Journal of Hazardous Materials 187 (2011) 517–525

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Decolorization of synthetic dyes by crude laccase from a newly isolated *Trametes trogii* strain cultivated on solid agro-industrial residue

Xiangkang Zeng[a], Yujie Cai[a], Xiangru Liao[a,*], Xianglong Zeng[a], Wenxiu Li[a], Dabing Zhang[b]

[a] The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, 1800 Lihu Road, Wuxi 214122, Jiangsu Province, China
[b] Hanbon Science & Technology Co. Ltd., Huaian 223001, China

ARTICLE INFO

Article history:
Received 8 August 2010
Received in revised form 14 January 2011
Accepted 14 January 2011
Available online 22 January 2011

Keywords:
Decolorization
Synthetic dyes
Laccase
Solid substrate fermentation
Trametes trogii

ABSTRACT

A new dye-decolorizing white-rot fungus was isolated and identified as *Trametes trogii* based on its ITS-5.8S rRNA gene sequence analysis and morphological characteristics. Laccase was the only lignolytic enzyme produced by this strain during solid substrate fermentation (SSF) in soybean cake, a solid agro-industrial residue used for the first time in enzyme production. The extracellular crude enzyme from *T. trogii* in solid substrate fermentation showed good activity in synthetic dye color removal, decolorizing 85.2% Remazol Brilliant Blue R (50 $mg\,l^{-1}$), 69.6% Reactive Blue 4 (35 $mg\,l^{-1}$), and 45.6% Acid Blue 129 (83.3 $mg\,l^{-1}$) without the addition of redox mediators, 90.2% Acid Red 1 (10 $mg\,l^{-1}$), and 65.4% Reactive Black 5 (18.3 $mg\,l^{-1}$) with the addition of 1 mM 1-hydroxybenzotriazole in 30 min. Native polyacrylamide gel electrophoresis (Native-PAGE) of the crude enzyme and effects of laccase inhibitors on decolorization corroborated the laccase as the major enzyme involved in the decolorization of dyes. The comparison of color removal by the crude culture filtrates and by the whole fungal culture on the solid substrate revealed the former was more advantageous.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Synthetic dyes, which are extensively used in many industries including paper, printing, cosmetics, and pharmaceuticals [1], are a major part of our life [2] and are especially of primary importance to textile industries [3]. Unfortunately, during textile procedures, inefficiencies in dyeing result in large amounts of the dyestuff being directly lost into the wastewater, which ultimately finds its way into the environment [3]. Dyes released by the textile industries pose a threat to environmental safety [4]. Some dyes and/or their degradation products are toxic and potentially carcinogenic [5]. Moreover, effluents from the textile industries containing dyes are highly colored, which reduce the amount of sunlight to photosynthetic organisms, resulting in decreased oxygen levels in aquatic ecosystems [6]. Thus it is necessary to find ecologically efficient solutions for colored textile effluents. Decolorization of these dyes by physical or chemical methods including adsorption and precipitation, chemical degradation or photodegradation is financially and also methodologically demanding, time-consuming and largely ineffective [7]. Recently, decolorization of dyes through biological means, especially through fungi, has gained momentum as they are economical and can be applied to a wide range of dyes [3,7–10]. Among known color-removal fungi, white-rot fungi (WRF) play an important role as they produce various isoforms of extracellular oxidases including laccase, Mn peroxidase (MnP) and lignin peroxidase (LiP), which are involved in the degradation of lignin in their natural lignocellulosic substrates [8]. The broad substrate specificity of these enzymes in ligninolytic systems of WRF makes it possible for them to degrade a variety of recalcitrant compounds and even complex pollutants including dyes.

Nowadays, most studies on dye decolorization by WRF have been demonstrated by using liquid culture conditions. Nevertheless, a main obstacle for the development of such systems is the huge volume of the effluent generated in daily use [11]. Moreover, for broad application, the cost of enzymes is also one of the main factors determining the economic viability of the process. Solid substrate fermentation may solve these problems. Solid substrate fermentation (SSF) is defined as a fermentation process in which the substrate itself acts as carbon/energy source, occurring in the absence or near-absence of free water [12]. Compared with liquid substrate fermentation, solid substrate fermentation can obtain higher product titers with low energy consumption, low volumes and low equipment costs, and most notably without effluents and less pollution [13].

A survey of recent literature reveals many examples of dye decolorization studies using enzymes, however, there has been a scarcity of literature addressing dye removal by enzymes

* Corresponding author. Tel.: +86 0510 85916372.
E-mail addresses: yu_jie_cai@yahoo.com.cn (Y. Cai), liaoxiangru@163.com (X. Liao).

0304-3894/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2011.01.068

produced in solid substrate fermentation. Since the enzymatic system secreted by white-rot fungi depends on strains and conditions, more white-rot fungi need to be screened for their ability to degrade dyes [14]. In addition, agro-industrial residues are generally better substrates for enzyme production in solid substrate fermentation processes [12]. Soybean cake, a food industry coproduct predominantly used as animal feed, is abundant, cheap and needs further exploration into its additional value. Therefore, the object of this study was to isolate new white-rot fungi strains and then use them to produce color-removal lignolytic enzymes in solid soybean cake substrate fermentation. To the best of our knowledge, our study represents the first time dye removal by culture filtrates from solid soybean cake substrate fermentation has been reported.

2. Materials and methods

2.1. Chemicals and raw materials

2, 6-Dimethoxyphenol (DMP), 1-hydroxybenzotriazole (HOBT), sodium azide (NaN_3) and synthetic dyes (Table 1) were all purchased from Sigma–Aldrich. Wood shavings and soybean cake were used as substrates for the growth of fungal mycelia under solid state conditions. Wood shavings were commercial Ono™ products used for feeding small pets. Soybean cake, a coproduct from the food industry, was collected from a local soybean oil factory. It was created when soybeans were cold pressed to extract oil. After dried to constant weight at 60 °C, the soybean cake was milled as soybean cake powder by using a homogenizer at a speed of 10,000 rpm for 2 min.

2.2. Culture media

A potato dextrose agar (PDA) plate containing 1 mM guaiacol was used for strain screening as described by Ryu et al. [15]. The culture medium for preinoculum contained ($g\,l^{-1}$):20 glucose, 5 peptone, and 3 yeast extract. A 50 ml liquid medium as specified by Garzillo et al. [16] in a 250 ml Erlenmeyer flask was used for a liquid culture. The solid substrate fermentation (SSF) culture medium was obtained as follows: in a 250 ml Erlenmeyer flask, 3 g wood shavings and 4 g soybean cake powder was mixed and moistened to 65% (w/w) water content using 13 ml nutrient solution. The basic nutrient solution contained ($g\,l^{-1}$): Glucose 5, $(NH_4)2SO_4$ 15, KH_2PO_4 2, and $MgSO4{\cdot}7H_2O$ 1, $CuSO_4{\cdot}5H_2O$ 0.014 and $CaCl_2$ 0.6. The “one factor at a time” method was used to optimize the medium for laccase production. Medium optimization by the “one factor at a time” method involves changing the independent variable (carbon source, nitrogen source, pH, water content and $CuSO_4{\cdot}5H_2O$ concentration in SSF medium) while fixing the others at certain levels in the basic nutrient solution. All the media were autoclaved at 121 °C for 20 min before inoculation.

