Desalination and Water Treatment
www.deswater.com

22 (2010) 22–29
October

doi: 10/5004/dwt.2010.1489

Screening of factors affecting reactive blue 19 decolorization by *Ganoderma* sp. using fractional factorial experimental design

Mehran Mohammadian Fazli[a], Ali Reza Mesdaghinia[a,*], Kazem Naddafi[a], Simin Nasseri[a], Masoud Yunesian[a], Mahnaz Mazaheri Assadi[b], Sasan Rezaie[c], Hossein Hamzehei[d]

[a]*Department of Environmental Health Engineering, School of Public Health and Center for Environmental Research, Tehran University of Medical Sciences, Tehran, Iran*
Tel. +98-21-88094036; e-mail: mhrnmoh@gmail.com
[b]*Biotechnology Center, Iranian Research Organization for Science and Technology, Tehran, Iran*
[c]*Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*
[d]*Research Laboratory, Zanjan University of Medical Sciences, Zanjan, Iran*

Received 12 August 2009; Accepted 8 April 2010

ABSTRACT

Synthetic dyes are extensively used in different industries; however, they have adverse impacts on human health and the environment. White rot fungi are capable of degrading dyes and various xenobiotics. In order to detect the important factors affecting decolorization, experimental screening is usually conducted to optimize the process. In the present investigation, fractional factorial design was used for identifying factors affecting reactive blue 19 decolorization by *Ganoderma* sp. among 10 initial factors. Implemented factors considered include media components and operating conditions of color removal process. Depicted results showed that experimental design is the appropriate approach to determine the main and interaction effects. The ANOVA showed that the significant main and interaction effects include temperature, type of carbon and energy source, pH and carbon source concentration. Screening procedure suggested that the optimization of decolorization process focused on glycerol concentration around 20 g/l as carbon source, temperature around 30 °C, and pH around 6. Other factors and their levels included yeast extract 0.4 g/l, copper sulfate 0.001 g/l, dye concentration 100 mg/l, ethanol 2%, volume of inoculation 5 ml and shaking speed 150 rpm.

Keywords: Dye; Decolorization; *Ganoderma* sp.; Screening experiments; Fractional factorial design

1. Introduction

Synthetic dyes are extensively used in many industries and the discharge of colored effluents has concerned both industrial and academic scientists [1]. Over 10000 dyes with a total annual production in excess of 7×10^5 tones worldwide are commercially available, and typically 5 to 10 percent of this amount is discharged as industrial effluents [2]. Dye-containing effluents are hardly decolorized by conventional biological treatments. In addition to their visual and adverse impact in terms of chemical oxygen demand, some synthetic dyes cause allergy, dermatitis, skin irritation and they are toxic, mutagenic and carcinogenic in humans [3–5]. Reactive blue 19 (Fig. 1), is an anthraquinone dye that constitutes the second most

*Corresponding author.

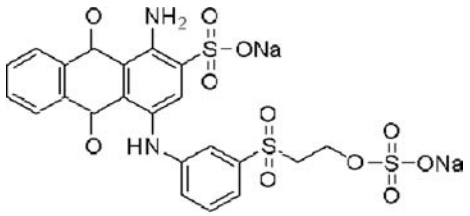

Fig. 1. Chemical structure of reactive blue 19.

important class of textile dye after azo dyes. It is an important class of toxic and recalcitrant organopollutant and usually used as a model dye for decolorization studies [4,6–7].

White rot fungi (WRF) are the most efficient ligninolytic organisms capable of degrading various types of dyes such as azo, heterocyclic, reactive and polymeric dyes. This capability is due to extracellular non-specific enzyme systems composed of lactases, lignin peroxidases and manganese peroxidases. This ligninolytic system of WRF is directly involved in the degradation of various xenobiotic compounds and dyes. It has been frequently reported that laccase is the main enzyme of *Ganoderma* sp. [8–12]. Use of WRF is the most common technology of bioremediation as their ability to degrade structurally diverse xenobiotic organopollutants is more efficient than other remediation technologies. Thus, more technically advanced research efforts are required for identifying and exploiting new fungal species and improvement of practical application to propagate the use of fungi for bioremediation of industrial effluents and contaminated soils [2,3,11,13–15].

