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Effect of Nutrients on the Production of Extracellular Enzymes for Decolorization of Reactive Blue 19 and Reactive Black 5

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Abstract Several white-rot fungi are able to produce extracellular lignin-degrading enzymes such as manganese peroxidase (MnP), lignin peroxidase (LiP), and laccase. In order to enhance the production of laccase and MnP using *Trametes versicolor* KCTC 16781 in suspension culture, the effects of major medium ingredients, such as carbon and nitrogen sources, on the production of the enzymes were investigated. The decolorization mechanism in terms of biodegradation and biosorption was also investigated. Among the carbon sources used, glucose showed the highest potential for the production of laccase and MnP. Ammonium tartrate was a good nitrogen source for the enzyme production. No significant difference in the laccase production was observed, when glucose concentration was varied between 5 g/l and 30 g/l. As the concentration of nitrogen source increased, a lower MnP activity was observed. The optimal C/N ratio was 25 for the production of laccase and MnP. When the concentrations of glucose and ammonium tartrate were simultaneously increased, the laccase and MnP activities increased dramatically. The maximum laccase and MnP activities were 33.7 U/ml at 72 h and 475 U/ml at 96 h, respectively, in the optimal condition. In this condition, over 90% decolorization efficiency was observed.

Key words: *Trametes versicolor*, laccase, manganese peroxidase (MnP), enzyme, decolorization

Color is the most obvious indicator of water pollution in textile wastewater. Very low concentration of dye (less than 1 ppm for some dyes) is sufficient to be detectable and cause for environmental concern. Several chemical and physical decolorization treatment methods have been used to deal with the diverse textile effluents, but these technologies are still inefficient in the removal of color. In addition, they are not economical and applicable to the treatment of various kinds of dye wastewaters and aromatic compounds [3, 23]. The biotechnological method has been considered as the most suitable alternative for the treatment of textile effluent, due to its potential for achieving total color removal, easy application, low cost, and eco-friendliness [1, 6, 10, 15].

White-rot fungi produce various isoforms of extracellular lignin-degrading enzymes including lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase. These enzymes have attracted wide attention because of their ability to partially or completely degrade recalcitrant organic pollutants such as chlorophenols, nitrotoluenes, and polycyclic aromatic hydrocarbons [14]. Laccase (benzenediol: oxygen oxidoreductases; EC 1.10.3.2), multicopper enzymes belonging to the blue oxidases, catalyzes the one-electron oxidation of various organic and inorganic substrates, including mono-, di-, and polyphenols, aminophenols, methoxyphenols, aromatic amines, and ascorbate, with concomitant four-electron reduction of oxygen to water [11]. MnP catalyzes the oxidation of Mn(II) to Mn(III), which in turn can oxidize several phenolic substrates [21].

These enzymes render phenolic compounds less toxic by means of polymerization reactions and cross-coupling of pollutant phenols with naturally occurring phenols. Earlier researchers have proposed a mechanism for the degradation of azo dyes with simple structures: Peroxidase or Mn(III)-malonate complex oxidizes the phenolic ring of the dyes

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with electrons to produce a carbonium ion on the carbon bearing the azo linkage. Water attacks the carbonium ion to cause hydrolytic cleavage of the azo linkage [20]. These enzymes have become important as industrially relevant enzymes, because of a number of diverse applications for biocatalytic purposes such as delignification of lignocellulosics and cross-linking of polysaccharides, for bioremediation applications such as detoxification of waste and transformation of textile dye, for food technological uses, for medical care applications, and for biosensor and analytical applications [9].

In a previous work, we reported that white-rot fungi could efficiently decolorize several dyes, based on the solid and liquid experiment, and showed the possibility of potential application of major enzymes for decolorization [17]. In this study, we examined the effects of different carbon and nitrogen sources on the production of laccase and MnP, and their optimal concentrations were determined. In order to find the decolorization mechanism, the trends of biodegradation and biosorption were investigated under optimal condition. The results are expected to help determine the design and operation parameters for an effective dye and textile wastewater treatment process.