2.3. Isolation and identification of fungi

Forty fungal fruit body samples from different groups of basidiomycetes were collected from decayed wood from a local forest in Wuxi, China (31°32′24″N and 120°12′24″E). For culture isolation, fragments of the basidiomata were subjected to surface sterilization with 75% alcohol under aseptic conditions for 30 s, thoroughly washed with sterilized water, followed by inoculation on screening plates, and incubated at 30 °C for several days. Isolated fungal strains were maintained at 4 °C in a PDA medium. The ITS-5.8S rRNA gene sequence of the fungal strain was obtained and used to establish its phylogenetic relationship. Amplification of the region flanking the 5.8S rRNA gene was carried out with universal primers ITS1 and ITS4 [17]. The ITS-5.8S rRNA gene sequence was compared and aligned with sequences deposited in the GenBank database by using CLUSTAL W version 1.8 [18]. Phylogenetic analysis and construction of an unrooted tree were performed by the MEGA version 3.0, and a neighbor-joining tree was constructed by the NEIGHBOR program [19].

2.4. Enzyme production and preparation

Five plugs (7 days of growth on PDA plates in darkness at 30 °C, with diameter of 5 mm) were transferred into a 250 ml shaken flask containing 50 ml preinoculum culture medium with 20 glass beads (diameter of 2 mm) and grown at 30 °C under continuous stirring at 200 rpm for 48 h. Homogeneous inocula (2 ml) were added to the SSF media and the liquid media for enzyme production. SSF culture was grown statically in the dark at 30 °C while liquid culture was at 30 °C, under static and continual agitation (200 rpm) conditions, respectively. For crude enzyme preparation, the substrate after fermentation was weighted followed by the addition of 80 ml distilled water and stirred at 200 rpm at 30 °C for 1 h. At the same time, 1 g substrate was collected and dried to constant mass for about 10 h at 60 °C to determine its water content. Crude enzyme solution from SSF as well as the submerged liquid culture were filtrated through cotton gauze (eight layers) followed by centrifugation (10,000 × g; 10 min) at 4 °C to separate the solids. Crude enzymes were obtained from supernatants and stored at 4 °C.

2.5. Enzyme and protein assays

Laccase (EC 1.10.3.2) activity was measured according to [20], modified by using 10 mM DMP in a citrate-phosphate buffer (20 mM pH 4.0) at 30 °C. Lignin peroxidase (EC 1.11.1.14; LiP) activity was determined using veratryl alcohol as described by Tien and Kirk [21]. Manganese peroxidase (EC 1.11.1.13; MnP) activity was assayed by the oxidation of 1 mM $MnSO_4$ in sodium malonate (50 mM pH 4.5), in the presence of 0.1 mM H_2O_2 [22]. One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 μmol of substrate per min. In liquid culture enzyme activity was expressed as U per milliliter liquid culture medium ($U\,ml^{-1}$) while activity in solid culture was reported as U per gram of dry substrate ($U\,g^{-1}$). Extracellular protein was determined using the Bradford method as specified by Bradford [23].

2.6. In vitro decolorization of dyes by crude enzyme

The decolorization of dyes by crude enzymes was carried out statically in a test tube at 30 °C. Three milliliters of reaction mixture containing citrate-phosphate buffer (20 mM pH 4.0), 0.5 ml crude enzyme (laccase activity 1.1 $U\,ml^{-1}$) and either RBBR (50 $mg\,l^{-1}$), RB 4 (35 $mg\,l^{-1}$), AB 129 (83.3 $mg\,l^{-1}$), AR 1 (10 $mg\,l^{-1}$) or RB 5 (18.3 $mg\,l^{-1}$). Dyes were added to these final concentrations in order to get an initial absorbance below 1.0 at their maximum wavelengths (Table 1). The absorbance of the mixture at maximum wavelength was recorded with a Mapada™ UV-1600 spectrophotometer. Decolorization was calculated by measuring the decrease in absorbance maximum according to the following expression: decolorization (%) = $A_0 - A/A_0 \times 100\%$, where A_0 was the initial absorbance and A was the final absorbance. A control test containing the same amount of a heat-denatured laccase was performed in parallel. The influence of parameters in this process on decolorization, such as laccase activity (0.11–3.3 $U\,ml^{-1}$), temperature (30–80 °C), pH (3–7) and redox mediator (HOBT) was also investigated. To further confirm whether laccase was involved in dye decolorization, laccase inhibitors (L-cystein and NaN_3), both at concentrations of 0.1 and 1 mM, were used to investigate their effects on dye decolorization.

Table 1
Characteristics of dyes used in this study.

Dye	Classification	Molecular Structure	Molecular Weight	λmax (nm)
Remazol Brilliant Blue R (RBBR)	Anthraquinone		626.54	592
Reactive Blue 4 (RB 4)	Anthraquinone		637.43	595
Acid Blue 129 (AB 129)	Anthraquinone		460.48	629
Acid Red 1 (AR 1)	Azo		509.42	506/532[a]
Reactive Black 5 (RB 5)	Azo		991.82	597

[a] Acid Red 1 has two λ_{max}, 506 nm is used for analysis in this study.

2.7. Decolorization of dyes by whole fungal culture

Dye decolorization by whole fungal cultures was performed by injecting RBBR into the nutrient solution of solid substrate fermentation culture media (Section 2.2) at various concentrations (100–500 mg l^{-1}) before autoclaving. The inoculation procedure was the same as in Section 2.4. The toxic effect of dye on fungal growth was investigated by inoculating a 5-mm-diameter PDA plug centrally on PDA plates containing different contents of RBBR (100–500 mg l^{-1}). Plates were incubated in the dark at 30 °C and diameters of fungal mycelium growth and decolorized zone were measured after 4 days.

