Journal of Hazardous Materials 192 (2011) 855–873

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

Decolorization of different dyes by a newly isolated white-rot fungi strain *Ganoderma* sp.En3 and cloning and functional analysis of its laccase gene

Rui Zhuo[a,b], Li Ma[a], Fangfang Fan[a,b], Yangmin Gong[b], Xia Wan[b], Mulan Jiang[b], Xiaoyu Zhang[a], Yang Yang[a,b,*]

[a] Key Laboratory of Molecular Biophysics of Ministry of Education, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, China
[b] Key Laboratory of Oil Crops Biology of Ministry of Agriculture in China, Oil Crops Research Institute of Chinese Academy of Agricultural Sciences, Wuhan 430064, China

ARTICLE INFO

Article history:
Received 14 September 2010
Received in revised form 31 May 2011
Accepted 31 May 2011
Available online 7 June 2011

Keywords:
White-rot fungi
Ganoderma sp.En3
Decolorization of dyes
Laccase gene
cis-acting elements

ABSTRACT

A laccase-producing white-rot fungi strain *Ganoderma* sp.En3 was newly isolated from the forest of Tzuchin Mountain in China. *Ganoderma* sp.En3 had a strong ability of decolorizing four synthetic dyes, two simulated dye bath effluents and the real textile dye effluent. Induction in the activity of laccase during the decolorization process indicated that laccase played an important role in the efficient decolorization of different dyes by this fungus. Phytotoxicity study with respect to *Triticum aestivum* and *Oryza sativa* demonstrated that *Ganoderma* sp.En3 was able to detoxify four synthetic dyes, two simulated dye effluents and the real textile dye effluent. The laccase gene *lac-En3-1* and its corresponding full-length cDNA were then cloned and characterized from *Ganoderma* sp.En3. The deduced protein sequence of LAC-En3-1 contained four copper-binding conserved domains of typical laccase protein. The functionality of *lac-En3-1* gene encoding active laccase was verified by expressing this gene in the yeast *Pichia pastoris* successfully. The recombinant laccase produced by the yeast transformant could decolorize the synthetic dyes, simulated dye effluents and the real textile dye effluent. The ability of decolorizing different dyes was positively related to the laccase activity. In addition, the 5′-flanking sequence upstream of the start codon ATG in *lac-En3-1* gene was obtained. Many putative *cis*-acting responsive elements were predicted in the promoter region of *lac-En3-1*.

1. Introduction

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) is a group of copper-containing polyphenol oxidases which can catalyze the four-electron reduction of O_2 to H_2O with the concomitant oxidation of phenolic compounds. Laccase is widespread in plants, fungi and bacteria, but the most important sources of this enzyme are white-rot fungi. White-rot fungi can degrade the lignin thoroughly. The lignin degradation enzyme of white-rot fungi, which exhibits unique biodegradation capabilities, is secreted extracellularly and unusually nonspecific. Laccase is one of the important ligninolytic enzymes playing crucial role in the highly-efficient degradation of lignin [1,2]. Besides the function of lignin degradation [3], fungal laccases have been found to play an important role in fungal morphogenesis and fruiting body development [4–6], fungal pathogenicity [7] and pigmentation [8,9]. Due to the advantage of this enzyme, such as substrate non-specific, directly oxidizing various phenolic compounds, using molecular oxygen as the final electron acceptor instead of hydrogen peroxide, showing a considerable level of stability in the extracellular environment [2], laccase has been widely applied in biotechnology and industry, such as delignification of lignocellulosics, paper pulping/bleaching, degradation of different recalcitrant compounds, bioremediation, sewage treatment, dye decolorization and biosensors [10–13]. Thus laccase is a kind of valuable enzyme which is widely used in the biotechnology and industry.

In order to obtain large amounts of enzyme for application, several laccase genes have been cloned from different fungal sources [14–18] and heterologously expressed in *Pichia pastoris* [19,20], *Pichia methanolica* [21], *Saccharomyces cerivisiae* [22–24], *Kluyveromyces lactis* [25], *Yarrowia lipolytica* [26], *Aspergillus nidulans* [27], *Aspergillus niger* [28], *Aspergillus oryze* [29], and *Trichoderma reesei* [30,31]. Previous research about the regulation of laccase gene expression demonstrates that the transcription of laccase gene can be regulated by metal ions [32,33], various aromatic compounds related to lignin or lignin derivatives [34,35], nutrient nitrogen [32] and carbon [33].

Isolating new white-rot fungi strain and new laccase gene from different sources is urgently needed for better utilizing laccase in the field of biotechnology and industry, such as decolorizing

* Corresponding author at: College of Life Science and Technology, Huazhong University of Science and Technology, Luoyu Road 1037, Wuhan 430074, China. Tel.: +86 27 87792108.
E-mail address: yangyang@mail.hust.edu.cn (Y. Yang).

0304-3894/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2011.05.106

and detoxifying industrial dyes. In this study, a laccase-producing white-rot fungi strain *Ganoderma* sp.En3 was newly isolated from the forest of Tzu-chin Mountain in China. *Ganoderma* sp.En3 was able to decolorize and detoxify the synthetic dyes, simulated dye bath effluents and real textile dye effluent efficiently. The laccase gene *lac-En3-1* and its corresponding full-length cDNA were cloned and characterized from *Ganoderma* sp.En3. The functionality of *lac-En3-1* gene encoding active laccase was verified by expressing this gene in the yeast *Pichia pastoris* successfully. The recombinant laccase produced by the yeast gene-engineering strain was found to possess the ability to decolorize different dyes. The capability of decolorizing different dyes exhibited positive correlation with the laccase activity. In addition, the 5′-flanking sequence upstream of the start codon ATG in *lac-En3-1* gene was obtained by Self-Formed Adaptor PCR (SEFA PCR). Many putative *cis*-acting responsive elements, which may play important role in the transcriptional regulation of *lac-En3-1* gene, were discovered in the promoter region of *lac-En3-1*. Our work suggested that *Ganoderma* sp.En3 had great potential for practical and useful application in environmental biotechnology, such as decolorizing and detoxifying industrial dyes and dye bath effluents.

2. Experimental

2.1. Strains and media

Ganoderma sp.En3 was isolated from the forest of Tzu-chin Mountain in China and preserved in Institute of Environment & Resource Microbiology, Huazhong University of Science & Technology, Wuhan, China. *Ganoderma* sp.En3 was maintained on potato-dextrose agar (PDA) medium. *Pichia pastoris* GS115 and plasmid pPIC3.5K were purchased from Invitrogen. MD, BMGY, BMMY and BMM media were prepared according to the instruction of the *Pichia* Expression Kit manual (Invitrogen). *Escherichia coli* DH5α was used in all of the cloning procedures.

2.2. Isolation of genomic DNA and total RNA from fungi

Ganoderma sp.En3 was grown in the GYP medium: 20 g glucose l^{-1}, 5 g yeast extract l^{-1}, 5 g peptone from casein l^{-1} and 1 g $MgSO_4 \cdot 7H_2O$ l^{-1}. The pH was adjusted to 5.0 with H_3PO_4 prior to sterilization [17]. The mycelium was harvested from liquid culture at the peak of laccase activity. Genomic DNA was extracted using the E.Z.N.A. Fungal DNA kit (Omega, USA). Total RNA was extracted using the TRIZOL Reagent (Invitrogen) according to the instructions, followed by RNase-Free DNase (Promega) digestion.

2.3. Cloning of the complete structural gene of lac-En3-1

The degenerate primers CuI and CuIV (sequence shown in Table 1) were designed according to the conserved amino acids sequences of the copper-binding region I (HWHGFFQ) and IV (HCHIDFH) in fungal laccases, respectively. Degenerate PCR was performed using the genomic DNA of *Ganoderma* sp.En3 as the template. A 1600bp PCR fragment was obtained and cloned to pMD18-T vector (TAKARA) for sequencing. It was confirmed that this 1600bp PCR fragment contained specific sequence of laccase gene by DNA sequencing. In order to obtain the complete structural gene encoding laccase, TAIL-PCR (thermal asymmetric interlaced PCR) [36] was performed to amplify the 5′ and 3′-flanking sequence of the known 1600bp laccase gene partial sequence using the genomic DNA of *Ganoderma* sp.En3 as the template. The specific nested primers for amplify the 5′-flanking sequence of the known 1600bp laccase gene were En3-5-1, En3-5-2 and En3-5-3 (primer sequence shown in Table 1). The specific nested primers for amplify the 3′-flanking sequence of the known 1600bp laccase gene were En3-3-1, En3-3-2 and En3-3-3 (primer sequence shown in Table 1). The arbitrary degenerate primers (AD primers) used in TAIL-PCR were designed according to Ref. [36] and show in Table 1. TAIL-PCR was performed according to the detailed method described in Ref. [36]. The TAIL-PCR product was cloned to pMD18-T vector (TAKARA) for DNA sequencing. After obtaining the 5′ and 3′-flanking sequence of the known 1600bp laccase gene, high fidelity PCR was performed to amplify the complete structural gene using the genomic DNA of *Ganoderma* sp.En3 as the template and En3-f1, En3-r1 as the specific primers (sequence shown in Table 1). The PCR product-2118bp complete structural gene encoding laccase was cloned to pMD18-T vector (TAKARA) for DNA sequencing and designated as *lac-En3-1*.

Table 1
Oligonuleotide primers used in this study. D = A/G/T, N = A/G/C/T, R = A/G, Y = C/T.