MATERIAL AND METHODS

Microorganisms and Culture Conditions

T. versicolor KCTC 16781 was used in this study [16, 17]. Mycelia were raised and stored on 1.5% agar plates with PDA (potato dextrose agar) medium. For inoculation, mycelia blocks were cut from a plate culture. Ten blocks were placed in 20-ml plates with glucose dextrin yeast peptone (GDYP) medium containing glucose, dextrin, yeast extract, peptone, KH_2PO_4, and $MgSO_4$, and incubated for 6 days at 28°C. After cultivation, the mycelia were harvested from plate, and homogenized at 2,000 rpm for 30 sec. In order to activate the strain, the mycelia were cultivated in the potato dextrose broth (PDB) for 3 days. Eight ml of the activated strain under the suspension condition were used to inoculate each flask, which contained 80 ml of Kirk's Basal Salts medium with 5 g/l of glucose, 0.22 g/l of ammonium tartrate, 0.2 g/l of KH_2PO_4, 0.05 g/l of $MgSO_4 \cdot 7H_2O$, 0.01 g/l of $CaCl_2$, 0.001 g/l of thiamin·HCl, and 10 ml of trace elements, but without tween solution and veratryl alcohol. Various carbon sources (glucose, sucrose, fructose, glycerol, lactose, and starch) and nitrogen sources (yeast extract, $NaNO_3$, peptone, asparagine, ammonium tartrate, tryptone, and ammonium nitrate) were used to obtain an optimal medium. Different concentrations of glucose (5–30 g/l) and ammonium tartrate (0.22–1.32 g/l) were tested to determine the optimal concentration for the enzyme production. Reactive blue 19 (RB 19) and reactive black 5 (RB 5) were added to the flask, and pH was adjusted to 4.5 using 2,2'-dimethyl succinic acid. Trace element solution had the following composition (g/l of distilled water): $CuSO_4 \cdot 7H_2O$, 0.08; H_2MoO_4, 0.05; $MnSO_4 \cdot 4H_2O$, 0.07; $ZnSO_4 \cdot 7H_2O$, 0.043 and $Fe_2(SO_4)_3$, 0.05. The culture flasks were shaken at 120 rpm in a shaking incubator for 3 days at 28°C under aerobic condition.

Analytical Methods

Samples were withdrawn from the culture at appropriate time intervals, centrifuged (10,000 rpm, 15 min), and the supernatant was analyzed for pH, glucose concentration, decolorization, and ligninolytic enzyme production. Dye decolorization was measured photometrically at maximum wavelength of each dye using UV/Visible spectrophotometery (Bio-Tek Instruments, Milano, Italy), and was calculated from the decrease of absorbance of maximum peak for each dye. The wavelengths showing maximum absorbance for RB 5 and RB 19 were 594 nm and 600 nm, respectively. In addition, in order to investigate the decolorization by a biosorption mechanism, the fungus was centrifuged, mixed with methanol by vortexing for 1 min, and then ultrasonicated (Branson, CT, U.S.A.) at 42 kHz for 30 min. The supernatant was then analyzed.

Laccase (EC. 1.10.3.2) activity was determined by oxidation of 4.47 mM syringaldazine in McIlvaine buffer (0.1 M citric acid and 0.2 M sodium phosphate, pH 4.6). The reaction mixture (3 ml) consisted of 2.5 ml of McIlvaine buffer (pH 4.6) containing 500 µl of culture filtrate. The reaction was initiated by adding 10 µl of syringaldazine (4.47 mM) solution. The oxidation rates of syringaldazine to quinone were photometrically measured at 525 nm at 40°C (ε=65,000 M^{-1} cm^{-1}). The results are expressed in unit defined as mol of quinone formed from syringaldazine per min [7]. MnP (EC 1.11.1.13) activity was determined by the oxidation of 2,2'-azinobis(3-ethyl-6-benzothiazoline sulfonic acid) (ABTS) in 100 mM sodium lactate buffer (pH 4.5) [13]. One milliliter sample mixture consisted of 500 µl of 50 mM sodium lactate buffer containing 0.2 mM $MnSO_4$, 80 µg/ml ABTS, and 500 µl of 100 µM H_2O_2. The reaction at room temperature was initiated by the addition of 10 µl of culture filtrate. The increase of absorbance was measured at 420 nm using ε_{420} of 36,000 $M^{-1}cm^{-1}$ to calculate MnP activity. One unit of MnP oxidizes 1 µmol of ABTS per min. LiP (EC 1.11.1.14) activity was assayed, based on the oxidation of azure B [2]. The reaction mixture contained 1 ml of 125 mM sodium tartrate buffer (pH 3.0), 500 µl of 0.16 mM azure B, 500 µl of the culture filtrate, and 500 µl of 2 mM H_2O_2. The reaction was initiated at 24°C by adding H_2O_2, and one unit of the enzyme activity was defined as the amount of enzyme to decrease 0.1 unit absorbance at 651 nm per min per ml of the culture filtrate.