2.8. Gel electrophoresis and staining

The concentrated crude enzyme samples (precipitated by 80% saturated ammonium sulfate, dissolved in an appropriate amount of 20 mM pH 6.0 citrate-phosphate buffer, and dialyzed overnight) were subjected to polyacrylamide gel electrophoresis on native PAGE (12%). The gels were stained using Coomassie

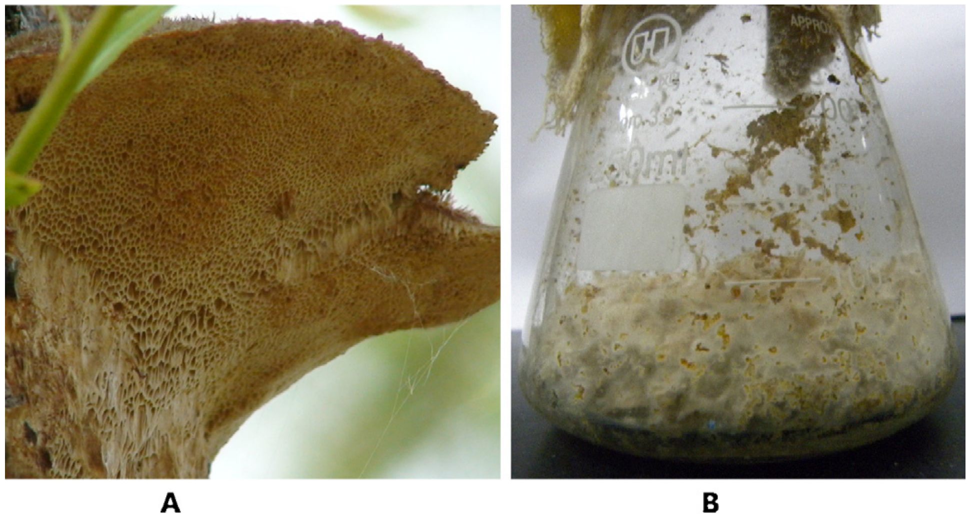


Fig. 1. (a) Morphological characteristics of the fruit body of *Trametes trogii* SYBC-LZ. (b) The growth of strain SYBC-LZ on solid soybean cake and wood shavings substrate.

Brilliant Blue R-250 to visualize protein bands or incubated in a citrate-phosphate buffer (20 mM pH 4.0) containing 5 mM DMP, 200 mg l^{-1} RB 4, or 200 mg l^{-1} RBBR to reveal laccase activity.

2.9. Data analysis

The data were obtained from triplicate experiments, unless mentioned particularly, and analyzed with the SPSS 11.5 software

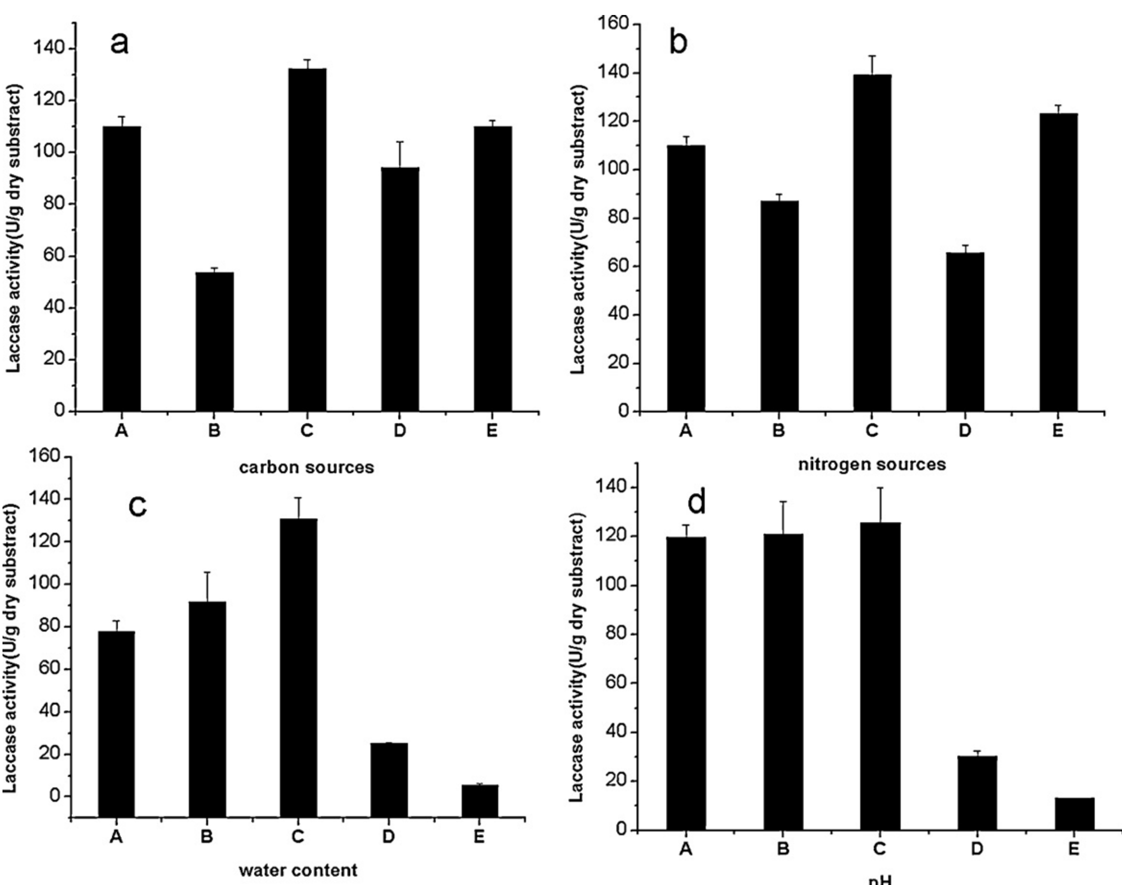


Fig. 2. Effects of nutrient and environmental factors on laccase production by *Trametes trogii* in solid substrate fermentation. (a) Effect of carbon sources on laccase production. From A to E the carbon source is glucose, maltose, sucrose, fructose, and starch. (b) Effect of nitrogen sources on laccase production. The nitrogen source (from A to E) is ammonium sulfate, ammonium chloride, potassium nitrate, ammonium tartrate, and peptone. (c) Laccase production in solid state fermentation with different water content. From A to E the water content is 50%, 55%, 60%, 65%, and 70% (w/w). (d) Laccase production in solid state fermentation with different initial pH values. The initial pH (from A to E) is 3.0, 4.0, 5.0, 6.0, and 7.0. All experiments are done by changing one independent variable while fixing others at certain levels in the basic nutrient solution. Results are significantly different ($p<0.05$).

Table 2
Enzymes produced by *Trametes trogii* strains and their activities.

Strain	Culture mode and medium	Culture time	Maximum activity	Reference
F. trogii 201 (DSM 11919)	SmF (synthetic medium)	10 days	Lac 0.8 U ml^{-1}	[16]
T. trogii (BAFC 463)	SmF (synthetic medium)	23 days	Lac 45.32 U ml^{-1} MnP 214.5 mU ml^{-1} Glox 116 mU ml^{-1}	[24]
T. trogii (BAFC 212)	SmF (synthetic medium)	26 days	Lac 22.75 U ml^{-1} MnP 0.34 U ml^{-1} Glox 0.20 U ml^{-1}	[25]
T. trogii CTM 10156	SmF (olive mill waste water)	12 days	Lac 25.12 U ml^{-1}	[26]
T. trogii strain B6J	SmF (synthetic medium)	10 days	Lac 20 U ml^{-1}	[27]
F. trogii ATCC 200800	SSF (wheat bran and soybean)	10 days	Lac 10.73 CU[a] peroxidase 10.79 CU	[28]
T. trogii BAFC 463	SSF (poplar wood)	43 days	Lac 901 U g^{-1} MnP 20 U g^{-1}	[29]
T. trogii SYBC-LZ	SSF (wood shavings and soybean cake)	6 days	Lac 218 U g^{-1}	

SmF: Submerged Fermentation; SSF: Solid-state fermentation; Lac: laccase MnP: Mn peroxidase GLOX: glyoxal oxidase.