There are many variables or factors that affect enzyme production and decolorization by different white rot fungi. These features are important in the process design and optimization of fungal treatment of effluents [3].

Statistical design of experiments refers to the process of planning the experiments so that appropriate data that can be analyzed by statistical methods will be collected, resulting in valid and objective conclusions. This technique has been applied for the enhancement of culture conditions and media composition and for various fermentation processes in biotechnology. The statistical approach to screening experiments are usually conducted in the early stages of a study, when it is likely that some of the factors initially considered have little or no effect on the process or there are some likely interactions between factors. The information from this screening or characterization experiment will be used to identify the critical process factors and to determine the direction of adjustment for these factors to reduce future experiments. The screening design also provides information about which factors should be more carefully controlled during optimization process. Typically, screening experiments make use of fractional factorial design, such as two-level fractional design [16,17].

The present work was undertaken to screen significant factors affecting reactive blue 19 (RB19) decolorization by *Ganoderma* sp. through two-level fractional factorial design of experiments.

2. Materials and methods

2.1. Microorganism

The organism used in this study, *Ganoderma* sp., was purchased from Persian Type Culture Collection (PTCC), Iranian Research Organization for Science and Technology, Tehran, Iran. The stock cultures were maintained on potato dextrose agar (PDA) slants at 4 °C and subcultured at monthly intervals [18].

2.2. Chemicals

All chemicals, including culture media, dye and enzyme substrate, were purchased from Merck and Sigma-Aldrich companies.

2.3. Growth conditions

Precultures were prepared in 250 ml capacity flasks containing 150 ml potato dextrose broth (PDB). Flasks were autoclaved at 121 °C for 15 min at 15 psi, cooled and inoculated by fungal mycelia, which were grown on PDA for 12 days. Inocula were prepared by washing the mycelia from surface of a PDA slant, and by addition of 15 ml sterile distilled water [19]. Inoculated flasks were incubated at 28 °C for 4 days at 150 rpm to obtain fungal pellets with 1–3 mm. Five milliliters of pellets under suspension condition were used to inoculate each main culture flask containing 45 ml Basal slt medium and 150 mg/l RB19 [20]. The initial concentration of biomass was 1.5 ± 0.165 mg/l in each flask.

The basal medium, to determine fungal growth, contained (g/l of distilled water): glucose 20, yeast extract 2.5, KH_2PO_4 1, Na_2HPO_4 0.05, $MgSO_4 \cdot 7H_2O$ 0.5, $CaCl_2$ 0.01, $FeSO_4 \cdot 7H_2O$ 0.01, $MnSO_4 \cdot 4H_2O$ 0.001, $ZnSO_4 \cdot 7H_2O$ 0.001 and $CuSO_4 \cdot 5H_2O$ 0.002. The pH was adjusted to 5.5 [8].

2.4. Analytical methods

2.4.1. Color removal measurement

Color as American Dye Manufacturer Institute (ADMI) value was measured according to EPA Method 110.1 [21]. This method is an extension of the Tristimulus

filter method. Tristimulus values are converted to an ADMI single number color difference, of the same magnitude assigned to platinum-cobalt standards, using the Adams Nickerson Color Difference (DE). Hach DR5000 spectrophotometer was used for ADMI values because standard curves and complex equations have been installed in this instrument [21].

Percentage of decolorization was calculated as follows:

$$\text{Decolorization (\%)} = \left(1 - \frac{ADMI}{ADMI_0}\right) \times 100$$

where $ADMI_0$ is initial solution color and ADMI is final solution color.