Primer	Nucleotide sequence
CuI	CAYTGGCAYGGNTTYTTYCA
CuIV	TGRAARTCDATRTGRCARTG
En3-5-1	GTGGCACGATAAGCATAGCTCTAAGGTGC
En3-5-2	TCGACATCGTAGAGCCATCCAAGAGGATC
En3-5-3	AGCCTGACCGGGAACTCGGAAGTC
En3-3-1	CGGAAGCGTCTATGCGCTCCCATCC
En3-3-2	CCCTTCCACATGCACGGCGTAAGTAC
En3-3-3	CAACTATCGCAACCCCGTCTGGCGC
En3-f1	atggtcagattccaatcattccttccctacctcg
En3-r1	ctactggtcctccggcgcgagcgc
En3-f1-BamHI	aaa***ggatcc***accatggtcagattccaatcattccttccctacctcg
En3-r1-NotI	ttt***gcggccgc***ctactggtcctccggcgcgagcgc
lac-En3-SP1	gagtgaagtacgagtcggagggagcggtac
lac-En3-SP2	GGTCGTCTTCAACATGGTGTGGTTTGTCATCC
lac-En3-SP3	tgcattggatatgaccagatNNNNNNNNNNtccgat
lac-En3-SP4	gcccagtctgagatgaaccaggatcaggtagggag
AD1	TG(A/T)GNAG(A/T)ANCA(G/C)AGA
AD2	AG(A/T)GNAG(A/T)ANCA(A/T)AGG
AD3	(G/C)TTGNTA(G/C)TNCTNTGC
AD4	NTCGA(G/C)T(A/T)T(G/C)G(A/T)GTT
AD5	NGTCGA(G/C)(A/T)GANA(A/T)GAA
AD6	(A/T)GTGNAG(A/T)ANCANAGA
AD7	(A/T)TCTGNCT(A/T)ANTANCT

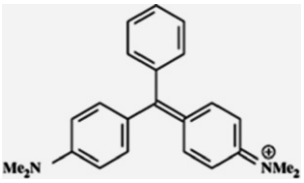
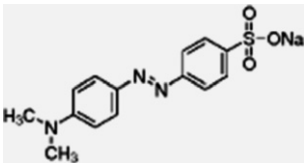
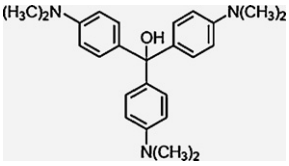
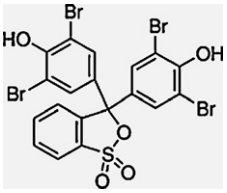
2.4. Cloning of the full-length cDNA of lac-En3-1

According to the known 5′ and 3′-end sequences of the laccase structural gene, primer En3-f1 was designed to match the start codon ATG region and primer En3-r1 was designed to match the sequence immediately downstream of the stop codon TAG (sequences of primer En3-f1 and En3-r1 were shown in Table 1). Using En3-f1 and En3-r1 as the specific primers, RT-PCR was then performed to amplify the full-length cDNA of *lac-En3-1* with PrimeSTAR™ HS DNA Polymerase (TAKARA). The 1566bp full-length cDNA was cloned to pMD18-T vector (TAKARA) for DNA sequencing, resulting in the recombinant plasmid pMD18-T-lac-En3-1.

2.5. Cloning of the 5′-flanking sequence upstream of the start codon of lac-En3-1

In order to obtain the promoter region of laccase gene, Self-Formed Adaptor PCR (SEFA PCR) [37] was performed to amplify the 5′-flanking sequence upstream of start codon of *lac-En3-1*. The nested primers used for Self-Formed Adaptor PCR (lac-En3-SP1, lac-En3-SP2, lac-En3-SP3 and lac-En3-SP4, the sequences of these primers were shown in Table 1) were designed based on the known structural gene sequence. lac-En3-SP1, lac-En3-SP2 and lac-En3-SP4 were gene-specific primers and had high annealing

Table 2
Characteristics of the synthetic dyes studied in this work.

Dyes	C.I.	Structure	λ_{max} (nm)	Type
Malachite green	42,000		618	Triphenylmethane
Methyl orange	13,025		462	Azo
Crystal violet	42,555		584	Triphenylmethane
Bromophemol blue			592	

temperatures (about 70 °C). lac-En3-SP3 was a partially degenerate primer. Self-Formed Adaptor PCR (SEFA PCR) was performed according to the detailed method described in Ref. [37].

2.6. Heterologous expression of lac-En3-1 gene in Pichia pastoris

Firstly the vectors for expression of *lac-En3-1* gene in *Pichia pastoris* were constructed. High fidelity PCR was performed to obtain the full-length *lac-En3-1* cDNA using the plasmid pMD18-T-lac-En3-1 as template and En3-f1-*Bam*HI, En3-r1-*Not*I as primers (sequences shown in Table 1, restriction sites for *Bam*HI and *Not*I were incorporated into upstream and downstream primers respectively). Then the PCR product was purified and digested with *Bam*HI and *Not*I. This digested fragment was inserted into the same sites of pPIC3.5K vector (Invitrogen), resulting in the recombinant plasmid pPIC3.5K-lac-En3-1 (containing the laccase native signal peptide).

The pPIC3.5K-lac-En3-1 and the control vector pPIC3.5K were linearized by *Sac*I digestion and transformed into *Pichia pastoris* GS115 (Invitrogen) respectively by the electroporation method described in the instruction of Multi-Copy *Pichia* Expression Kit (Invitrogen). The electroporated cells were plated onto MD agar plates for selecting the His^+ transformants. Some His^+ transformants were selected randomly and grown on the BMGY agar plates at 28 °C for two days and then inoculated onto the BMM agar plates containing $CuSO_4$ (0.1 mmol/l) and ABTS (0.2 mmol/l). Under the induction of methanol, secretion of active laccase was identified by the presence of a dark green zone around transformant colonies.

After selection of positive transformants which could produce active laccase on ABTS plates, the positive transformants were then fermented with BMM liquid medium at 20 °C. The yeast transformants were inoculated into 20 ml BMG media in 250-ml Erlenmeyer flasks and incubated at 30 °C to OD600 of 10 with shaking at 200 rpm. Then the cultures were centrifuged at 3000 × *g* for 5 min and the cell pellets were suspended to OD600 of 2.0 with 30 ml BMM media (pH 6.0) containing 0.3 mM $CuSO_4$ and 0.8% alanine. The cultures were grown at 20 °C with shaking at 200 rpm, with 0.5% (v/v) methanol being added daily. Secreted laccase activities in cultures were measured daily. Laccase activity was measured by means of ABTS method as described by Ref. [38]. Native-PAGE was also performed according to the method described in Ref. [39]. All experiments were performed in triplicate.

2.7. Decolorization of four synthetic dyes-methyl orange, malachite green, bromophenol blue and crystal violet by Ganoderma sp.En3

2.7.1. Decolorization of four synthetic dyes by fungal whole cultures-decolorization of dyes in submerged cultures operated in batch mode

Ganoderma sp.En3 was precultured on PDA (potato-dextrose agar) plates at 28 °C for 1 week. Flasks containing 50 ml liquid medium (1 l distilled water: glucose 20 g, yeast extract 2.5 g, KH_2PO_4 1 g, Na_2HPO_4 0.05 g, $MgSO4{\cdot}7H_2O$ 0.5 g, $CaCl_2$ 0.01 g, $FeSO_4{\cdot}7H_2O$ 0.01 g, $MnSO_4{\cdot}4H_2O$ 0.001 g, $ZnSO_4{\cdot}7H_2O$ 0.001 g, $CuSO_4{\cdot}5H_2O$ 0.002 g. The pH of the medium was adjusted to 5.5) [40] were each inoculated with five plugs obtained from the edge of actively growing mycelia of *Ganoderma* sp.En3 and incubated at 28 °C in a shaking incubator (150 rpm) for 5 days. Then different dyes were added into the 5-days cultures at various concentrations: 25, 50, 100, 200 mg l^{-1} respectively and incubated at 28 °C with shaking at 150 rpm continuously. An aliquot (3 ml) of the culture media was withdrawn at different time intervals. Decolorization was monitored by measuring the absorbance of the culture supernatant at 618 nm for malachite green, 462 nm for methyl orange, 592 nm for bromophenol blue, 584 nm for crystal violet.

More information about these dyes: CI, structure, type, λ_{max} were shown in Table 2. The decolorization of dye, expressed as dye decolorization (%), was calculated as the formula: decoloriza-

tion (%) = $[(A_i - A_t)/A_i] * 100$, where, A_i: initial absorbance of the dye, A_t: absorbance of the dye along the time. All experiments were performed in triplicate.

The extracellular activity of lignin modifying enzymes, such as laccase, manganese peroxidase, lignin peroxidase and total organic carbon (TOC) before and after decolorization were also measured as following. Laccase activity was measured by means of ABTS method as described by Ref. [38]. Manganese peroxidase activity was measured as described by Ref. [41]. Lignin peroxidase activity was measured as described by Ref. [42]. The total organic carbon (TOC) content was analyzed using a TOC Analyzer (O.I. Analytical 1010 Total Organic Carbon Analyzer).

2.7.2. Decolorization of four synthetic dyes by the culture supernatants operated in batch mode

The culture supernatants prepared from *Ganoderma* sp.En3 were used to decolorize four synthetic dyes. The assays were carried out at 30 °C. The reaction mixture in a total volume of 1 ml contained (final concentration): acetate buffer (25 mM, pH 4.5), dyes (methyl orange, malachite green, bromophenol blue: 50 mg l^{-1}; crystal violet: 20 mg l^{-1}) and 100 μl culture supernatant (containing 0.03 U laccase). Decolorization was monitored by measuring the absorbance of the reaction mixture at 618 nm for malachite green, 462 nm for methyl orange, 592 nm for bromophenol blue, 584 nm for crystal violet.

The decolorization of dye, expressed as dye decolorization (%), was calculated as the formula: decolorization (%) = $[(A_i - A_t)/A_i] * 100$, where, A_i: initial absorbance of the dye, A_t: absorbance of the dye along the time [43,44]. All experiments were performed in triplicate.

Kojic acid was added into the culture supernatants of *Ganoderma* sp.En3 at various concentrations. Then the decolorization of dyes was performed again as described above. All experiments were performed in triplicate.