[a] In this article, one unit of laccase and peroxidase activity (colorimetric unit: CU) is defined as the amount of enzyme that caused an increase in absorbance (at 465 nm) of 0.1 h^{-1}.

and the experimental results were expressed as mean ± standard deviation.

3. Results and discussion

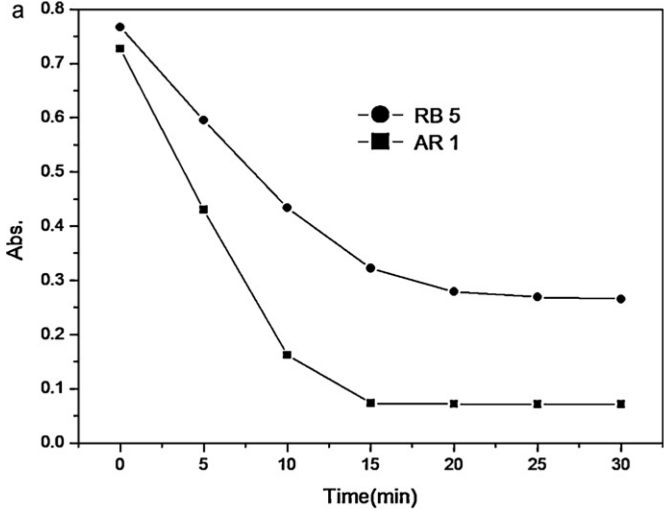
3.1. Isolation and identification of fungi

Four strains were isolated from 40 fungal samples and the molecular analysis was done with all of them. A very promising white rot fungus strain (named SYBC-LZ) was obtained based on the redness around the strain colony and used in this study. Its ITS-5.8S rRNA gene sequence (GenBank accession number HQ000043) with a length of 649 bp was amplified and showed 99% identity with that of two *Trametes suaveolens* strains (strains FJ810184.1, FJ478094.1) and four *Trametes trogii* strains (strains EU790491.1, EU273516.1, EF546236.1, EU661876.1) after BLAST. The morphological characteristics of its fruit body (Fig. 1a) and mycelium were more similar to those of *T. trogii* reported previously. Therefore, this strain was designated as *T. trogii* SYBC-LZ (Fig. 2).

3.2. Laccase production by Trametes trogii SYBC-LZ

The newly isolated strain *T. trogii* SYBC-LZ was cultivated by three culture processes to obtain an optimum method for enzyme production. Enzyme assays were done every 24 h from day 3 after inoculation. Only laccase activity in ligninolytic systems was observed in the media designed in this article. A maximum laccase activity was reached on 6 days after inoculation in solid substrate fermentation (100.0 ± 3.9 U g^{-1}), on 9 days under shaking condition (4.31 ± 0.13 U ml^{-1}), and on 10 days under static condition (1.25 ± 0.05 U ml^{-1}). As a high laccase activity could be obtained 6 days after inoculation under solid substrate fermentation, in this study solid substrate fermentation was selected for laccase production in the following experiments.

Laccase production by fungi might be influenced by critical nutritional factors, such as carbon and nitrogen sources and their concentrations and the ratio between them, as well as other factors, e.g. the nature and concentration of the inducers and conditional indices [4]. In the present study, sucrose was found to be the optimum carbon source for laccase production among the five carbon sources (Fig. 4a). The result that glucose was not the most favorable carbon source for laccase production might be explained by the findings from Galhaup et al. [30], who had demonstrated a repression of laccase gene transcription by glucose. Among the five inorganic nitrogen sources tested here, potassium nitrate was more suitable for producing laccase (Fig. 4b). Copper atoms served as cofactors in the catalytic core of laccase [31]; thus, a minimum concentration (millimolar range) of copper ions was necessary for production of the active enzyme [4]. Also copper ion was demonstrated to be an inducer for laccase in *T. trogii* [37]. However, excess copper had a toxic effect on biomass and thus inhibited laccase production. In this strain, laccase activity (78.7 ± 5.3 U g^{-1}) was rather low without $CuSO_4 \cdot 5H_2O$ in the nutrient solution and the highest level of laccase (219.0 ± 13.0 U g^{-1}) was achieved when $CuSO_4 \cdot 5H_2O$ was 1 mM. The initial moisture and pH of the solid state substrates were of importance for the growth of mycelium. In solid state fermentation, an ideal solid support must sustain colonization and good growth [4]. Appropriate water content (65%) and pH value (3.0–5.0) of the solid substrate made the raw materials in this solid state fermentation become ideal supporters of



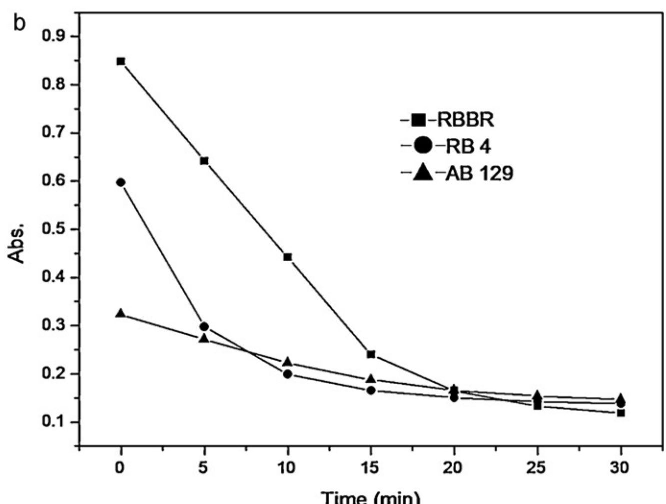


Fig. 3. Absorbance changes of reaction mixture during the color-removal process using crude enzyme produced by *Trametes trogii* SYBC-LZ. (a) Decolorization of AR 1 and RB 5 by crude enzyme with 1 mM HOBT. (b) Decolorization of RBBR, RB 4 and AB 129 by the crude enzyme without HOBT.