RESULTS AND DISCUSSION

Effect of Carbon and Nitrogen Sources on the Production of Laccase and MnP

In the previous study, *T. versicolor* KCTC 16781 was selected as a good strain that could decolorize several dyes and produce useful enzymes for decolorization [16, 17]. In order to optimize the parameters of enzyme production with *T. versicolor* KCTC 16781, different culture conditions with various carbon and nitrogen sources were tested, because carbon sources could influence the formation of biomass and the production of primary or secondary metabolites [5]. As shown in Fig. 1, *T. versicolor* KCTC 16781 was able to produce laccase on 5 g/l of all carbon sources tested, but the enzyme activities were different from each other: Glucose and lactose were efficiently and rapidly utilized by the fungus, and they resulted in the high levels of laccase activity (approximately 23–28 U/ml) and MnP activity (approximately 80–92 U/ml). The maximum laccase activity (approximately 28 U/ml) was obtained after 4 days with lactose as carbon source, whereas other carbon sources such as glucose, fructose, and glycerol showed relatively lower enzyme activities (approximately 15–24 U/ml). The maximum MnP activity (approximately 85 U/ml) was obtained using glucose, and lactose and fructose also resulted in good activities (78–83 U/ml). Glucose was chosen as the readily consumed carbon source for the next test, because activities of laccase and MnP with glucose showed values similar to lactose. In addition, it is cheaper than other nutrients and can be used as an available carbon source. From a similar point of view, several carbon sources have been studied as the effective co-substrate on the decolorization of cotton bleaching effluent by an unidentified white-rot fungus, and the results show that glucose, starch, maltose, and cellobiose were good carbon sources, whereas sucrose, lactose, xylan, xylose, methanol, and glyoxal were not proper carbon sources [25].

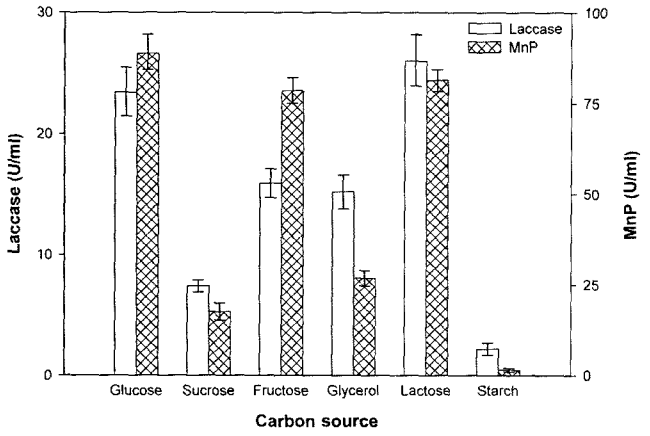

Fig. 1. Effects of various carbon sources on the production of laccase and MnP.

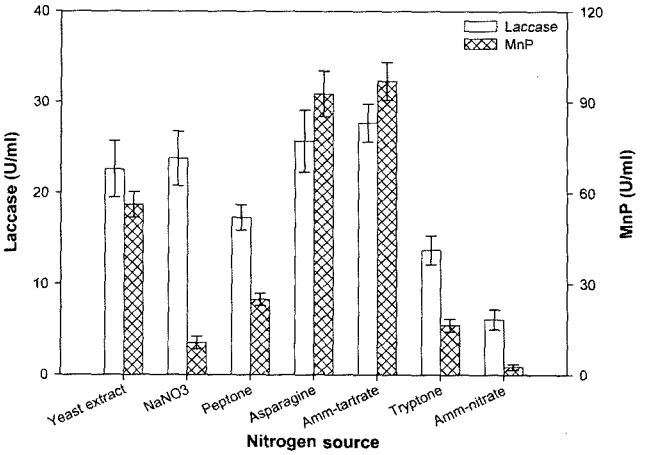

Fig. 2. Effects of various nitrogen sources on the production of laccase and MnP.