2.4.2. Enzyme assay

Laccase activity was measured using 0.216 mM syringaldazine as the substrate. The assay mixture (3 ml) contains 2200 µl of phosphate buffer (pH 6.5), 500 µl supernatant, and 300 µl syrigaldazine solution. The absorbance increase of assay mixture was monitored at 530 nm at environment ambient temperature [22].

2.4.3. Biomass measurement

Dried-weight biomass was measured by gravimetric method through centrifuging submerged culture at 11000 rpm for 10 minutes, followed by incubation of the fungal biomass at 65 °C for 48 h [23].

2.5. Experimental design for screening factors

To elucidate the main effects and two-factor interaction effects, two-level fractional factorial design was used. The experimental design was analyzed using statistical software MiniTab 15.1. Ten factors and two levels for each factor were considered. These factors were selected according to studies which demonstrated and mentioned these factors to be of importance and criteria affecting enzyme production or decolorization [3,8,18,20,23]. Table 1 lists the factors and natural values used in this experiment. By using the software and base on 2^{10-5} fractional factorial design, 32 runs were designed. Table 2 represents the combination matrix of runs and factors. All runs were duplicated.

Table 1
Factors and levels used for screening variables affecting decolorization of RB19 by *Ganoderma* sp.

Factor	Unit	Level	
		Low	High
Type of carbon source	–	glycerol	starch
Carbon source concentration	g/l	20	40
Nitrogen source concentration (Yeast extract)	g/l	0.2	0.4
Temperature	°C	25	30
$CuSO_4$ concentration	g/l	0.001	0.003
Ethanol	V/V	0	2
Inoculum volume	ml	5	10
pH	–	5	6
Shaker speed	rpm	100	150
Dye concentration	mg/l	100	200

3. Results and discussion

3.1. Preliminary decolorization investigation

To study the time course of color removal and fungal growth rate, 13 duplicated samples of basal medium incorporated to 150 mg/L RB19 dye were inoculated by 5 ml suspension of fungal mycelium pellets as mentioned above. Percent of color removal and laccase activity by *Ganoderma* sp. are depicted in Fig. 2. In the basal medium, maximum color removal was achieved after 5 days, and was equal to 75.4%. Decolorization rate of RB19 based on first order equation obtained $k = -0.009\ h^{-1}$, which indicates a half-life of 3.2 days at 28 °C. Another study reported that the half-life of hydrolyzed RB19 is about 46 years at pH 7 and 25 °C [4]. It has been reported that *Pleurotus ostreatus* at the end of 48 h incubation period, decolorized 16.81% and 44.99% of 20 mg/l and 100 mg/l initial RB19 concentrations respectively. However, approximately 90 mg/l of 100 mg/l RB19 was decolorized by *F. trogii* at the end of 48 hours [24]. Toh et al. also clearly showed the difference in decolorization ability of different fungi for different dyes [25]. Kirby et al. reported that *Phlebia tremellosa* took 14 days to achieve 57.5–100% decolorization in diverse range of synthetic dyes [26]. On the other hand, Fig. 2 shows increase of laccase activity, which means it is responsible for dye decolorization. Teerapatsakul reported that *Ganoderma* sp. has high laccase activity in the removal of lignin and it improves by optimization [18]. Silva et al. decolorized RB19 by two *Ganoderma* species and reported that the enzyme responsible for decolorization in both fungi is laccase [11].

Fig. 3 shows laccase production starts during the secondary growth phase of fungus. Merwe has reported various cultivation parameters that influence laccase production and activity. These factors include carbon limitation, nitrogen source and concentration and microelements [27]. Further, Heinzkill and Wesenberg report ligninolytic systems of WRF were mainly activated during secondary metabolic phase and triggered by nitrogen concentration or when carbon or sulfur became limiting [3,28]. Teerapatsakul observed that

Table 2
Ten factors in two levels fractional factorial design for screening factors affecting decolorization of RB19 by *Ganoderma* sp.