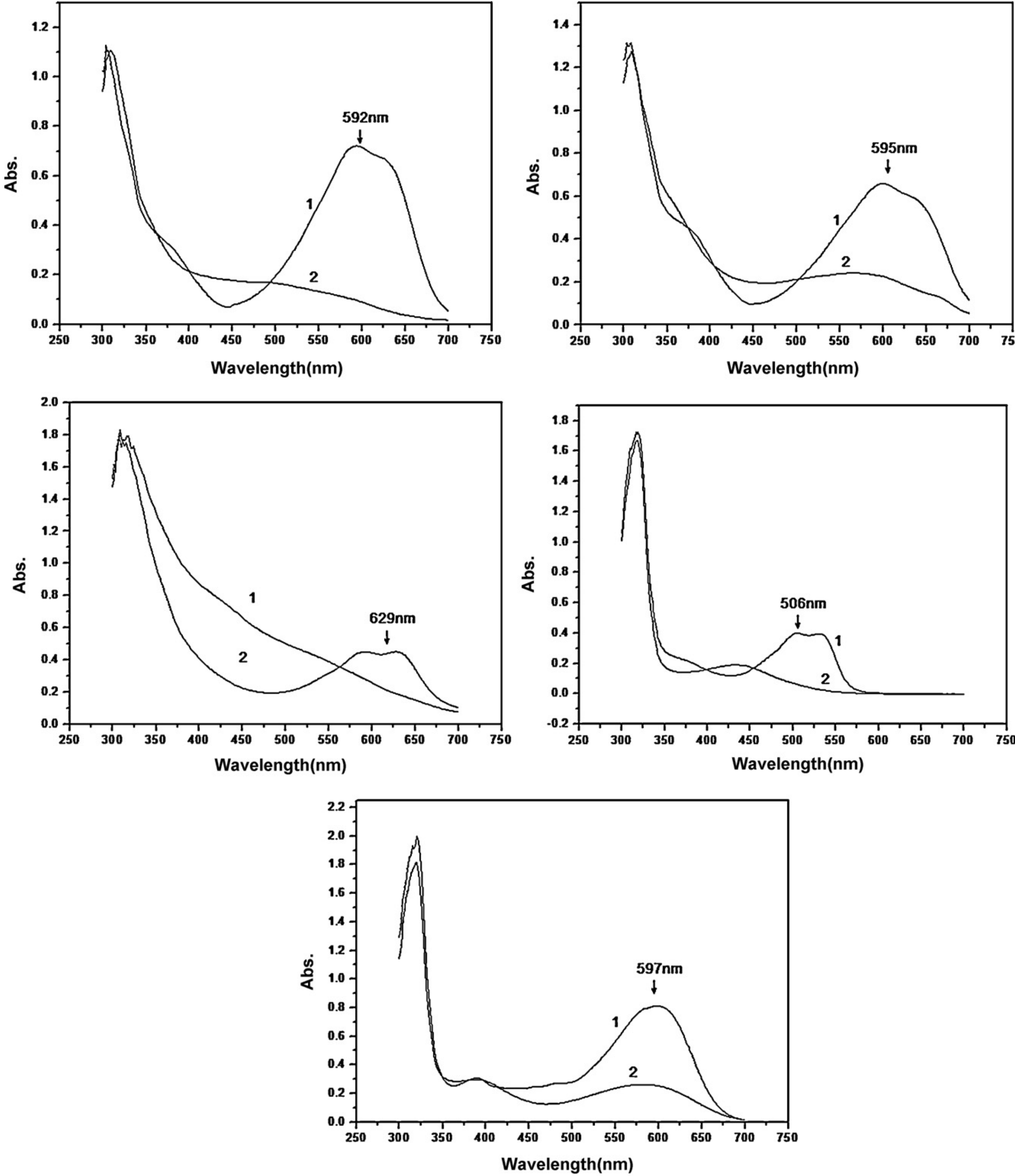


Fig. 4. UV–visible absorbance spectra of dye reaction mixture before and after treatment with crude laccase from *Trametes trogii* SYBC-LZ (a) RBBR, (b) RB 4, (c) AB 129, (d) AR 1, and (e) RB 5.

fungal growth and secrete high levels of laccase (Fig. 4c and d). In general, a relatively higher level of laccase activity was obtained just by optimizing nutritional and environmental factors in solid state fermentation.

White-rot fungi are involved in wood decay worldwide, and a few strains such as *T. trogii* have been reported as outstanding strains for ligninolytic enzyme production, including laccases as well as lignin peroxidases, manganese peroxidases and even cellobiose dehydrogenases [16,26,27,32,33]. Under the conditions described here, *T. trogii* SYBC-LZ only produced a phenol oxidase (laccase), which was in accordance with previous reports of *T. trogii* [16,27,33]. It had been reported SSF was able to reproduce the natural growth conditions of filamentous fungi or closely resembled the natural processes of filamentous fungi [4,34]. Therefore, it had

more advantages in the production of some enzymes [35]. Laccase production from *T. trogii* strains is shown in Table 2, and to the best of our knowledge, this study represents the first report on utilizing soybean cake as a solid substrate for laccase production. This method has the potential to be very efficient, as a high laccase activity was reached within just 6 days, much shorter than previous studies.

3.3. In vitro decolorization of dyes using crude enzyme

Dyes such as anthraquinone dyes are good substrates of laccases and their decolorization is proportional to enzyme activity [36]. Moreover, high potential laccases have been shown to decolorize anthraquinone dyes more efficiently than other classes of dyes [37]. In this paper, the decolorization of dyes with two structural patterns was investigated using crude extracellular culture extract. Dye decolorization efficiency depended upon the dye structure, enzymes used, and system conditions. As shown in Fig. 3, anthraquinone dyes (RB 4, RBBR, and AB 129) could be decolorized directly utilizing the crude enzyme produced by *T. trogii* SYBC-LZ, while the decolorization of azo dyes (AR 1 and RB 5) occurred only by employing 1 mM of redox mediator HOBT. The absorbance decreased sharply in the first 15 min but changed little in the following 15 min. Fig. 4 illustrates the UV–visible absorbance spectrum for five dyes. For each dye, an absorbance peak decreased after treatment with the enzyme, which was associated with decolorization. It is worth mentioning that after treatment the absorbance of the mixture at other wavelengths did not increase significantly (except AB 129), which meant no products with other colors existed while the initial color was removed. Moreover, results in Table 5 show the crude extracellular culture extract of this strain yielded high decolorization of anthraquinone dyes (RB 4 69.6% and RBBR 85.2%) but not of AB 129 (only 45.6%) in 30 min. Likewise, AR 1 was largely decolorized (90.2%) whereas RB 5 was more recalcitrant to degradation (65.4%).