Seven nitrogen sources (yeast extract, $NaNO_3$, peptone, asparagines, ammonium tartrate, tryptone, and ammonium nitrate; 0.22 g/l) were also tested for more efficient enzyme production with *T. versicolor* KCTC 16781, and the results obtained are shown in Fig. 2. When yeast extract, asparagine, and ammonium tartrate were used, laccase and MnP activity showed higher values than the other sources, and ammonium tartrate was chosen as the best nitrogen source for the production of laccase and MnP. Their nature and concentration employed have also been shown to be of considerable importance [12].

Effects of Carbon and Nitrogen Concentration on the Production of Laccase and MnP

Among the nutritional factors, sugar plays a significant role in controlling secondary metabolism. The effects of glucose concentrations (5–25 g/l) on the enzyme production were investigated. As listed in Table 1, no significant differences on the enzyme production were observed when glucose concentration varied between 5 g/l and 25 g/l. With the increase of glucose concentration, the cell growth increased, but the enzyme activity did not. An increase of glucose concentration (up to 25 g/l) did not further increase MnP activity, but showed somewhat lower activity (93 U/ml), indicating that the high glucose concentration may repress MnP production. Glucose repression can occur in fungi and yeasts and it is thought to be an energy-saving response [19]. Cell growth increased when the glucose concentration increased, but the laccase production was not associated with cell growth. The enzyme production was also distinctly affected by the concentration of nitrogen source. According to the increase of ammonium tartrate concentration (from 0.22 g/l to 1.50 g/l), laccase and MnP activities gradually decreased from 24.8 U/ml to 14.4 U/ml, and from 43.4 U/ml to 29.7 U/ml, respectively. The regulatory mechanisms in controlling the use of nitrogen sources in bacteria, yeasts,

Table 1. Effects of glucose and ammonium tartrate concentrations on the enzymes production at 96 h.

Glucose conc. (g/l)	Enzyme activity (U/ml)		Ammonium tartrate conc. (g/l)	Enzyme activity (U/ml)	
	Laccase	MnP		Laccase	MnP
5	30.6±2.5	137.6±5.8	0.22	24.8±1.3	43.4±3.2
10	32.8±2.4	105.3±7.3	0.44	22.8±1.9	53.1±2.6
15	32.2±3.8	113.2±5.5	0.88	19.0±0.6	30.6±1.9
20	31.6±2.1	103.3±3.4	1.00	15.0±1.1	34.8±2.6
25	31.8±1.6	93.0±8.4	1.50	14.4±2.1	29.7±1.9

and molds are now partially understood: Ammonia (or some readily used nitrogen source) represses enzymes involved in the use of other nitrogen sources, and these enzymes include nitrite reductase, nitrate reductase, nicotinamide adenine dinucleotide (NAD)-dependent glutamate dehydrogenase, arginase, ornithine transaminase, extracellular protease, acetamidase, threonine dehydrase, allantoinase, and those enzymes dealing with purine degradation, transport of urea and glutamate, and histidine usage [8].

Once the amount of one ingredient was fixed, the more addition of the other carbon or nitrogen sources did not affect the enzyme activity. However, under the optimal ratio (25:1) of carbon and nitrogen, the increase of the substrate concentration could enhance the enzyme activity. Therefore, the effect of simultaneous increase of glucose and ammonium tartrate concentration on the enzyme production was investigated. Glucose and ammonium tartrate concentrations containing Kirk's medium were 5 g/l and 0.22 g/l, respectively. As shown in Table 2, when the concentration of glucose and ammonium increased 5-fold, the activity of laccase increased 1.5-fold and that of MnP increased 2-folds. Laccase activity reached the maximum value at 72 h and then decreased owing to the shortage of the substrate or the consumption of glucose with the ratio of 5:0.22 of glucose and ammonium tartrate. Increase of carbon and nitrogen concentration maintained the high laccase activity until the final stage of cultivation. The high concentration of glucose and ammonium tartrate (30 g/l and 1.32 g/l) resulted in a dramatic increase of MnP concentration. The maximum value of the MnP activity was over 450 U/ml. Based on the result, the optimal concentration of glucose/ammonium tartrate was determined as 30.0 g/l and 1.32 g/l, respectively (Table 2). It has also been reported that the increase of glucose concentration from 10 g/l to 40 g/l resulted in 5-fold increase of the laccase production in *T. pubescens* [11].