Run	Factors										
	Carbon type	Carbon concentration	Nitrogen concentration	Shaking speed	Temperature	$CuSO_4$ concentration	Ethanol v/v	Inoculum volume	pH	Dye concentration	Color removal, %
1	glycerol	20	0.2	100	25	0.003	2	10	6	200	92.07
2	starch	20	0.2	100	25	0.001	0	5	5	200	88.18
3	glycerol	40	0.2	100	25	0.001	0	5	6	100	95.81
4	starch	40	0.2	100	25	0.003	2	10	5	100	71.11
5	glycerol	20	0.4	100	25	0.001	0	10	5	100	94.74
6	starch	20	0.4	100	25	0.003	2	5	6	100	97.47
7	glycerol	40	0.4	100	25	0.003	2	5	5	200	95.38
8	starch	40	0.4	100	25	0.001	0	5	6	200	87.28
9	glycerol	20	0.2	150	25	0.001	2	5	5	100	94.14
10	starch	20	0.2	150	25	0.003	0	10	6	100	90.13
11	glycerol	40	0.2	150	25	0.003	0	10	5	200	92.83
12	starch	40	0.2	150	25	0.001	2	5	6	200	87.86
13	glycerol	20	0.4	150	25	0.003	0	10	6	200	96.77
14	starch	20	0.4	150	25	0.001	2	10	5	200	96.31
15	glycerol	40	0.4	150	25	0.001	2	10	6	100	97.22
16	starch	40	0.4	150	25	0.003	0	5	5	100	72.07
17	glycerol	20	0.2	100	30	0.003	0	5	5	100	93.42
18	starch	20	0.2	100	30	0.001	2	10	6	100	92.52
19	glycerol	40	0.2	100	30	0.001	2	10	5	200	95.84
20	starch	40	0.2	100	30	0.003	0	5	6	200	97.72
21	glycerol	20	0.4	100	30	0.001	2	5	6	200	95.59
22	starch	20	0.4	100	30	0.003	0	10	5	200	96.92
23	glycerol	40	0.4	100	30	0.003	0	10	6	100	97.00
24	starch	40	0.4	100	30	0.001	2	5	5	100	97.31
25	glycerol	20	0.2	150	30	0.001	0	10	6	200	93.78
26	starch	20	0.2	150	30	0.003	2	5	5	200	94.74
27	glycerol	40	0.2	150	30	0.003	2	5	6	100	98.65
28	starch	40	0.2	150	30	0.001	0	10	5	100	96.63
29	glycerol	20	0.4	150	30	0.003	2	10	5	100	94.84
30	starch	20	0.4	150	30	0.001	0	5	6	100	95.14
31	glycerol	40	0.4	150	30	0.001	0	5	5	200	93.26
32	starch	40	0.4	150	30	0.003	2	10	6	200	95.76

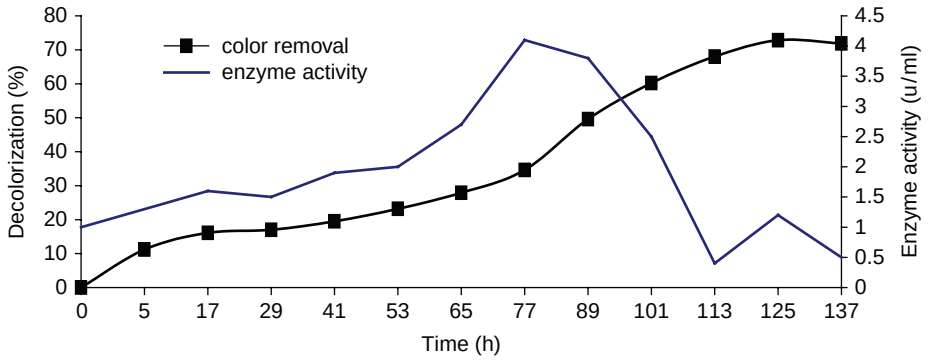

Fig. 2. Color removal and laccase activity in basal medium by Ganoderma sp.