3.4. Detection of enzyme(s) involved in dye decolorization

Laccase was the only enzyme involved in decolorization since there were no other ligninolytic enzymes detected in the culture extract. The involvement of laccase in decolorization was also demonstrated by native PAGE. As shown in Fig. 5, the laccase activity band was detected and a clear white band of decolorized zone was observed against a blue background after staining with RBBR or RB 4. This was consistent with the recent finding in *Ganoderma lucidum* which secreted only laccase contributing for decolorizing RBBR in solid substrate fermentation [11]. Further work was done to corroborate this fact by employing laccase inhibitors. It is known that Cystein and NaN_3 are laccase inhibitors [40]. Results in Table 3 show that after adding laccase inhibitors, the decolorization ratios changed. Decolorizations of RB 4, AR 1, and RB 5 were all inhibited when Cystein was at a concentration of 1 mM, but not at 0.1 mM; RBBR was inhibited at both concentrations. For all dyes, 1 mM NaN_3 completely inhibited the decolorization and decolorization ratios

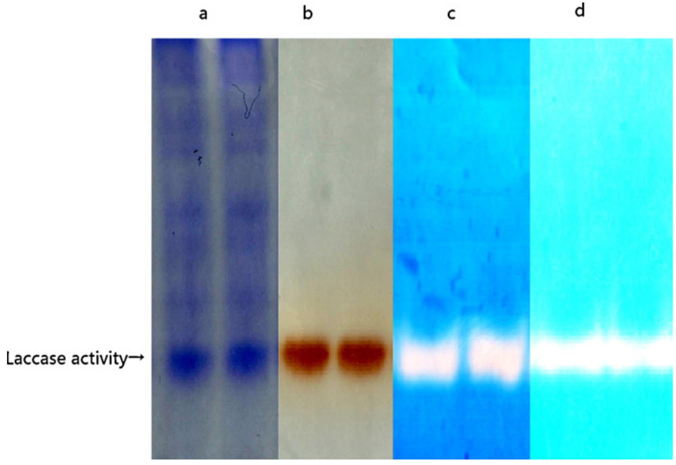


Fig. 5. Results of Native-PAGE of crude enzyme staining with (a) Commassie Blue R-250, (b) 5 mM DMP, (c) 200 mg l^{-1} RB 4,and (d) 200 mg l^{-1} RBBR. Duplicated samples are run in Native-PAGE.

significantly decreased even when NaN_3 was at a low concentration (0.1 mM). Therefore, this result also suggests that laccase was the main enzyme of the crude enzyme involved in decolorization.

3.5. Effect of various parameters on dye decolorization

Parameters of this dye decolorization process would affect the dye-removal reaction directly. The effect of laccase activity on decolorization was performed by utilizing diluted crude enzyme (laccase activity 0.11–3.3 U ml^{-1}). Optimum laccase activity for the decolorization of all the four dyes was 1.1 U ml^{-1} and the decolorization changed little although laccase activity was higher (data not shown). Therefore, the subsequent experiments were carried out with 1.1 U ml^{-1} laccase. Temperature served in affecting reaction rates as well as laccase stability. In this study, dyes were decolorized to a maximum between 40 and 60 °C, and a sharp reduction of decolorization occurred at temperatures above 60 °C. The pH values played a key role in decolorization by crude enzyme. As shown in Fig. 5a, maximum decolorization rates for all dyes occurred at 4.0 or 5.0. When pH was 7.0, the decolorization could almost be omitted. This result was in agreement with that from Mechichi et al. [27] and an explanation might be that laccase achieves optimum activity at pH 4.0–5.0, and no activity at pH 7.0. Fig. 5b establishes the effect of HOBT on dye decolorization and optimum HOBT concentration for AR 1 and RB 5 at 0.5–1 mM. In this study, HOBT had no effect on the anthraquinone dye decolorization, just as the result yielded in the reference [27], however, it greatly enhanced decolorization of azo dyes, which was consistent with the results from Couto et al. [41] and Soares et al. [42].

Table 3
Effect of laccase inhibitors on decolorization by laccase from *Trametes trogii* SYBC-LZ.

Dye decolorization (%)	Control	0.1 mM Cystein	1 mM Cystein	0.1 mM NaN_3	1 mM NaN_3
RBBR[a]	86.9 ± 0.6	69.3 ± 1.2	4.8 ± 0.2	2.8 ± 0.1	0
RB 4[a]	77.6 ± 0.1	77.4 ± 0.3	29.5 ± 1.1	10.9 ± 0.7	0
AR 1[b]	90.5 ± 0.3	91.5 ± 1.7	9.1 ± 0.8	39.1 ± 3.0	0
RB 5[b]	71.6 ± 0.6	71.8 ± 0.7	10.6 ± 2.4	58.8 ± 4.7	0

Results are expressed as means of triplicate experiments ± the standard deviation and are significantly different ($p < 0.01$).
[a] The decolorization is measured after 1 h and RBBR and RB4 concentration is 100 mg l^{-1} and 70 mg l^{-1} respectively.
[b] The decolorization is measured after 30 minutes in the presence of 1 mM HOBT and AR 1 and RB 5 concentration is 10 mg l^{-1} and 18.3 mg l^{-1} respectively.

Table 4
The decolorization of dyes by *Trametes trogii* strains.

Strain	Dyes	Culture Time	Decolorizaion mode	pH/temperature/ agitation	Decolorization (%)	Reference
T. trogii (BAFC 212)	Xylidine (8.7 mg l^{-1})	26 days	Culture filtrates	30 °C Statically	1.59 (Δ*A*/min per l) 2.03 (Δ*A*/min per l) 7889(Δ*A*/min per l)	[25]
	Malachite Green (2.7 mg l^{-1})					
	Anthraquinone Blue (60 mg l^{-1})					
T. trogii CTM 10156	Blue Tubantin GLL 300 (50 mg l^{-1})	12 days	Culture filtrates	30 °C Statically	85% (6 h)	[26]
	Black Tubantin VSF 600 (50 mg l^{-1})				85% (6 h)	
	Blue Solophenyl (50 mg l^{-1})				45% (6 h)	
T. trogii strain B6J	Remazol Brilliant Blue R(200 mg l^{-1})	12 days	Culture filtrates	30 °C 200 rpm	Almost 100% (8 h)	[27]
	Remazol Brilliant Blue R (100 mg l^{-1})		Purified laccase	30 °C pH 5 statically	97% (50 min)	
F. trogii ATCC 200800	Reactive Black 5(150 mg l^{-1})	21 days	Whole fungal mycelia culture	30 °C pH 5 statically	99.0% (21 days)	[28]
	Reactive Black 5 (61 mg l^{-1})	12 days	Immobilized fungal pellets		-	[38]
	Astrazone dyes (264 mg l^{-1})	4 days	Fungal pellets	30 °C 150 rpm	75% (24 h)	[39]
T. trogii SYBC-LZ	Remazol Brilliant Blue R (50 mg l^{-1})	6 days	Culture filtrates	30 °C pH 4.0 statically	85.2% (30 min)	
	Reactive Blue 4 (35 mg l^{-1})				69.6% (30 min)	
	Acid Blue 129 (83.3 mg l^{-1})				45.6% (30 min)	
	Acid Red 1 (10 mg l^{-1})				90.2% (30 min)[a]	
	Reactive Black 5 (18.3 mg l^{-1})				65.4% (30 min)[a]	

[a] The culture filtrates could not decolorize these dyes directly; decolorization rates were measured after adding 1 mM HOBT in the reaction solution.