Decolorization of Reactive Dyes Under Optimal Medium

The ability of *T. versicolor* KCTC 16781 to decolorize reactive dyes was assessed under the optimal medium that was shown to be favorable for the enzyme production. Table 3 shows the decolorization of RB 5 and RB 19 vs. the different initial concentrations (100 mg/l, 300 mg/l, and 500 mg/l). RB 19 (100 mg/l) was completely decolorized within 24 h, and 300 mg/l and 500 mg/l of RB 19 was nearly decolorized (over 99%) within 72 h. Over 95% of decolorization were observed during the early cultivation period (24 h).

Decolorization of RB 5 started relatively slowly than RB 19 decolorization: Approximately 65% of RB 5 (100 mg/l and 300 mg/l) were removed after 48 h, and 95% decolorization was observed after 96 h. Those results suggest that the efficiency and extent of fungal decolorization of media may drastically be reduced at RB 5 concentrations exceeding 300 mg/l. Reduction of color removal rate may result from toxicity of the dyes to fungus through the inhibition of metabolic activities, decreased growth rate, and inadequate biomass concentration for the uptake of higher quantities of dyes [22]. It has been reported that azo dye concentration of 300 mg/l is able to exert toxic effect on the white-rot fungus *Phanerochete chrysosporium* [18]. The negative effect on the rates of ligninase-catalyzed decolorization can happen, because higher concentrations of dyes inhibit the substrate [24].

LiP was not detected with and without of the dye-containing cultures. Table 4 shows the maximum enzyme activities of laccase and MnP as a function of different initial concentrations. Low levels of MnP and laccase activities

Table 2. Relationship between enzyme production and glucose and ammonium tartrate concentration.

Glucose:Ammonium tartrate (g/l:g/l)	Laccase activity (U/ml)		MnP activity (U/ml)	
	72 h	96 h	72 h	96 h
5:0.22	21.0±0.9	3.5±0.5	66.9±4.5	11.0±1.2
10:0.44	32.5±1.5	16.5±0.9	141.2±7.6	53.0±3.2
15:0.66	30.0±2.1	28.5±1.2	122.5±8.2	118.5±6.4
20:0.88	30.5±2.8	28.0±1.9	170.5±5.6	384.0±10.8
25:1.10	32.7±1.4	25.6±1.4	160.0±7.2	387.7±9.8
30:1.32	36.8±3.1	31.7±2.7	142.0±9.9	475.4±8.8

Table 3. Decolorization with different initial concentrations.

	Decolorization at time (%)			
	24 h	48 h	72 h	96 h
100 mg/l of RB 19	100	100	100	100
300 mg/l of RB 19	98.2	98.8	99.1	99.2
500 mg/l of RB 19	96.9	98.5	99.0	99.1
100 mg/l of RB 5	58.5	66.6	85.4	96.9
300 mg/l of RB 5	52.2	64.8	82.9	93.9
500 mg/l of RB 5	48.5	63.4	74.0	86.9

Table 4. Maximum enzyme activity with different initial concentrations.

	Maximum activity at time (U/ml)	
	Laccase	MnP
100 mg/l of RB 19	24.6 at 72 h	466.4 at 96 h
300 mg/l of RB 19	25.7 at 60 h	638.9 at 60 h
500 mg/l of RB 19	19.1 at 60 h	613.0 at 60 h
100 mg/l of RB 5	26.3 at 84 h	821.3 at 84 h
300 mg/l of RB 5	21.9 at 60 h	578.7 at 96 h
500 mg/l of RB 5	19.4 at 60 h	593.0 at 84 h

were detected in RB 5-containing cultures in the early cultivation time, and these enzyme activities peaked at about 84 h, at which time about 26.0 U/ml (100 mg/l, RB 5) was detected. MnP activity continuously increased until the final stage of cultivation. During decolorization of three RB 5 concentrations, laccase and MnP increased gradually. However, since RB 19 rapidly decolorized over 95% of the dye within 24 h, it is quite apparent that the enzymes were dramatically produced and reached the maximum levels in 60–72 h. The decolorization of RB 19 was more active than that of RB 5 owing to high enzyme activity.