2.8. Decolorization of simulated dye bath effluent by Ganoderma sp.En3 operated in batch mode

Firstly, two kinds of simulated dye bath effluent were prepared as following. Simulated dye effluent-I was prepared as described by Vijayaraghavan et al. [45]. It mimic the effluent produced by typical reactive dye industries. The composition of the mixture was decided according to a cotton fiber dyeing procedure used at an integrated textile manufacturing plant [45,46]. The simulated dye effluent-I consisted of Remazol black B (0.768 g), Remazol orange 16 (0.006 g), Remazol brilliant blue R (0.369 g), Remazol brilliant violet 5R (0.194 g), acetic acid (0.79 g), sodium chloride (41.0 g), sodium carbonate (13.0 g) and sodium hydroxide (0.51 g) per liter of deionized water. The dye bath mixture was boiled for 3 h and then cooled for 12 h. The effluent sample was scanned using UV–Vis Spectrophotometry, which showed the maximum absorbance at 579 nm. Simulated dye effluent-II was prepared as described by Refs. [47,48]. The composition of simulated dye effluent-II was based on instructions of the manufacturer Bezema AG (Montlingen, Switzerland) for reactive dyes [47,48]. It consisted of 0.5 g/l Reactive Black 5, 30 g/l NaCl, 5 g/l Na_2CO_3 and 1.5 ml/l of 32.5% (w/v) NaOH in deionized water. The pH was adjusted to 4.5 with HCl. The effluent sample was scanned using UV–Vis Spectrophotometry, which showed the maximum absorbance at 582 nm.

Ganoderma sp.En3 was precultured on PDA (potato-dextrose agar) plates at 28 °C for 1 week. Flasks containing liquid medium (1 l distilled water: glucose 20 g, yeast extract 2.5 g, KH_2PO_4 1 g, Na_2HPO_4 0.05 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, $CaCl_2$ 0.01 g, $FeSO_4{\cdot}7H_2O$ 0.01 g, $MnSO_4{\cdot}4H_2O$ 0.001 g, $ZnSO_4{\cdot}7H_2O$ 0.001 g, $CuSO_4{\cdot}5H_2O$ 0.002 g. The pH of the medium was adjusted to 5.5) [40] were each inoculated with five plugs obtained from the edge of actively growing mycelia of *Ganoderma* sp.En3 and incubated at 28 °C in a shaking incubator (150 rpm) for 6 days. Then simulated dye effluent-I and II were added into the 6-days cultures (final concentration was 60%) respectively and incubated at 28 °C with shaking at 150 rpm continuously. An aliquot (3 ml) of the culture media was withdrawn at different time intervals. Decolorization was monitored by measuring the absorbance of the culture supernatant at 579 nm for simulated dye effluent-I, 582 nm for simulated dye effluent-II. The decolorization of dye effluent, expressed as decolorization (%), was calculated as the formula: decolorization (%) = $[(A_i - A_t)/A_i] * 100$, where, A_i: initial absorbance of the dye effluent, A_t: absorbance of the dye effluent along the time. All decolorization experiments were performed in three sets.

2.9. Decolorization of the real textile dye effluent by Ganoderma sp.En3

The real textile industry effluent containing indigo dyes was collected from Puqi Textile Dyeing Factory in Hubei Province of China, which was designated as the real indigo effluent. The characteristics of this wastewater with high concentration of salts, high ionic strength and other auxiliary chemicals was described as follows: COD = 24813.5 mg l^{-1}; TOC = 8445.3 mg l^{-1}; pH 9.2; the initial absorbance at 680 nm = 2.0 (the effluent sample was scanned using UV–Vis Spectrophotometry, which showed the maximum absorbance at 680 nm).

The ability of the fungus to decolorize the real indigo effluent was assayed in the Kirk medium: ammonium tartrate, 0.2 g/l; potassium dihydrogen phosphate, 2 g/l; $MgSO_4{\cdot}7H_2O$, 0.5 g/l; $CoCl_2{\cdot}2H_2O$, 0.1 g/l; glucose, 10 g/l; vitamin B, 0.01 g/l; trace elements solution, 10 ml/l. The medium contained the textile dye effluent instead of distilled water in equal volume. Flasks containing above liquid medium were inoculated with five plugs obtained from the edge of actively growing mycelia of *Ganoderma* sp.En3 and incubated at 28 °C in a shaking incubator (150 rpm). An aliquot (3 ml) of the culture media was withdrawn at different time intervals. Decolorization was monitored by measuring the absorbance of the culture supernatant at 680 nm. The decolorization of dye effluent, expressed as decolorization (%), was calculated as the formula: decolorization (%) = $[(A_i - A_t)/A_i] * 100$, where, A_i: initial absorbance of the dye effluent, A_t: absorbance of the dye effluent along the time. All decolorization experiments were performed in three sets.

2.10. Decolorization of four synthetic dyes by the yeast transformants which could produce active laccase

The *Pichia pastoris* transformants which could produce active laccase were used to decolorize four synthetic dyes: methyl orange, malachite green, bromophenol blue and crystal violet. Cell-free supernatants prepared from the culture of GS115(pPIC3.5K-lac-En3-1) at the peak of laccase activity and GS115(pPIC3.5K) as the negative control were used to decolorize different dyes. The reaction mixture respectively contained 50 mg l^{-1} methyl orange, malachite green, bromophenol blue or 20 mg l^{-1} crystal violet, acetate buffer (25 mM, pH 4.5) and 100 μl yeast culture supernatant. During incubation at 30 °C, the time course of decolorization was detected every 3 h by measuring the absorbance at 618 nm for malachite green, 462 nm for methyl orange, 592 nm for bromophenol blue, 584 nm for crystal violet. The decolorization of dye, expressed as dye decolorization (%), was calculated as the formula: decolorization (%) = $[(A_i - A_t)/A_i] * 100$, where, A_i: initial absorbance of the dye, A_t: absorbance of the dye along the time.

Kojic acid was added into the reaction mixture at a final concentration of 5, 10, 20, 50 mM respectively. Then the decolorization of dyes was performed again as described above. All experiments were performed in triplicate.

2.11. Decolorization of the simulated dye bath effluent and real textile dye effluent by the purified recombinant laccase produced by Pichia pastoris transformant

Firstly, the recombinant laccase produced by *Pichia pastoris* transformants was purified as following. The *Pichia Pastoris* transformant in which *lac-EN3-1* gene was successfully expressed was cultivated in BMG medium at 28 °C, 250 rpm for two days (OD600: 20). The cells were centrifugated at the speed of 1500 × *g* for 5 min and then resuspended in BMM medium (containing 0.3 mM Cu^{2+} and 0.8% alanine). The cultures were grown at 20 °C with shaking at 200 rpm, with 0.5% (v/v) methanol being added daily for ten days. 200 ml of culture was centrifugated at 10,000 × *g* for 20 min. The supernatant was concentrated into 10 ml using PEG20000 and then dialyzed against PBS buffer (pH 6.8, 0.05 mol/l) for 24 h by ice bath. The precipitate was abandoned by centrifugation (12,000 × *g*, 20 min). The supernatant was then applied to a Q-sepharose Fast flow column (10 mm × 150 mm, GE Healthcare), which was pre-equilibrated with 0.05 mol/l PBS, pH 6.8. The column was washed at a flow rate of 3 ml min^{-1} with 2 l PBS buffer to remove unbound protein. Bound laccase was eluted from the column with salt gradient (0.05, 0.15, 0.25, 0.35, 0.45 mol/l NaCl, dissolved in 0.05 mol/l, pH 6.8 PBS) in the same buffer with a flow rate of 3 ml min^{-1}. Elution was simultaneously monitored at 280 nm for protein detection. Active fractions were pooled, desalted, filter-sterilized, and stored at 4 °C. Protein concentrations was measured using the Bradford dye-binding assay (Coomassie brilliant blue) and bovine serum albumin as the standard. The homogeneity of the purified recombinant laccase was detected by SDS–PAGE.

The purified recombinant laccase produced by *Pichia pastoris* transformant was then used to decolorize the simulated dye effluent as following. The decolorization of simulated dye effluent-I and II by purified recombinant laccase was determined over a 36 h period. The reaction mixture respectively contained 6% simulated dye effluent-I (pH was adjusted to 5.0) and 30% simulated dye effluent-II, acetate buffer (50 mM, pH 5.0) and purified enzyme (0.04 U). During incubation at 30 °C, the time course of decolorization was detected every 3 h by measuring the absorbance at 579 nm for simulated dye effluent-I, 582 nm for simulated dye effluent-II. The decolorization of dye effluent, expressed as decolorization (%), was calculated as the formula: decolorization $(\%) = [(A_i - A_t)/A_i] * 100$, where, A_i: initial absorbance of the dye effluent, A_t: absorbance of the dye effluent along the time. All experiments were performed in triplicate.

The purified recombinant laccase produced by *Pichia pastoris* transformant was also used to decolorize the real textile dye effluent as following. The decolorization of the real indigo effluent by purified recombinant laccase was determined over a 36 h period. The reaction mixture respectively contained 80% real indigo effluent, acetate buffer (50 mM, pH 5.0) and purified enzyme (0.1 U). During incubation at 30 °C, the time course of decolorization was detected every 3 h by measuring the absorbance at 680 nm. The decolorization of dye effluent, expressed as decolorization (%), was calculated as the formula: decolorization $(\%) = [(A_i - A_t)/A_i] * 100$, where, A_i: initial absorbance of the dye effluent, A_t: absorbance of the dye effluent along the time. All experiments were performed in triplicate. Kojic acid was added into the reaction mixture at a final concentration of 100 mM. Then the decolorization of dye effluent was performed again as described above.

2.12. Phytotoxicity study of different synthetic dyes, simulated dye effluents and real indigo effluent before and after fungal treatment

The relative sensitivities towards the original dyes and their degradation products in relation to *Triticum aestivum* and *Oryza sativa* seeds were evaluated according to the method described by Ref. [49]. The phytotoxicity study was performed (at room temp) in relation to *Triticum aestivum* and *Oryza sativa* (10 seeds of each) by watering separately 10 ml sample of original dyes and their decolorization products per day. Seeds were also treated with distilled water at the same time as the control. The length of shoot and root of *Triticum aestivum* treated under different conditions was measured after 4 days. The length of shoot and root of *Oryza sativa* treated under different conditions was measured after 7 days. Values are mean of germinated seeds of three experiments.