3.6. Enzymatic decolorization versus whole fungal culture decolorization

In the present study, we tried to decolorize RBBR using whole fungal cultures on solid substrate. Results (data not shown) showed that fungal mycelium grew normally when dye concentration was from 100 mg l^{-1} to 200 mg l^{-1}, and the dye was decolorized. However, fungal mycelium began to grow slowly when dye concentration was at/above 300 mg l^{-1}, and there was little decolorization. A similar result is listed in Table 5 when the strain was cultured on plates containing various concentrations of RBBR. These results suggest a high concentration of RBBR would be toxic to fungal growth, thus creating a restriction in its utilization in dye decolorization by whole fungal cultures (Fig. 6).

Recently *T.trogii* strains showed ability in decolorization of dyes whether in the form of fungal pellets, crude filtrate cultures or its purified laccase (Table 4). Considering the toxicity of dyes which might make the decolorization process by whole fungal cultures become more time-consuming, the use of whole fungal cultures for decolorization was not easily applied on a large scale. Moreover, the use of purified or immobilized enzymes would increase the cost of decolorization processes [14]. Therefore, studies of *in vitro* color removal of synthetic dyes by crude enzymes have become more important [14]. Crude culture filtrates have several advantages. Their production process is not expensive, they might include natural laccase-mediators secreted by the fungus [43], and in addition, factors present in the medium such as proteins, residual macronutrients, and soluble extracellular metabolites could stabilize crude

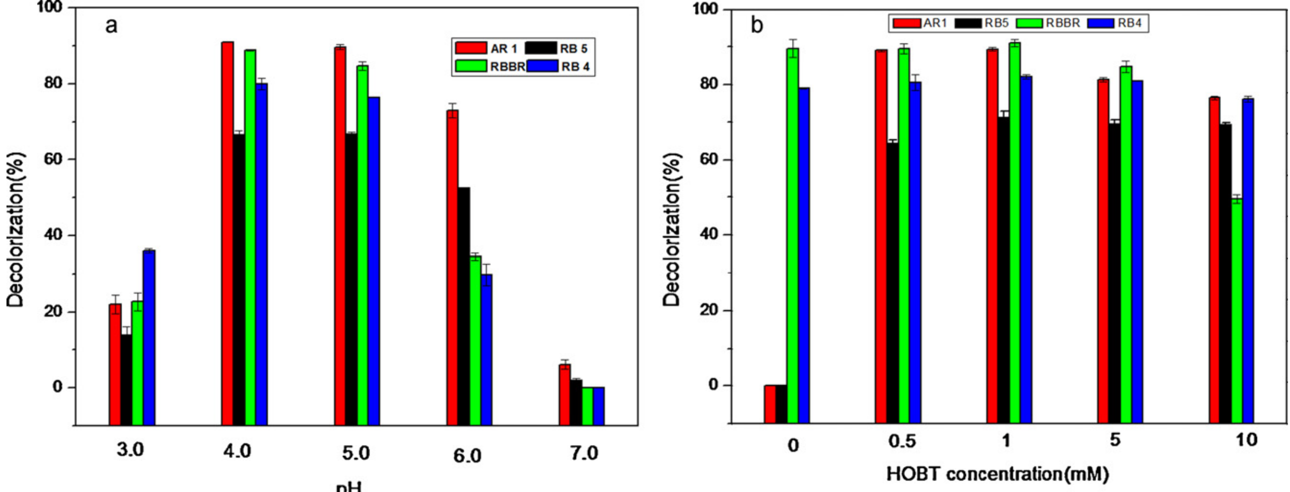


Fig. 6. Effect of pH (a) and HOBT (b) on decolorization of dyes. Results are significantly different ($p < 0.01$).

Table 5
The growth of *T. trogii* SYBC-LZ on PDA plates containing various content of RBBR.

RBBR concentration ($mg\,l^{-1}$)	Diameter of fungal mycelium (mm)	Diameter of decolorized zone (mm)
0	73.5 ± 3.5	–
100	60.0 ± 2.8	51.5 ± 0.7
200	54.0 ± 2.6	49.0 ± 1.5
300	46.0 ± 1.3	44.5 ± 2.1
400	34.5 ± 0.7	31.0 ± 1.4
500	28.5 ± 0.5	25.0 ± 0.6

Results are expressed as means of duplicate experiments± the standard deviation and are significantly different ($p < 0.01$).

enzymes [44]. Here the color removal by the crude culture filtrates could be efficiently completed in 30 min (Table 4), which is much shorter than by whole fungal cultures (about 6 days). In addition, the decolorization of dyes using crude enzyme could avoid inhibition of dyes on fungal growth. Thus the decolorization of dyes using crude enzyme is more advantageous.

4. Conclusions

In view of the results obtained in our present study, laccase production by newly isolated *T. trogii* SYBC-LZ on solid agro-industrial residue is much shorter than previous studies. In addition, the crude culture filtrates from solid substrate fermentation could decolorize textile dyes efficiently. Therefore, this strain has great potential to be used in bioprocesses to remove color from textile effluents. However, further research is required to study the color removal by the purified laccase to explore the mechanisms at work.

Acknowledgments

This work was financially supported by the National High Technology and Development Program of China (863 Program, Grant No. 2010AA101501) and Education Department of Jiangsu Province (No. 1016010241090570).