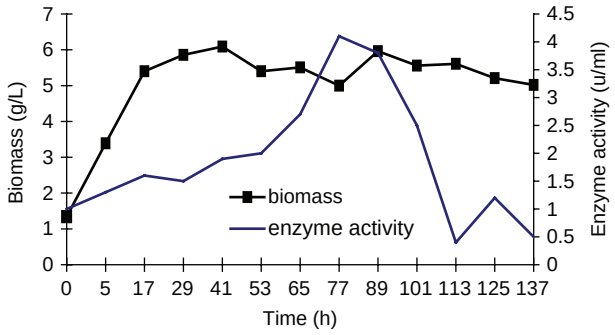

Fig. 3. *Ganoderma* sp. growth curve and laccase activity in basal medium.

laccase production occurred on day 5 and in the secondary phase. The maximal laccase production, 20 U/ml, was observed on day 9 at a later time but a greater quantity than in this study [18]. Further, the growth of fungus was studied as first order equation, and the specific growth rate was 0.113 h^{-1} equivalent to a 6.1 h generation time.

3.2. Screening factors through experimental design

The statistical combination of the factors and the decolorization measurements are shown in Table 2 for the total 32 sets of experiments. The color removal assay was performed after 5 days. The minimum and maximum decolorization was 71.11 and 98.65% respectively. These results demonstrate that *Ganoderma* sp. has good potential to be used for color removal. However, the aim of these experiments was to investigate main effects and two factor interactions that influence the rate of color removal. Table 3 shows the analysis of variance for color removal. It was found that both main effects and two-factor interactions are significant ($P < 0.0001$). Important and significant factors and two factor interactions in terms of estimated effect and P value are presented in Table 4. Temperature, type of carbon source, pH and carbon source concentration are significant but the magnitude of their effects are different. The increase of temperature and pH improve the color removal efficiency, but a decrease of carbon concentration increases decolorization efficiency. Meanwhile, results showed glycerol as a carbon source is better than using starch for color removal. These findings were similar to those reported by Teerapatsakul but were in contrast to Revankar's finding that starch is better [8,18]. Ethanol as inducer improved decolorization, but was not significant. While Merwe has reported ethanol under 4% level resulted in a significant increase in laccase production by *Pycnoporus sanguineus* [27].

Table 3
The analysis of variance of screening experimental design for decolorization of RB19 by *Ganoderma* sp.

Source	DF	Seq SS	Adj SS	Adj MS	F	*P*
Main effects	10	993.45	993.45	99.345	12.29	0.0001
2-way interactions	21	1479.18	1479.18	70.437	8.71	0.0001
Residual error	32	258.69	258.69	8.084		
Pure error	32	258.69	258.69	8.084		
Total	63	2731.32				