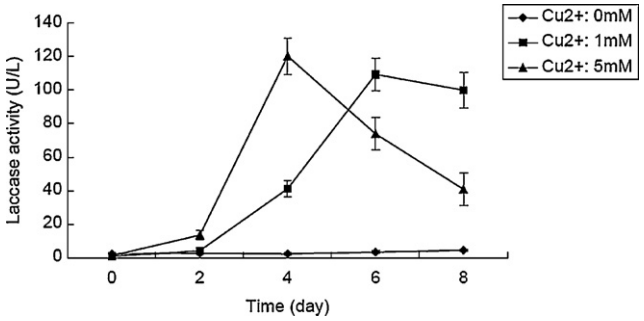


Fig. 1. The laccase production of *Ganoderma* sp.En3 grown in submerged medium supplemented with different concentrations of Cu^{2+}. All experiments were conducted in triplicates. Data are the mean ± SD of triplicate experiments.

2.13. Bioinformatic analysis of gene sequence

Analysis of the homology between the protein encoded by *lac-En3-1* and other known laccase proteins was performed using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The molecular weight and isoelectric point of protein were predicted with Compute pI/Mw tool (http://www.expasy.org/tools/pi_tool.html). N-glycosylation sites (Asn-X-Ser/Thr) were identified with ScanProsite program (http://www.expasy.ch/tools/scanprosite/). Signal peptides were predicted with SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). The Conserved Domains of protein were predicted and analyzed with Conserved Domain Database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Alignments of multiple DNA and amino acid sequences were generated with ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Based on this alignment, the neighbor joining phylogenetic tree was constructed using MEGA version 4. Bootstrapping was carried out with 500 replications. The putative *cis*-acting elements in the promoter region of laccase gene were predicted and identified with SoftBerry-NSITE/Recognition of Regulatory motifs (http://www.softberry.ru/berry.phtml?topic=nsite&group=programs&subgroup=promoter). The putative *cis*-acting elements in the promoter region of laccase gene were also examined using the MatInspector software (http://www.genomatix.de/products/MatInspector/).

3. Results and discussion

3.1. Decolorization of different synthetic dyes by Ganoderma sp.En3 isolated from the forest of Tzu-chin Mountain in China

3.1.1. Decolorization of different synthetic dyes by fungal whole cultures-decolorization of dyes in submerged cultures

Ganoderma sp.En3 was a white-rot fungi isolated from the forest of Tzu-chin Mountain in China. The laccase production of *Ganoderma* sp.En3 grown in liquid medium was measured continuously. The secreted laccase activity of *Ganoderma* sp.En3 could be enhanced significantly by Cu^{2+} (Fig. 1). Neither manganese peroxidase nor lignin peroxidase was detected during the growing

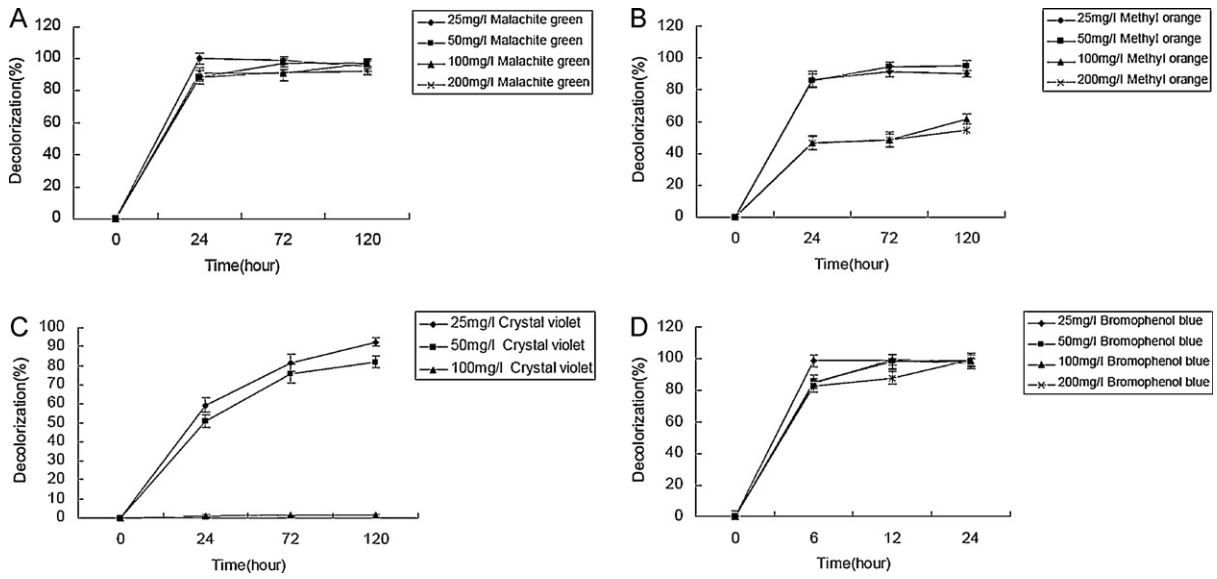


Fig. 2. Decolorization of four synthetic dyes at various increasing concentration of dye by *Ganoderma* sp.En3 whole cultures. All experiments were conducted in triplicates. Data are the mean ± SD of triplicate experiments.

process. Thus laccase was the most dominant lignin modifying enzyme produced by this fungus with undetectable activities of manganese peroxidase and lignin peroxidase. To investigate the ability of *Ganoderma* sp.En3 to decolorize dyes, four different synthetic dyes: methyl orange, malachite green, bromophenol blue and crystal violet were decolorized by fungal whole cultures as described in Section 2. Our research revealed that *Ganoderma* sp.En3 could decolorize these four dyes efficiently. Malachite green (50 mg l^{-1}), methyl orange (50 mg l^{-1}) and crystal violet (50 mg l^{-1}) were respectively decolorized up to 98.9%, 96.7% and 75.8% by *Ganoderma* sp.En3 within 72 h. Bromophenol blue (50 mg l^{-1}) was decolorized more rapidly. It could be decolorized up to 98.3% by *Ganoderma* sp.En3 within 12 h (Fig. 2).

In order to examine the effect of dye concentration on the decolorization, the decolorization of these four dyes was performed at various increasing concentration of dye for different time intervals (Fig. 2). As for malachite green and bromophenol blue, the efficiency of decolorization was not significantly affected by increasing concentration of dye (Fig. 2A and D). 91.2% decolorization of malachite green was observed at 200 mg l^{-1} concentration within 72 h. 90.9% decolorization of bromophenol blue was observed at 200 mg l^{-1} concentration within 12 h. But as for methyl orange and crystal violet, the efficiency of decolorization was decreased with increasing concentration of dye (Fig. 2B and C). When the concentration of crystal violet was increased to 100 mg l^{-1}, little decolorization was observed within 120 h. As the initial methyl orange concentration increased from 50 to 200 mg l^{-1}, the decolorization of methyl orange decreased from 96.7% to 48.7% after 72 h. In summary, above results indicated that the capacity of *Ganoderma* sp.En3 to decolorize malachite green and bromophenol blue were more powerful than decolorizing methyl orange and crystal violet. Compared with methyl orange and crystal violet, malachite green and bromophenol blue were more easily decolorized by *Ganoderma* sp.En3. The difference of dye structure may result in the difference of efficiency of decolorization.

We further compared the decolorization capability of *Ganoderma* sp.En3 with that of other strains reported previously. Liu et al. [50] have reported that malachite green (15 mg l^{-1}) was decolorized up to 66.5% by *Fome lignosus* within 240 h (10 days). Another white-rot fungi *Trametes versicolor* showed 99.5% decolorization of malachite green (15 mg l^{-1}) within 240 h (10 days). In our research, we found that the maximum decolorization (%) of malachite green (25 mg l^{-1}) could reach 99.8% within 24 h (1 day) as for *Ganoderma* sp.En3 (Fig. 2A). Compared with the result of Liu et al. [50], the time required for complete decolorization of malachite green by *Ganoderma* sp.En3 was far less than *Trametes versicolor*. The decolorization efficiency of malachite green by *Ganoderma* sp.En3 was also higher than *Fome lignosus*. Thus the decolorizing capacity of *Ganoderma* sp.En3 for malachite green was stronger than that of *Fome lignosus* and *Trametes versicolor* reported by Ref. [50]. The effect of dye concentration on the decolorization of malachite green by *Ganoderma* sp.En3 was also compared with other strains reported previously [49,51]. The research about the biodegradation of malachite green by *Kocuria rosea* demonstrated that the rate of decolorization was decreased with increasing concentration of malachite green. The rate of decolorization was decreased beyond the 50 mg l^{-1} dye concentration. Only 13% and 6% decolorization was observed at 70 and 100 mg l^{-1} dye concentration respectively. These results indicate toxicity of malachite green at higher dye concentration [49]. The research about the biodegradation of malachite green by *Sphingomonas paucimobilis* also revealed the reduction in decolorization with increase in dye concentration. As the initial malachite green concentration increased from 2.5 to 50 mg l^{-1}, the decolorization of malachite green decreased from 99% to 75% after 24 h [51]. In our research, we found that the efficiency of decolorization of malachite green by *Ganoderma* sp.En3 was not significantly reduced by increasing concentration of dye (Fig. 2A). *Ganoderma* sp.En3 showed 96.8%, 90.6% and 91.2% decolorization of malachite green at the concentration of 50, 100 and 200 mg l^{-1} within 72 h. This result suggested that white-rot fungus strain *Ganoderma* sp.En3 could tolerate higher concentrations of malachite green compared with other bacterial strains reported by Refs. [49,51]. The capability of *Ganoderma* sp.En3 for decolorizing high concentrations of malachite green was more powerful than that of some bacterial strains reported previously [49,51]. This was the advantage of *Ganoderma* sp.En3 as for its useful application in the environmental biotechnology, such as decolorizing industrial dyes with high concentrations. Methyl orange was a model azo dye. Previous research has reported that azo dyes were more recalcitrant to decolorization or could only be decolorized to a limited extent [52]. But our research demonstrated that *Ganoderma* sp.En3 could decolorize methyl orange efficiently. 25 mg l^{-1} and

Table 3
The change of the activity of lignin modifying enzymes, TOC before and after decolorization of four synthetic dyes by *Ganoderma* sp.En3. ND: not detected, LiP: lignin peroxidases, MnP: manganese peroxidase, TOC: total organic carbon.