References

[1] D. Marmion, Handbook of US Colorants: Foods, Drugs, Cosmetics, and Medical Devices, Wiley-Interscience, 1991.
[2] M.A. Rauf, S.S. Ashraf, Radiation induced degradation of dyes: an overview, J. Hazard. Mater. 166 (2009) 6–16.
[3] G. McMullan, C. Meehan, A. Conneely, N. Kirby, T. Robinson, P. Nigam, I. Banat, R. Marchant, W. Smyth, Microbial decolourisation and degradation of textile dyes, Appl. Microbiol. Biotechnol. 56 (2001) 81–87.
[4] J.A. Majeau, S.K. Brar, R.D. Tyagi, Laccases for removal of recalcitrant and emerging pollutants, Bioresour. Technol. 101 (2010) 2331–2350.
[5] H. Moawad, W.M. Abd El-Rahim, M. Khalafallah, Evaluation of biotoxicity of textile dyes using two bioassays, J. Basic Microb. 43 (2003) 218–229.
[6] P.P. Champagne, J.A. Ramsay, Dye decolorization and detoxification by laccase immobilized on porous glass beads, Bioresour. Technol. 101 (2010) 2230–2235.
[7] T. Robinson, G. McMullan, R. Marchant, P. Nigam, Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative, Bioresour. Technol. 77 (2001) 247–255.
[8] D. Wesenberg, I. Kyriakides, S.N. Agathos, White-rot fungi and their enzymes for the treatment of industrial dye effluents, Biotechnol. Adv. 22 (2003) 161–187.
[9] Y.Z. Fu, T. Viraraghavan, Fungal decolorization of dye wastewaters: a review, Bioresour. Technol. 79 (2001) 251–262.
[10] M. Saparrat, E. Hammer, Decolorization of synthetic dyes by the deuteromycete *Pestalotiopsis guepinii* CLPS no. 786 strain, J. Basic Microb. 46 (2006) 28–33.
[11] K. Murugesan, I.H. Nam, Y.M. Kim, Y.S. Chang, Decolorization of reactive dyes by a thermostable laccase produced by *Ganoderma lucidum* in solid state culture, Enzyme Microb. Technol. 40 (2007) 1662–1672.
[12] A. Pandey, C. Soccol, D. Mitchell, New developments in solid state fermentation: I-bioprocesses and products, Process Biochem. 35 (2000) 1153–1169.
[13] M. Raimbault, General and microbiological aspects of solid substrate fermentation, Electron. J. Biotechnol. 1 (1998) 174–188.
[14] L. Levin, E. Melignani, A. Ramos, Effect of nitrogen sources and vitamins on ligninolytic enzyme production by some white-rot fungi.Dye decolorization by selected culture filtrates, Bioresour. Technol. 101 (2010) 4554–4563.
[15] W.Y. Ryu, M.Y. Jang, M.H. Cho, The selective visualization of lignin peroxidase, manganese peroxidase and laccase, produced by white rot fungi on solid media, Biotechnol. Bioprocess Eng. 8 (2003) 130–134.
[16] A.M. Garzillo, M.C. Colao, C. Caruso, C. Caporale, D. Celletti, V. Buonocore, Laccase from the white-rot fungus *Trametes trogii*, Appl. Microbiol. Biotechnol. 49 (1998) 545–551.
[17] M. Gardes, T. Bruns, ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts, Mol. Ecol. 2 (1993) 113–118.
[18] J. Thompson, D. Higgins, T. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Res. 22 (1994) 4673–4680.
[19] S Kumar, K. Tamura, M. Nei, MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment, Brief. Bioinformatics 5 (2004) 150–163.
[20] M.C.N. Saparrat, F. Guillen, A.M. Arambarri, A.T. Martinez, M.J. Martinez, Induction, isolation, and characterization of two laccases from the white rot basidiomycete *Coriolopsis rigida*, Appl. Environ. Microb. 68 (2002) 1534–1540.
[21] M. Tien, T. Kirk, Lignin-degrading enzyme from *Phanerochaete chrysosporium*: purification, characterization, and catalytic properties of a unique H_2O_2-requiring oxygenase, Proc Natl. Acad. Sci. U.S.A. 81 (1984) 2280–2284.
[22] H. Wariishi, K. Valli, M. Gold, Manganese (II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*.Kinetic mechanism and role of chelators, J. Biol. Chem. 267 (1992) 23688–23695.
[23] M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[24] L. Levin, F. Forchiassin, A. Viale, Ligninolytic enzyme production and dye decolorization by *Trametes trogii*: application of the Plackett-Burman experimental design to evaluate nutritional requirements, Process Biochem. 40 (2005) 1381–1387.
[25] S. Trupkin, L. Levin, F. Forchiassin, A. Viale, Optimization of a culture medium for ligninolytic enzyme production and synthetic dye decolorization using response surface methodology, J. Ind. Microbiol. Biotechnol. 30 (2003) 682–690.
[26] H. Chakroun, S. Sayadi, T. Mechichi, A. Dhouib, High level of laccases production by *Trametes trogii* culture on olive mill wastewater-based media, application in textile dye decolorization, J. Chem. Technol. Biotechnol. 84 (2009) 1527–1532.
[27] T. Mechichi, N. Mhiri, S. Sayadi, Remazol Brilliant Blue R decolourization by the laccase from *Trametes trogii*, Chemosphere 64 (2006) 998–1005.
[28] T. Deveci, A. Unyayar, M. Mazmanci, Production of Remazol Brilliant Blue R decolourising oxygenase from the culture filtrate of *Funalia trogii* ATCC 200800, J. Mol. Catal. B: Enzym. 30 (2004) 25–32.
[29] L. Levin, C. Herrmann, V. Papinutti, Optimization of lignocellulolytic enzyme production by the white-rot fungus *Trametes trogii* in solid-state fermentation using response surface methodology, Biochem. Eng. J. 39 (2008) 207–214.
[30] C. Galhaup, S. Goller, C. Peterbauer, J. Strauss, D. Haltrich, Characterization of the major laccase isoenzyme from *Trametes pubescens* and regulation of its synthesis by metal ions, Microbiology 148 (2002) 2159–2169.
[31] L. Lenin, F. Forchiassin, A.M. Ramos, Copper induction of lignin-modifying enzymes in the white-rot fungus *Trametes trogii*, Mycologia 94 (2002) 377–383.
[32] L. Levin, F. Forchiassin, Ligninolytic enzymes of the white rot basidiomycete *Trametes trogii*, Acta Biotechnol 21 (2001) 179–186.
[33] I. Ciullini, S. Tilli, A. Scozzafava, F. Briganti, Fungal laccase, cellobiose dehydrogenase, and chemical mediators: combined actions for the decolorization of different classes of textile dyes, Bioresour. Technol. 99 (2008) 7003–7010.
[34] R. Tengerdy, G. Szakacs, Bioconversion of lignocellulose in solid substrate fermentation, Biochem. Eng. J. 13 (2003) 169–179.
[35] S.R. Couto, M.A. Sanroman, Application of solid-state fermentation to ligninolytic enzyme production, Biochem. Eng. J. 22 (2005) 211–219.
[36] Y. Wong, J. Yu, Laccase-catalyzed decolorization of synthetic dyes, Water Res. 33 (1999) 3512–3520.
[37] P.P. Champagne, J.A. Ramsay, Contribution of manganese peroxidase and laccase to dye decoloration by *Trametes versicolor*, Appl. Microbiol. Biotechnol. 69 (2005) 276–285.
[38] M. Mazmanci, A. Ünyayar, Decolourisation of Reactive Black 5 by *Funalia trogii* immobilised on Luffa cylindrica sponge, Process Biochem. 40 (2005) 337–342.
[39] O. Yesilada, D. Asma, S. Cing, Decolorization of textile dyes by fungal pellets, Process Biochem. 38 (2003) 933–938.
[40] C. Johannes, A. Majcherczyk, Laccase activity tests and laccase inhibitors, J. Biotechnol. 78 (2000) 193–199.
[41] S.R. Couto, M. Sanroman, G.M. Gubitz, Influence of redox mediators and metal ions on synthetic acid dye decolourization by crude laccase from *Trametes hirsuta*, Chemosphere 58 (2005) 417–422.
[42] G.M.B. Soares, M.T.P. de Amorim, M. Costa-Ferreira, Use of laccase together with redox mediators to decolourize Remazol Brilliant Blue R, J. Biotechnol. 89 (2001) 123–129.
[43] C. Johannes, A. Majcherczyk, Natural mediators in the oxidation of polycyclic aromatic hydrocarbons by laccase mediator systems, Appl. Environ. Microb. 66 (2000) 524–528.
[44] L. Papinutti, P. Dimitriu, F. Forchiassin, Stabilization studies of *Fomes sclerodermeus* laccases, Bioresour. Technol. 99 (2008) 419–424.