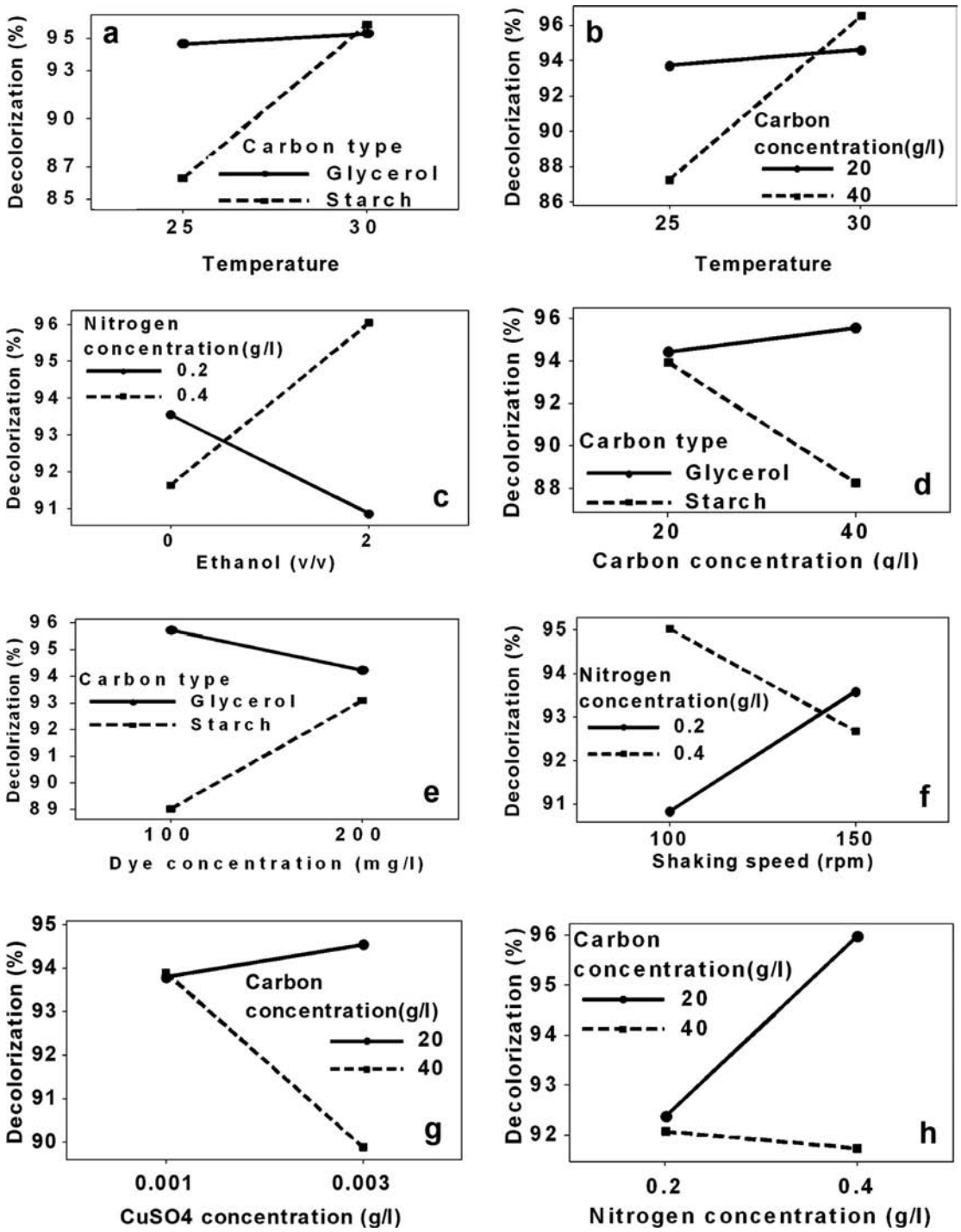

Fig. 4. Important interactions affecting RB19 decolorization by *Ganoderma* sp. a) Type of carbon source × Temperature; b) Carbon source concentration × Temperature; c) Nitrogen source concentration × Ethanol; d) Type of carbon source × Carbon source concentration; e) Type of carbon source × Dye concentration; f) Nitrogen source concentration × Shaker speed; g) Carbon source concentration × CuSO4 concentration; h) Carbon source concentration × Nitrogen source concentration.

Table 4
Factor and interaction effects and significance for screening experimental design for decolorization of RB19 by *Ganoderma* sp.

Term	Effect	*P* value
Temperature	5.078	0.0001
Type of carbon source × Temperature	4.460	0.0001
Carbon source concentration × Temperature	4.183	0.0001
Type of carbon source	–3.920	0.0001
Nitrogen source concentration × Ethanol	3.550	0.0001
Type of carbon source × Carbon source concentration	–3.423	0.0001
pH	2.783	0.0001
Type of carbon source × Dye concentration	2.765	0.0001
Nitrogen source concentration × Shaker speed	–2.553	0.001
Carbon source concentration × $CuSO_4$	–2.387	0.002
Carbon source concentration	–2.285	0.003
Carbon source concentration × Nitrogen source concentration	–1.969	0.009

Eight interactions were significant ($P < 0.01$) and are depicted in Fig. 4. Interactions are both positive (e.g. carbon type × temperature) and negative (e.g. nitrogen concentration × shaking speed). Negative effect of interaction means high levels of factors cause less efficient decolorization [23]. Results obtained in this study supported observations elsewhere because they reported these factors and interactions are important in laccase production or color removal [8,18,20,29]. Wesenberg reported laccase production is often enhanced by agitation but it was not observed in this study [3].