Dye (concentration)	Time (h)	Decolorization (%)	Laccase activity ($U l^{-1}$)	LiP activity ($U l^{-1}$)	MnP activity ($U l^{-1}$)	TOC reduction (%)
Malachite green ($50 mg l^{-1}$)	0	0	33.2 ± 4.77	ND	ND	
	72	98.9 ± 1.27	130.2 ± 7.26	ND	ND	92.7
Methyl orange ($50 mg l^{-1}$)	0	0	27.96 ± 4.71	ND	ND	
	72	96.7 ± 1.22	120.5 ± 7.92	ND	ND	84.2
Bromophenol blue($50 mg l^{-1}$)	0	0	41.11 ± 13.03	ND	ND	
	12	98.3 ± 4.01	135.37 ± 7.26	ND	ND	86.1
Crystal violet ($25 mg l^{-1}$)	0	0	30.3 ± 9.43	ND	ND	
	120	92.4 ± 2.4	112.5 ± 13.02	ND	ND	63.1

$50 mg l^{-1}$ methyl orange could be decolorized up to 92.0% and 96.7% respectively by *Ganoderma* sp.En3 within 72 h. Therefore, our research indicated that *Ganoderma* sp.En3 showed high potential of decolorizing azo dyes effectively. The study on decolorizing other azo dyes by *Ganoderma* sp.En3 was going on in our laboratory. The previous research about the decolorization of crystal violet by white-rot fungi has revealed that *Fome lignosus* showed 72% decolorization of crystal violet ($25 mg l^{-1}$) within 240 h (10 days) [50]. A marine fungal isolate NIOCC # 2a, producing laccase as the major lignin-degrading enzyme, was used to decolorize crystal violet. The decolorization of crystal violet by this marine fungus was very low. Crystal violet was decolorized up to about 60% by this marine fungus within 144 h (6 days) [53]. In our research, *Ganoderma* sp.En3 showed 92.4% decolorization of crystal violet ($25 mg l^{-1}$) within 120 h (5 days). Thus, our work suggested that *Ganoderma* sp.En3 possess stronger capacity for decolorizing crystal violet compared with some other fungal strains reported previously [50,53]. But as for *Ganoderma* sp.En3, the decolorization capability for crystal violet was still relatively lower than that for the other three dyes: malachite green, bromophenol blue and methyl orange. Among the four synthetic dyes studied in this work, bromophenol blue was decolorized by *Ganoderma* sp.En3 most efficiently. The decolorization rate of bromophenol blue was much faster than the other three dyes (Fig. 2). The maximum decolorization (%) of bromophenol blue (25, 50, 100 and 200 $mg l^{-1}$) could respectively reach 98.6%, 98.2%, 99.4% and 90.9% within only 12 h (Fig. 2D). The efficiency of decolorization of bromophenol blue by *Ganoderma* sp.En3 was also not significantly affected by increasing concentration of dye (Fig. 2D), which indicated that *Ganoderma* sp.En3 could tolerate higher concentrations of bromophenol blue. This character may be very valuable for applying *Ganoderma* sp.En3 to decolorize industrial dyes with high concentrations. In summary, the advantage of *Ganoderma* sp.En3 used for decolorizing industrial dyes compared with some other strains reported previously [49–53] was summarized as below: Firstly, the decolorizing capability of *Ganoderma* sp.En3 was more powerful than that of some other strains reported previously [49–53]; Secondly, *Ganoderma* sp.En3 could tolerate higher concentrations of malachite green and bromophenol blue, which may be very helpful for using *Ganoderma* sp.En3 to decolorize industrial dyes with high concentrations; Thirdly, the decolorization rate of *Ganoderma* sp.En3 was faster than that of some other strains reported previously [50,53]. *Ganoderma* sp.En3 decolorized bromophenol blue very rapidly. Short incubation times required for the complete decolorization suggests that *Ganoderma* sp.En3 offers practical and economical opportunity for decolorization of some industrial dyes.

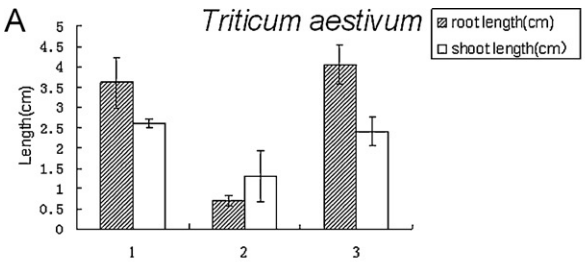
The change of the activity of lignin modifying enzymes, such as laccase, manganese peroxidase, lignin peroxidase before and after decolorization was studied and shown in Table 3. Laccase, manganese peroxidase, lignin peroxidase activities were assayed in the supernatant medium before and after decolorization. Extracellular laccase activities were significantly induced by 292.1%, 330.9%, 229.3% and 271.3% respectively after decolorization of malachite green, methyl orange, bromophenol blue and crystal violet by *Ganoderma* sp.En3 (Table 3). Neither manganese peroxidase nor lignin peroxidase was detected during the decolorization process. Induction in the activity of laccase during the decolorization process suggested that laccase was involved in the decolorization of these four dyes.

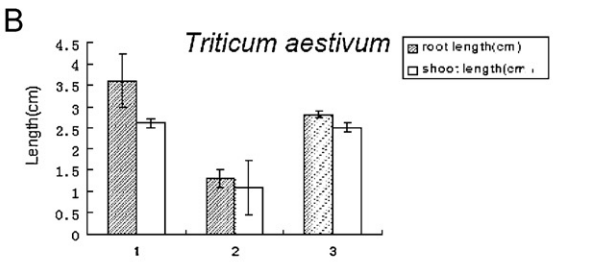
The change of total organic carbon (TOC) before and after decolorization was also studied and shown in Table 3. Significant reductions in TOC were observed after decolorization of these four dyes by *Ganoderma* sp.En3. After decolorization, 92.7%, 84.2%, 86.1% and 63.1% reduction of TOC was observed respectively for malachite green, methyl orange, bromophenol blue and crystal violet (Table 3).

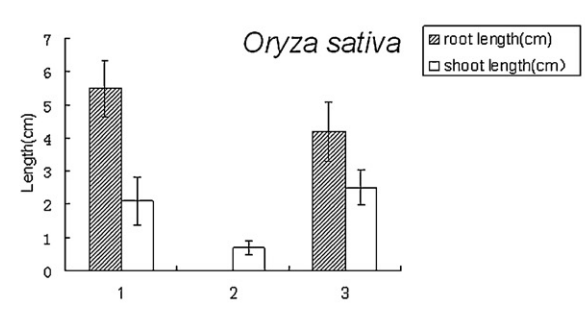
In order to prove effectiveness of the fungal decolorization process, not only decolorization but also the toxicity analysis of the decolorization products was required. Because disposal of the untreated dyeing effluent without any treatment into water bodies was harmful to agriculture, it was of concern to assess the phytotoxicity of the dye before and after decolorizaton [54]. Therefore, the phytotoxicity of these four dyes and their degradation product after fungal treatment were further studied as described in Section 2. The relative sensitivities towards the four dyes and their degradation products in relation to *Triticum aestivum* and *Oryza sativa* seeds were shown in Fig. 3. The results of phytotoxicity study demonstrated that the untreated original dyes could greatly inhibit the growth of shoot and root of *Triticum aestivum* and *Oryza sativa* seeds. The original dyes were toxic to the germination of crop seeds. On the contrary, the decolorization products showed lower phytotoxicity than the original one with respect to *Triticum aestivum* and *Oryza sativa* (Fig. 3). This result indicated that biodegradation of the dyes by *Ganoderma* sp.En3 resulted in its detoxification. Previous research about biodegradation of malachite green by *Kocuria rosea*, *Sphingomonas paucimobilis*, *Fomes sclerodermeus* have revealed the degradation of malachite green into less toxic product [49,51,55]. In our work, the result of phytotoxicity study of malachite green and its degradation product was in agreement with previous research [49,51,55]. As for methyl orange, bromophenol blue and crystal violet, there was no report about the phytotoxicity study of these dyes before and after decolorization previously. In our work, the result of phytotoxicity study firstly demonstrated that *Ganoderma* sp.En3 not only decolorized but also detoxified theses three dyes. To the best of our knowledge, this was the first report about the detoxification of methyl orange, bromophenol blue and crystal violet by fungal treatment. In summary, in the case of the dyes malachite green, methyl orange, bromophenol blue and crystal violet, a significant decrease in the phytotoxicity was achieved after the fungal treatment.

