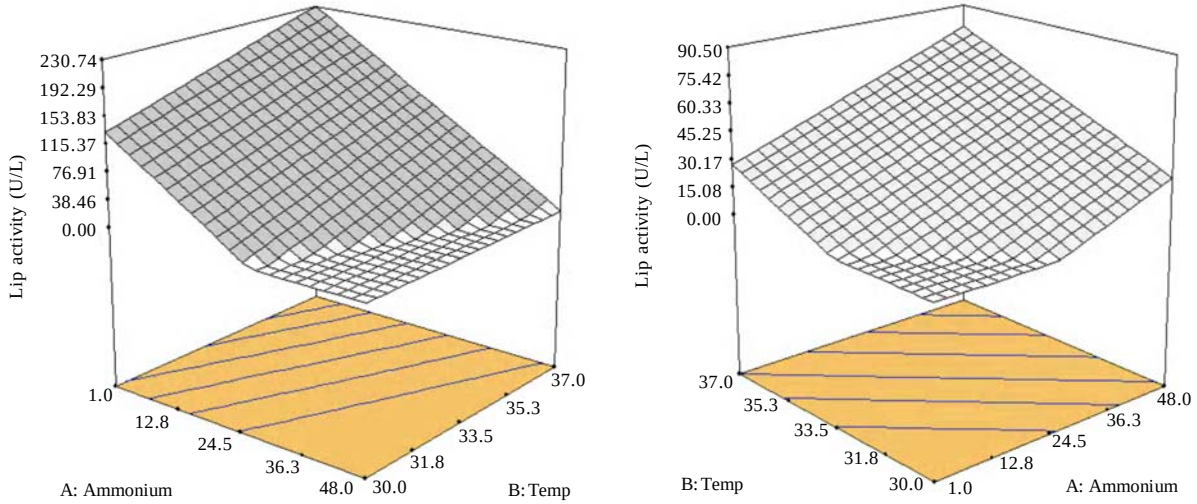

Fig. 1: Three dimensional plan of the LiP activity (U/L) according to different amount of ammonium and temperature in presence of: (i) succinate buffer and (ii) acetate buffer

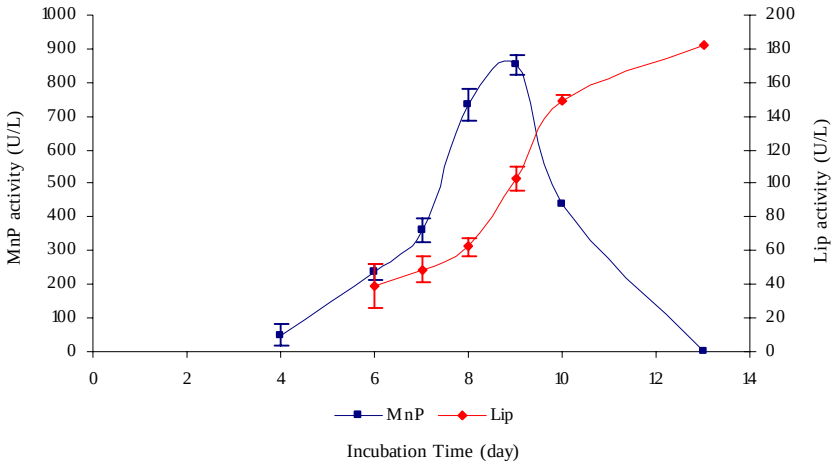

Fig. 2: Time profile of LiP and MnP activities under optimum conditions

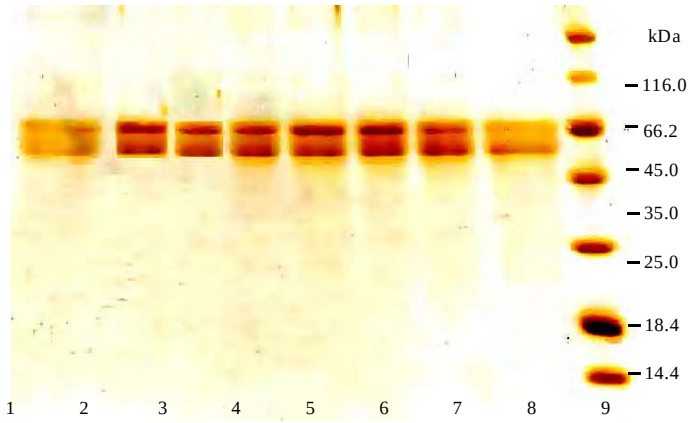

Fig. 3: The extracellular expression profile of the *P. chrysosporium* RP78 in the presence of different azo dyes after 13 days incubation. Lanes 1: without dye, 2: Reactive Orange 16, 3: Reactive Black 5, 4: Direct Violet 51, 5: Bismarck Brown R, 6: Basic Orange II, 7: Acid Red 114, 8: Acid Red 88, 9: Molecular weight marker

Fig. 4: 2D gel electrophoresis of the extracellular protein expression of the *P. chrysosporium* RP78 under optimal conditions

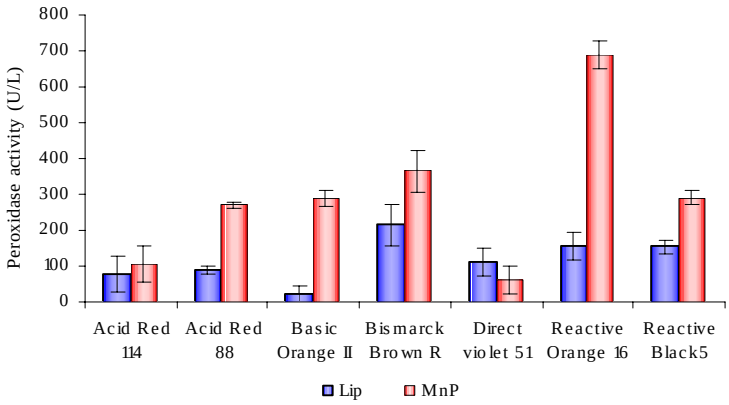

Fig. 5: LiP and MnP activities in the presence of different azo dyes

MnP activities were assayed quantitatively, the spots are probably related to H1-H5 ligninase isoenzymes according to their molecular weights and isoelecteric pH's. The recent works that had been performed under different substrates and growth conditions had identified different patterns for extracellular proteome of *P. chrysosporium* in different media (Sato *et al.*, 2007).

CONCLUSION

Effective mycodecolorization of some azo dyes by *P. chrysosporium* RP78 at the optimum condition has been recorded in the present study. The lignolytic activity was optimized under the nitrogen limitation condition in presence of succinate buffer at 37 °C. It was concluded that the efficiency of dyes decolorization depends on the structure and complexity of azo dyes. The results showed that complex and large structure of these compounds are more sensitive to enzymatic decolorization. Furthermore, the dyes which adsorbed to mycelia required more time for decolorization like Direct Violet 51 and Acid Red 114. SDS-PAGE showed that there is no difference in the extracellular expression pattern in the presence of different azo dyes. Thereupon, it can be concluded that the type of dye has no significant effect on the extracellular enzyme production. Since *P. chrysosporium* RP78 can efficiently decolorize a wide range of textile azo dyes under optimized conditions, further studies on scaling up and using this fungus for treatment of textile waste water are suggested. Meanwhile, this study serves as another example for the application of the Taguchi DOE methodology for improvement of biological process.

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