4. Conclusions

Analyzing of data from decolorization of RB19 by *Ganoderma* sp. performed using fractional factorial design experiment, indicated that four factors and eight interactions are important for improving and optimizing color removal process. Statistical analysis method showed that factors, including temperature, type of carbon and energy source, carbon source concentration and pH were significant variables and optimization of process should be based on these factors. Proper media components and operating conditions for optimization is suggested, type of carbon source: glycerol, carbon source concentration: around 20 g/l, nitrogen source concentration: 0.4 g/l, temperature: around 30 °C, pH: around 6, copper sulfate concentration: 0.001 g/l, dye concentration: 100 mg/l, ethanol: 2 volumetric percent, volume of inoculation: 5 ml and shaking speed: 150 rpm.

Acknowledgment

This research was supported by Tehran and Zanjan University of Medical Sciences, which is gratefully acknowledged.

References

[1] N. M. Mahmoodi, M. Arami and K. Gharanjig, Laboratory studies and CFD modeling of photocatalytic degradation of colored textile wastewater by titania nanoparticles, Desalin. Water Treat., 1 (2009) 312–317.
[2] Y. Fuand T. Viraraghavan, Fungal decolorization of dye wastewaters: a review, Bioresource Technol., 79 (2001) 251–262.
[3] D. Wesenberg, I. Kyriakidesand S.N. Agathos, White-rot fungi and their enzymes for the treatment of industrial dye effluents, Biotechnol. Advan., 22 (2003) 161–187.
[4] Dos Santos A.B., F.J. Cervantes and J.B.V. Lier, Review paper on current technologies for decolourisation of textile wastewater: perspectives for anaerobic biotechnology, Bioresource Technol., 98 (2007) 2369–2385.
[5] A.E. Ofomaja, Equilibrium sorption of methylene blue using mansonia wood sawdust as biosorbent, Desalin. Water Treat., 3 (2009) 1–10.
[6] A. Rezaee, M.T. Ghaneian, A. Khavanin, S. J. Hashemian, Moussavi Gh., Ghanizadeh Gh. and E. Hajizadeh, Photochemical oxidation of reactive blue 19 dye in textile wastewater by UV/$K_2S_2O_8$ process, Iranian J. Environ. Health, Sci. and Eng., 5 (2) (2008) 95–100.
[7] G. Palmieri, G. Cennamo and G. Sannia, Remazol brilliant blue R decolourisation by the fungus Pleurotus ostreatus and its oxidative enzymatic system, Enzyme and Microbial Technol., 36 (2005) 17–24.
[8] M.S. Revankar and S.S. Lele, Synthetic dye decolorization by white rot fungus, *Ganoderma* sp. WR-1, Bioresource Technol., 98 (2007) 775–780.
[9] K. Murugesan, I. Nam, Y. Kim and Y. Chang, Decolorization of reactive dyes by a thermostable laccase produced by *Ganoderma lucidum* in solid state culture, Enzyme and Microbial Technol., 40 (2007) 1662–1672.