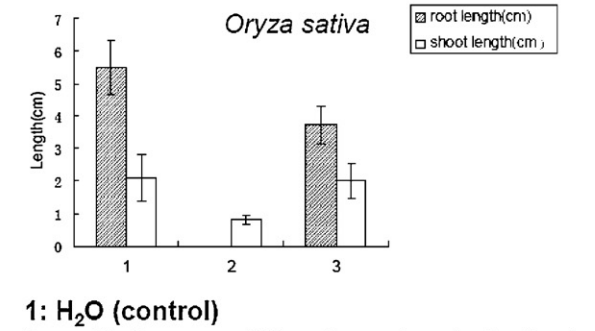
3.1.2. Decolorization of different synthetic dyes by culture supernatants

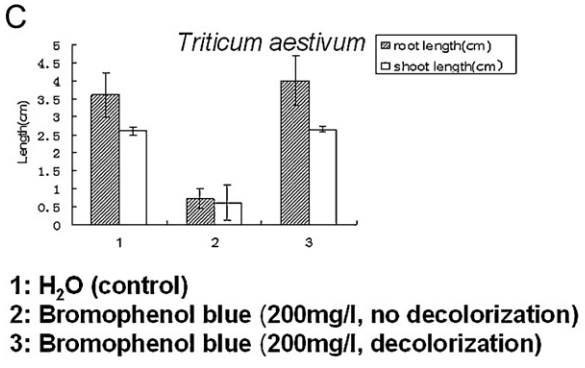
For the practical application, the use of crude laccase is more economical and cost effective. Thus, the exploration of crude laccase for decolorizing four synthetic dyes was investigated. The

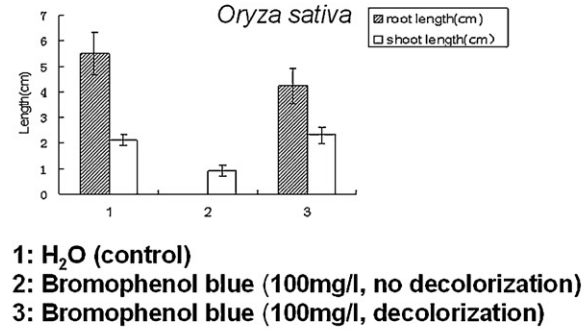


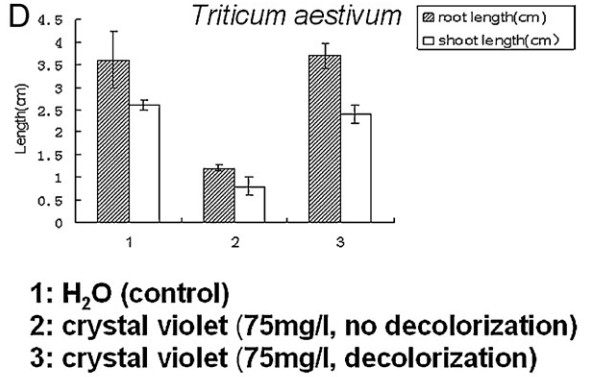












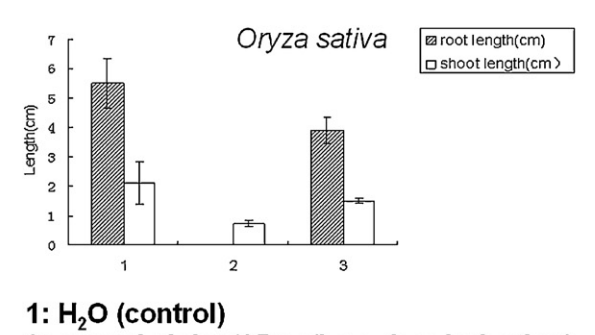


Fig. 3. Phytotoxicity study of four synthetic dyes and their decolorization product after fungal treatment. The relative sensitivities towards the four dyes and their decolorization products in relation to *Triticum aestivum* and *Oryza sativa* seeds were evaluated. Values are mean of germinated seeds of three experiments. The error bars represent SD (standard deviation). A: malachite green, B: methyl orange, C: bromophenol blue, D: crystal violet.

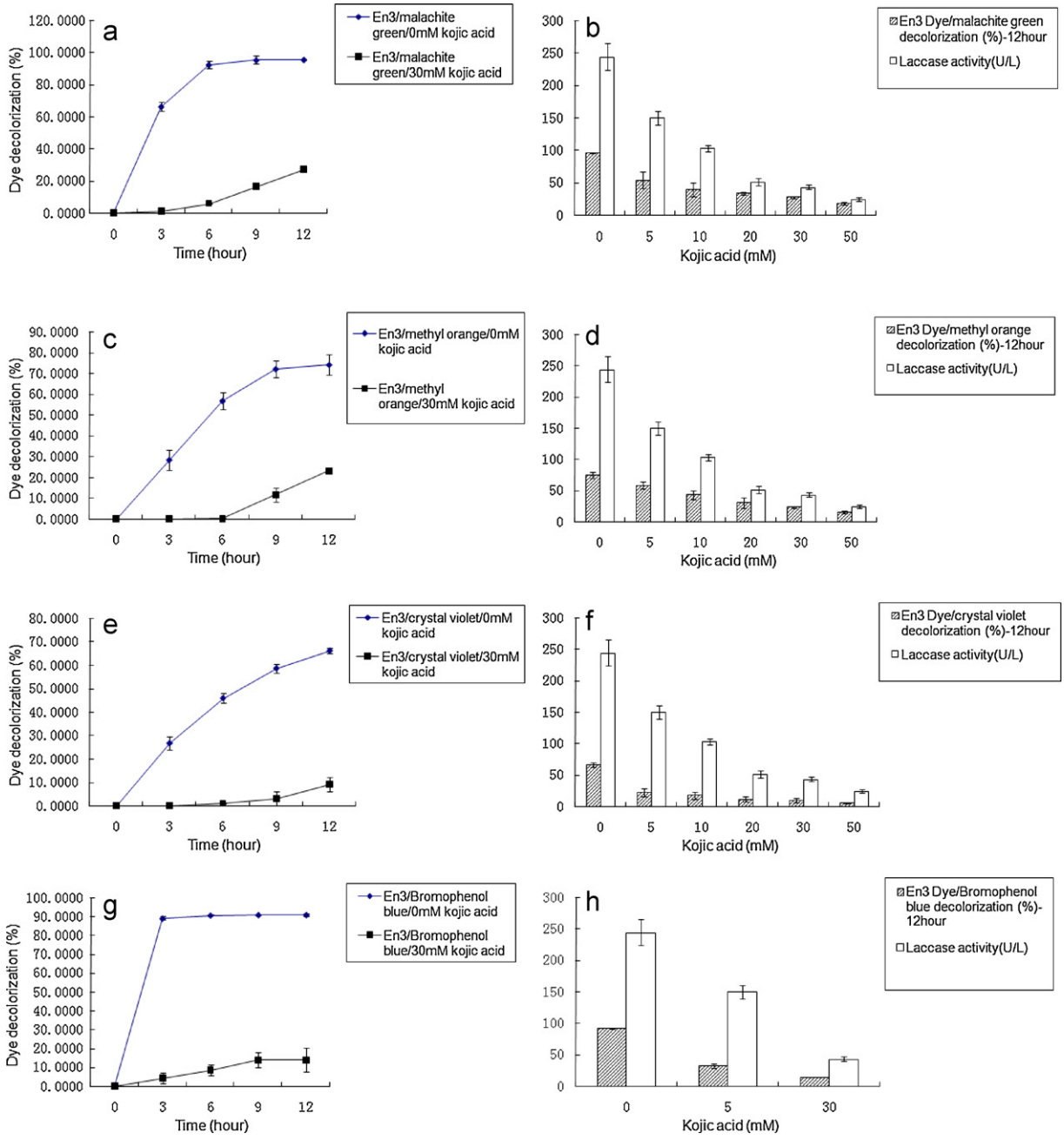


Fig. 4. Decolorization of four synthetic dyes by the crude enzymes produced by *Ganoderma* sp.En3. The culture supernatants prepared from *Ganoderma* sp.En3 grown in GYP medium were used to decolorize four dyes: malachite green, methyl orange, bromophenol blue and crystal violet. The ability to decolorize four dyes could be inhibited by adding kojic acid (30 mM) which was a specific inhibitor of fungal laccase. In this figure, En3/different dyes/30 mM kojic acid means that 30 mM kojic acid was added into the culture supernatants of *Ganoderma* sp.En3 and decolorization was then performed. En3/different dyes/0 mM kojic acid means that no kojic acid was added (a, c, e and g). Various concentrations of kojic acid were added respectively (0, 5, 10, 20, 30, 50 mM). Following the increase of the concentration of kojic acid, the laccase activity decreased. Following the decrease of laccase activity, the decolorization efficiency of four dyes (shown as the dye decolorization % for 12 h of incubation) were also inhibited obviously (b, d, f and h). All experiments were conducted in triplicates. Data are the mean ± SD of triplicate experiments.

culture supernatants prepared from *Ganoderma* sp.En3 grown in GYP medium were further used to decolorize four different dyes as described in Section 2. The highest decolorization (%) for 12 h of incubation was detected to be 95.45% for malachite green, 74.03% for methyl orange, 66.0% for crystal violet, and 90.88% for bromophenol blue (Fig. 4a, c, e and g). The diversity of the chemical structures of dyes might result in the differences in the decolorization efficiencies toward these four synthetic dyes. Small structural differences among the dyes may significantly influence their decolorization efficiency. The differences in electron distribution, charge density, steric factors or dye substituents may result in the differences in the decolorization extent [50,56].

However, it was also found that the ability to decolorize each dye could be inhibited by adding kojic acid into the culture supernatants of *Ganoderma* sp.En3. Fig. 4a, c, e and g indicated that the decolorization efficiency of four dyes was obviously inhibited by adding 30 mM kojic acid. Previous research has proved that kojic acid was a specific inhibitor of fungal laccase. Laccase was a group of multi-copper oxidases which contain four copper atoms in the catalytic centre per molecule. Copper ion was necessary for the

activity of laccase. Complexation of kojic acid with copper ion in the catalytic centre could inhibit the laccase activity [57,58]. Our result also confirmed that the laccase activity of *Ganoderma* sp.En3 could be significantly inhibited by kojic acid. The laccase activity decreased when the concentration of kojic acid increased. Following the decrease of laccase activity, the decolorization efficiency of four dyes (12 h of incubation) was also inhibited obviously (Fig. 4b, d, f and h). The laccase activity was 243.94 U l^{-1} when no kojic acid was added. The decolorization (%) for 12 h of incubation was detected to be 95.45% for malachite green, 74.03% for methyl orange, 66.0% for crystal violet, and 90.88% for bromophenol blue; When 30 mM kojic acid was added, the laccase activity was reduced to 42.9 U l^{-1}, the decolorization (%) for 12 h of incubation was reduced to be 27.0% for malachite green, 23.1% for methyl orange, 9.7% for crystal violet, and 13.89% for bromophenol blue. Thus it indicated that the capability of decolorizing different dyes by *Ganoderma* sp.En3 was positively related to the laccase activity (Fig. 4b, d, f and h).