The mechanisms for decolorization, including biodegradation and biosorption, were investigated. Decolorizations of RB 19 and RB 5 with *T. versicolor* KCTC 16781 were accompanied by major changes in their absorption spectra. In the case of RB 19, there was a rapid decrease of the O.D. peak at 595 nm. Likewise, in cultures containing RB 5, there was a continuous decrease and shift in absorbance peak from 594 to about 510 nm [17]. This suggests that dye transformation took place through a series of intermediates, although it should be noted that mineralization did not take place, as indicated by the absorbance peak in the UV region. During the reaction, biodegradation by enzyme and biosorption by cell were observed as the different mechanism for decolorization. In order to confirm that adsorption of dye to the mycelium takes place, the filtered mycelia of cultures incubated with dyes was resuspended in methanol, extracted, and centrifuged. Color was not detected in the extracted supernatant of all concentrations of RB 19. The lowest concentration of RB 5 (100 mg/l) did not show any adsorption, whereas color was detected in the extracted supernatant of 300 mg/l and 500 mg/l of RB 5. It is thought that the high concentration of RB 5 inhibited the growth of fungi, decreasing the activity of fungi. Then, the adsorption occurred at that point: In the case of 100 mg/l of RB 5, color change was clearly visible throughout the experiment. During 2 days, it changed into a dark purple color, which changed several tones until it became a light red wine color at 4 days. About 67% of decolorization was achieved within 2 days, and increased to about 90% after 84 h.

The decolorization mechanism with *T. versicolor* KCTC 16781 after 96 h is shown in Table 5. We found that over 80% of 100 mg/l of RB 19 was removed within the first few hours, and the maximum value was attained in about 12 h, which indicates that a small amount of enzymes was able to react very fast. Although a higher concentration of RB 19 was added, *T. versicolor* KCTC 16781 could effectively decolorize over 99% of the dye without biosorption in the range of dye concentrations used. The decolorization mechanism of RB 5 is thought to be due to biodegradation and biosorption: The fungal strain was not able to grow any more owing to high initial dye concentration (≥300 mg/l). After 4 days, about 8–11% adsorption was observed in the range of between 85% and 90% color removal. Other study of color adsorption by *T. versicolor* mycelium reported that the adsorption accounted for only 5–10% of the total color removal [4].

T. versicolor KCTC 16781 is an efficient fungal strain to produce an enzyme for decolorization, and two major enzymes can be obtained in very high yield with glucose and ammonium-based medium. Although biosorption does not sometimes happen, the investigation on the decolorization mechanism by *T. versicolor* KCTC 16781 indicated that it proceeded primarily by biodegradation, accompanied with a minor biosorption on the cell surface. The reaction depends greatly on the complex reaction of enzymes and the different structure of dyes. For more efficient decolorization, various factors have to be considered, such as development of good

Table 5. Comparison of the decolorization mechanism of different dyes at 96 h.

Dyes / Mechanism	RB 19 (mg/l)			RB 5 (mg/l)		
	100	300	500	100	300	500
Biosorption (%)	0	0	0	0	11.3	8.7
Biodegradation (%)	100	99.2	99.1	96.9	82.6	78.2
Decolorization (%)	100	99.2	99.1	96.9	93.9	86.9

strains, observation of fungal growth, increase of enzyme activity, investigation of decolorization mechanisms, study of different dye structures, and so on. Further investigations should be focused on the immobilization of selected enzymes for industrial application of the enzymatic decolorization. By adopting their immobilized forms, the adequate characteristics, including high resistance to thermal denaturation, significant improvement of the enzymatic activity, and their preservation for long periods, would be required to develop practical applications.

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