[10] T.M. D'souza, C.S. Merritt and C.A. Reddy, Lignin-Modifying enzymes of the white rot basidiomycete *Ganoderma lucidium,* Appl. and Environ. Microbiol., 65(12) (1999) 5307–5313.
[11] C.M.M. Silva, I.S. Melo, P.R. Oliveira, Ligninolytic enzyme production by *Ganoderma* spp., Enzyme and Microbial Technol., 37 (2005) 324–329.
[12] S. Sarnthima and S. Khammuang, Evaluation of dyes decolourisation by the crude enzyme from *Pleurotus sajor-caju* grown on sorghum seed media, Pakistan J. Biological Sciences, 11(1) (2008) 62–67.
[13] M. Tavcar K. Svobodova, J. Kuplenk, C. Novotny A. Pavko, Biodegradation of azo dye R016 in different reactors by immobilized Irpex lacteus, Acta Chim. Slov., 53 (2006) 338–343.
[14] S. Tachibana, Y. Kiyota and M. Koga, Bioremediation of dioxin-contaminated soil by fungi screened from nature, Pak. J. Biol. Sci., 10(3) (2007) 486–491.
[15] A.K. Tripathi, N.S.K. Harsh and N. Gupta, Fungal treatment of industrial effluents: a mini-review, Life Sci. J., 4(2) (2007) 78–81.
[16] D.C. Montgomery, Design and analysis of experiments, John Wiley & Sons, 2001, pp. 9,303,427–510.
[17] Z. Zanirun, S. Abd-Aziz, F.T. Ling and M.A. Hassan Optimization of lignin peroxidase production using locally isolated *Pycnoporus* sp. through fractional design, Biotechnol., 8(3) (2009) 296–305.
[18] Teerapatsakul Ch., R. Parra, Bucke Ch. and L. Chitradon, Improvement of laccase production from *Ganoderma* sp. KU-ALK4 by medium engineering, World J. Microbial. Biotechnol., 23 (2007) 1519–1527.
[19] M. Azin, R. Moravej and D. Zareh Production of xylanase by *Trichoderma longibrachiatum* on a mixture of wheat bran and wheat straw: Optimization of culture condition by Taguchi method, Enzyme and Microbial Technol., 40 (2008) 801–805.
[20] Park Ch., J. Lim, Y. Lee, B. Lee, S. Kim, J. Lee, S. Kim, Optimization and morphology for decolorization of reactive black 5 by *Funalia trogii,* Enzyme and Microbial Technol., 40 (2007) 1758–1764.
[21] C.M. Kao, M.S. Chou, W.L. Fang, B.W. Liu, B.R. Huang, Regulating colored textile wastewater by 3/31 wavelength admi methods in Taiwan, Chemosphere, 44 (2001) 1055–1063.
[22] J.P. Ride, Physiological plant pathology, 16 (1980)187.
[23] A. L. Demain and J.E. Davis, Manual of industrial microbiology and biotechnology, ASM press, (1999) 57–58, 80–93.
[24] E.A. Erkurt, A. Unyayarand H. Kumbur, Decolorization of synthetic dyes by white rot fungi, involving laccase enzyme in the process, Process Biochemistry, 42 (2007) 1429–1435.
[25] Y. Toh, J.J.Y. Yen, J.P. Obbard and Y. Ting, Decolourisation of azo dyes by white-rot fungi (WRF) isolated in Singapore, Enzyme and Microbial Technol, 33 (2003) 569–575.
[26] N. Kirby, R. Marchantand G. McMullan, Decolourisation of synthetic textile dyes by *Phlebia tremellosa,* FEMS Microbiology Letters, 188 (2000) 93–96.
[27] J.J.V. Merwe, Production of laccase by the white-rot fungus *Pycnoporus saguinus,*. Thesis of Master Sciences, (2002) 16–17.
[28] M. Heinzkill, L.Bech, T. Halkier, P. Schneiderand T. Anke Characterization of laccase and peroxidase from wood-rotting fungi, Appl. Environ. Microbiol., 64(5) (1998) 1601–1606.
[29] R. Gayazov and J. Rodakiewicz-Nowak, Semi-continuous production of laccase by *Phelebia radiate* in different culture media, Folia Microbiol., 41(6) (1996) 480–484.