Because we used the crude culture supernatants to perform the decolorization test, there were perhaps other enzymes besides laccase that may be involved in the decolorization of different dyes by *Ganoderma* sp.En3. But above results obtained by using the specific inhibitor of fungal laccase-kojic acid have clearly demonstrated that laccase played a very important role in the decolorization of different dyes by *Ganoderma* sp.En3. Laccase was at least one of the important enzymes responsible for the efficient decolorization by this fungi.

3.2. Decolorization of simulated dye bath effluent by Ganoderma sp.En3

While many studies were devoted to decolorization of the textile dyes, few work have been reported on decolorization of dye effluents in which the presence of salts, very high ionic strength, extreme pH values and high dye concentration may be inhibitory to biological agents. Thus, in spite of the high efficiency in dye decolorization by some strains, decolorizing industrial effluent was quite troublesome [47,59]. Although above research have revealed that *Ganoderma* sp.En3 showed effective for decolorization of synthetic dyes, it was required for studying its decolorization capability in the presence of high concentration of salts, high ionic strength, and high dye concentration. Therefore, the ability of *Ganoderma* sp.En3 to decolorize the simulated dye bath effluents was further assessed. In this study, *Ganoderma* sp.En3 was used to decolorize two kinds of simulated dye bath effluent with a complex composition. One was designated as simulated dye effluent-I, which was comprised of four different reactive dyes and other auxiliary chemicals. It mimics the dye effluent produced by typical reactive dye industries [45,46]. Another was designated as simulated dye effluent-II, which was based on instructions of the manufacturer Bezema AG (Montlingen, Switzerland) for reactive dyes [47,48]. Detailed compositions of these two simulated dye effluents were described in Section 2. The capability of *Ganoderma* sp.En3 to decolorize and detoxify these complex reactive dye effluents was investigated as following.

Simulated dye effluent-I and simulated dye effluent-II were decolorized by fungal whole cultures as described in Section 2.

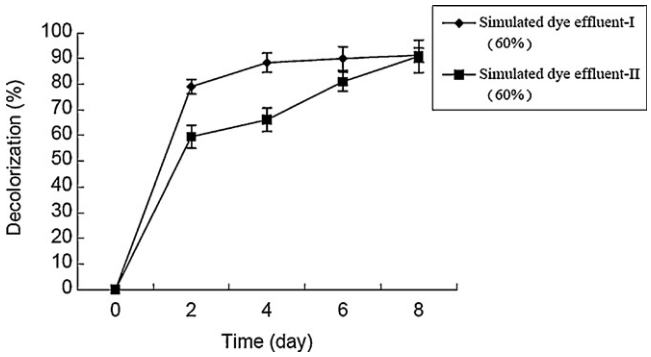


Fig. 5. Decolorization of simulated dye effluent by *Ganoderma* sp.En3 whole cultures. Simulated dye effluent-I and simulated dye effluent-II were decolorized by fungal whole cultures. All experiments were conducted in triplicates. Data are the mean ± SD of triplicate experiments.

Our research demonstrated that *Ganoderma* sp.En3 could cause an effective decolorization of simulated dye effluent-I and II (60%), reaching about 91.3% and 90.9% decolorization within 8 days respectively (Fig. 5). We further compared the capability of *Ganoderma* sp.En3 for decolorizing simulated dye effluent with that of other strains reported previously. Verma et al. [60] have studied the decolorization and detoxification of textile effluent B containing a mixture of reactive dyes by four marine-derived fungi. Textile effluent B (50%) could be decolorized up to 42.8% by NIOCC # 2a (*Cerrena unicolor*), 32.4% by NIOCC # 15V (*Coriolopsis byrsina*), 53.5% by NIOCC # 16V (*Diaporthe* sp.), 58.1% by NIOCC # C3 (*Pestalotiopsis maculans*) within 6 days [60]. Our research found that simulated dye effluent-I, which was comprised of four different reactive dyes and other auxiliary chemicals, could be decolorized up to 90% by *Ganoderma* sp.En3 within 6 days (Fig. 5). Considering the different complexity of dye effluents, the capability of *Ganoderma* sp.En3 to decolorize complex reactive dye effluent was comparable with that of fungal strains reported previously [60]. As for simulated dye effluent-II, Mohorcic et al. [47] have studied the decolorization of simulated dye effluent-II by some fungal strains. Simulated dye effluent-II (30%) could be decolorized up to about 90% by *Bjerkandera adusta*, 90% by *Geotrichum candidum*, 80% by *Trametes versicolor*, 60% by *Phanerochaete chrysosporium*, 60% by *Schizophyllum commune* within 17 days [47]. Our research demonstrated that *Ganoderma* sp.En3 showed 90.9% decolorization of the same simulated dye effluent-II (60%) within 8 days (Fig. 5). Compared with previous research [47], *Ganoderma* sp.En3 could decolorize the simulated dye effluent-II faster. *Ganoderma* sp.En3 could also tolerate higher concentrations of simulated dye effluent, which was more advantageous to decolorize the dyeing effluent with high concentrations. Thus our result suggested that *Ganoderma* sp.En3 had stronger ability to decolorize the simulated dye effluent-II than some other fungal strains reported previously [47].

The change of the activity of lignin modifying enzymes, such as laccase, manganese peroxidase, lignin peroxidase before and after decolorization was studied and shown in Table 4. Lignin modifying

Table 4
The change of the activity of lignin modifying enzymes, TOC before and after decolorization of simulate dye effluent by *Ganoderma* sp.En3. ND: not detected, LiP: lignin peroxidases, MnP: manganese peroxidase, TOC: total organic carbon.

Dye (concentration)	Time (day)	Decolorization (%)	Laccase activity (U l^{-1})	LiP activity (U l^{-1})	MnP activity (U l^{-1})	TOC reduction (%)
Simulate dye effluent-I (60%)	0	0	5.66 ± 2.27	ND	ND	
	8	91.3 ± 2.74	56.85 ± 10.34	ND	ND	73.1
Simulate dye effluent-II(60%)	0	0	7.17 ± 2.42	ND	ND	
	8	90.9 ± 6.34	63.27 ± 6.32	ND	ND	78.0

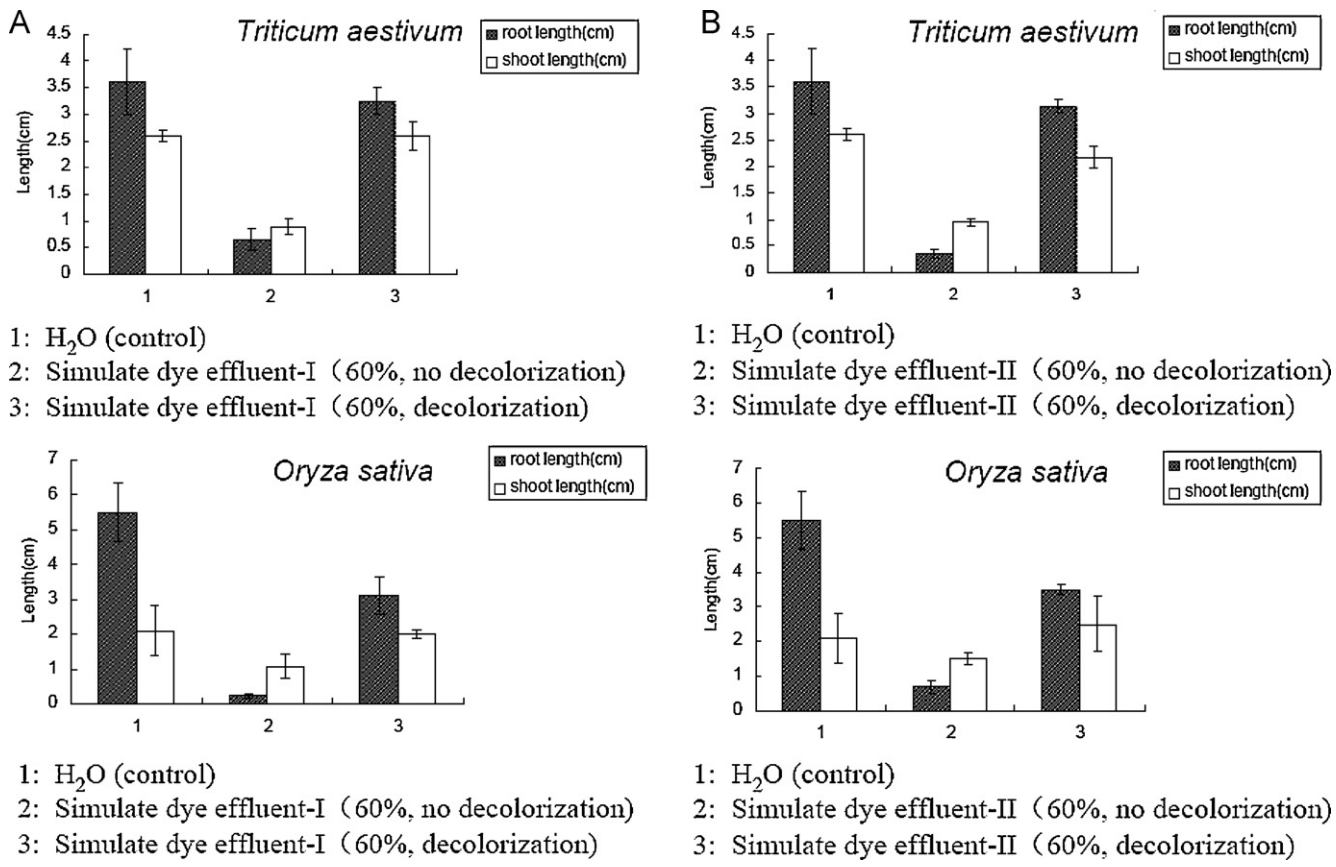


Fig. 6. Phytotoxicity study of two simulated dye effluents before and after fungal treatment. The relative sensitivities towards the original simulated dye effluent and their decolorization products in relation to *Triticum aestivum* and *Oryza sativa* seeds were evaluated. A: simulated dye effluent-I, B: simulated dye effluent-II. Values are mean of germinated seeds of three experiments. The error bars represent SD (standard deviation).

enzyme activities were assayed in decolorized samples treated by *Ganoderma* sp.En3. Extracellular laccase activity was significantly increased by 904.4% and 782.4% respectively after decolorization of simulated dye effluent-I and II (Table 4). Neither manganese peroxidase nor lignin peroxidase was detected during the decolorization process. Induction in the activity of laccase during the decolorization process indicated that laccase was involved in decolorizing these two simulated dye effluents by *Ganoderma* sp.En3. Previous research has found that laccase played a major role in decolorization of dye effluent by basidiomycetes [60]. Our result was in agreement with it.

The change of total organic carbon (TOC) before and after decolorization was also studied and shown in Table 4. Reduction of TOC of these two simulated dye effluents after fungal treatment could be detected. 73.1% and 78.0% reduction of TOC were observed respectively after decolorization of simulated dye effluent-I and II by *Ganoderma* sp.En3 (Table 4).

The high decolorization was not enough to solve the environmental problem caused by dye effluent. It was necessary to evaluate the toxicity of the effluent obtained after the fungal treatment. Thus the phytotoxicity of these two simulated dye effluents before and after fungal treatment were further assessed as described in Section 2. The relative sensitivities towards these two simulated dye effluents and their decolorization products in relation to *Triticum aestivum* and *Oryza sativa* seeds were shown in Fig. 6. The results of phytotoxicity study revealed that the original simulated dye effluent were toxic to the germination of crop seeds. When the seeds were treated with the original simulated dye effluent, the growth of shoot and root of *Triticum aestivum* and *Oryza sativa* seeds were inhibited greatly. But the decolorized effluent showed lower phytotoxicity than the original one with respect to *Triticum aestivum* and *Oryza sativa* (Fig. 6). This result suggested that *Ganoderma* sp.En3 was able to decolorize and detoxify these complex reactive dye effluents. Previous research about the biodegradation of simulated dye effluent-II by immobilised-coated laccase revealed that the decolorized effluent showed lower phytotoxicity than the original one [48]. In our work, the result of phytotoxicity study of simulated dye effluent-II after fungal treatment was in agreement with previous research [48]. As for simulated dye effluent-I, although some study about decolorization have been conducted [46], there were no report about the phytotoxicity study of this effluent before and after decolorization previously. Our work firstly demonstrated that *Ganoderma* sp.En3 not only decolorized but also detoxified simulated dye effluent-I. As far as we know, this was the first report about the detoxification of simulated dye effluent-I by fungal treatment.

3.3. Decolorization and detoxification of the real textile dye effluent by Ganoderma sp.En3

The capability of *Ganoderma* sp.En3 for decolorizing and detoxifying the real textile dye effluent was further investigated. The real textile industry effluent containing indigo dyes was collected from Puqi Textile Dyeing Factory in Hubei province of China, which was designated as real indigo effluent. The characteristics of this wastewater were described in Section 2.

Our research demonstrated that the real indigo effluent could be decolorized efficiently by the white rot fungus, *Ganoderma* sp.En3. The real indigo effluent (100%) could be decolorized up to 91.38% by *Ganoderma* sp.En3 within 14 days (Fig. 7). Considering that the real

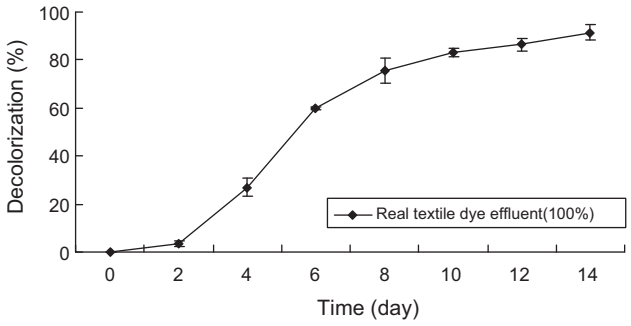


Fig. 7. Decolorization of the real textile dye effluent by *Ganoderma* sp.En3 whole cultures. The real indigo effluent was decolorized by fungal whole cultures. All experiments were conducted in triplicates. Data are the mean ± SD of triplicate experiments.

textile dye effluent treated by fungal cultures was not diluted in this study, our result suggested that *Ganoderma* sp.En3 had strong ability to decolorize the real indigo effluent produced by the textile dyeing factory.

The change of the activity of lignin modifying enzymes, such as laccase, manganese peroxidase, lignin peroxidase before and after decolorization was studied and shown in Table 5. Extracellular laccase activity was increased by 659.6% after decolorization of the real indigo effluent (Table 5). Neither manganese peroxidase nor lignin peroxidase was detected during the decolorization process. Induction in the activity of laccase during the decolorization process suggested that laccase was involved in decolorizing the real indigo effluent by *Ganoderma* sp.En3.

The change of total organic carbon (TOC) and chemical oxygen demand (COD) before and after decolorization was also studied and shown in Table 5. Reduction of TOC and COD of the real indigo effluent after fungal treatment could be detected. 60.1% reduction of TOC and 65.1% reduction of COD were observed respectively after decolorization of the real textile dye effluent by *Ganoderma* sp.En3 (Table 5).

The phytotoxicity of the real indigo effluent before and after fungal treatment was further evaluated as described in Section 2. The relative sensitivities towards the real indigo effluent and its decolorization products in relation to *Triticum aestivum* and *Oryza sativa* seeds were shown in Fig. 8. The results of phytotoxicity study revealed that the untreated wastewater were toxic to the germination of crop seeds. When the seeds were treated with the original real indigo effluent, the growth of shoot and root of *Triticum aestivum* and *Oryza sativa* seeds were inhibited greatly. But the decolorized effluent exhibited lower phytotoxicity than the original one (Fig. 8). This result suggested that the decolorization treatment by *Ganoderma* sp.En3 could also detoxify the real indigo effluent. Decolorization of the real indigo effluent was accompanied by reduction in phytotoxicity.

In summary, our research indicated that *Ganoderma* sp.En3 could decolorize and detoxify the real textile dye effluent containing indigo dyes effectively. Indigo dyes are extensively used by textile industries and largely employed on cellulosic fibers like cotton [61]. World consumption of dyes for cellulosic fibers represents 60,000 tonne year^{-1}, being 5% of this amount for indigo

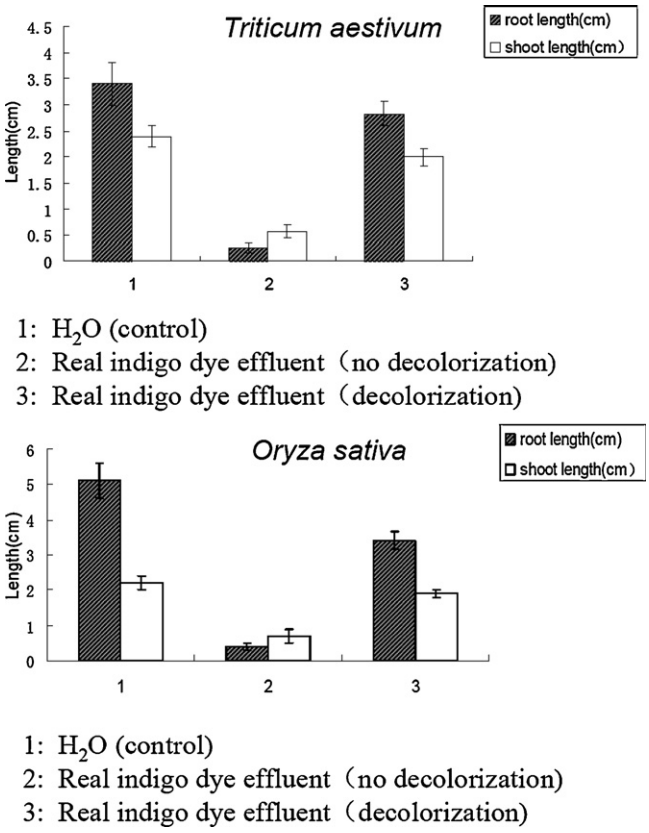


Fig. 8. Phytotoxicity study of the real textile dye effluent before and after fungal treatment. The relative sensitivities towards the original dye effluent and their decolorization products in relation to *Triticum aestivum* and *Oryza sativa* seeds were evaluated. Values are mean of germinated seeds of three experiments. The error bars represent SD (standard deviation).

[61,62]. Although previous research has found that the synthetic indigo dye could be decolorized by some ligninolytic basidiomycete fungi [61], there were few report about the fungal treatment of the real textile effluent containing indigo dyes. Our research suggested that the white-rot fungi strain *Ganoderma* sp.En3 had the practical application value for decolorizing and detoxifying the real textile effluent containing indigo dyes.

3.4. Cloning of full-length cDNA of the laccase gene from *Ganoderma* sp.En3 and bioinformatic analysis

Above results revealed that laccase produced by *Ganoderma* sp.En3 played an important role in the decolorization of four synthetic dyes and dye effluents. In order to better use *Ganoderma* sp.En3 for environmental biotechnology such as decolorization of different industrial dyes, the laccase gene of *Ganoderma* sp.En3 and its full-length cDNA were cloned and characterized in the following work.

The 1566bp full-length cDNA of the laccase gene containing intact ORF was cloned from *Ganoderma* sp.En3. This laccase gene

Table 5
The change of the activity of lignin modifying enzymes, TOC, COD before and after decolorization of the real textile dye effluent by *Ganoderma* sp.En3. ND: not detected, LiP: lignin peroxidases, MnP: manganese peroxidase, TOC: total organic carbon, COD:chemical oxygen demand.

Dye (concentration)	Time (day)	Decolorization (%)	Laccase activity (U l^{-1})	LiP activity (U l^{-1})	MnP activity (U l^{-1})	TOC reduction (%)	COD reduction (%)
Real indigo dye effluent(100%)	0	0	5.12 ± 0.59	ND	ND		
	14	91.38 ± 3.09	38.89 ± 2.61	ND	ND	60.1	65.1

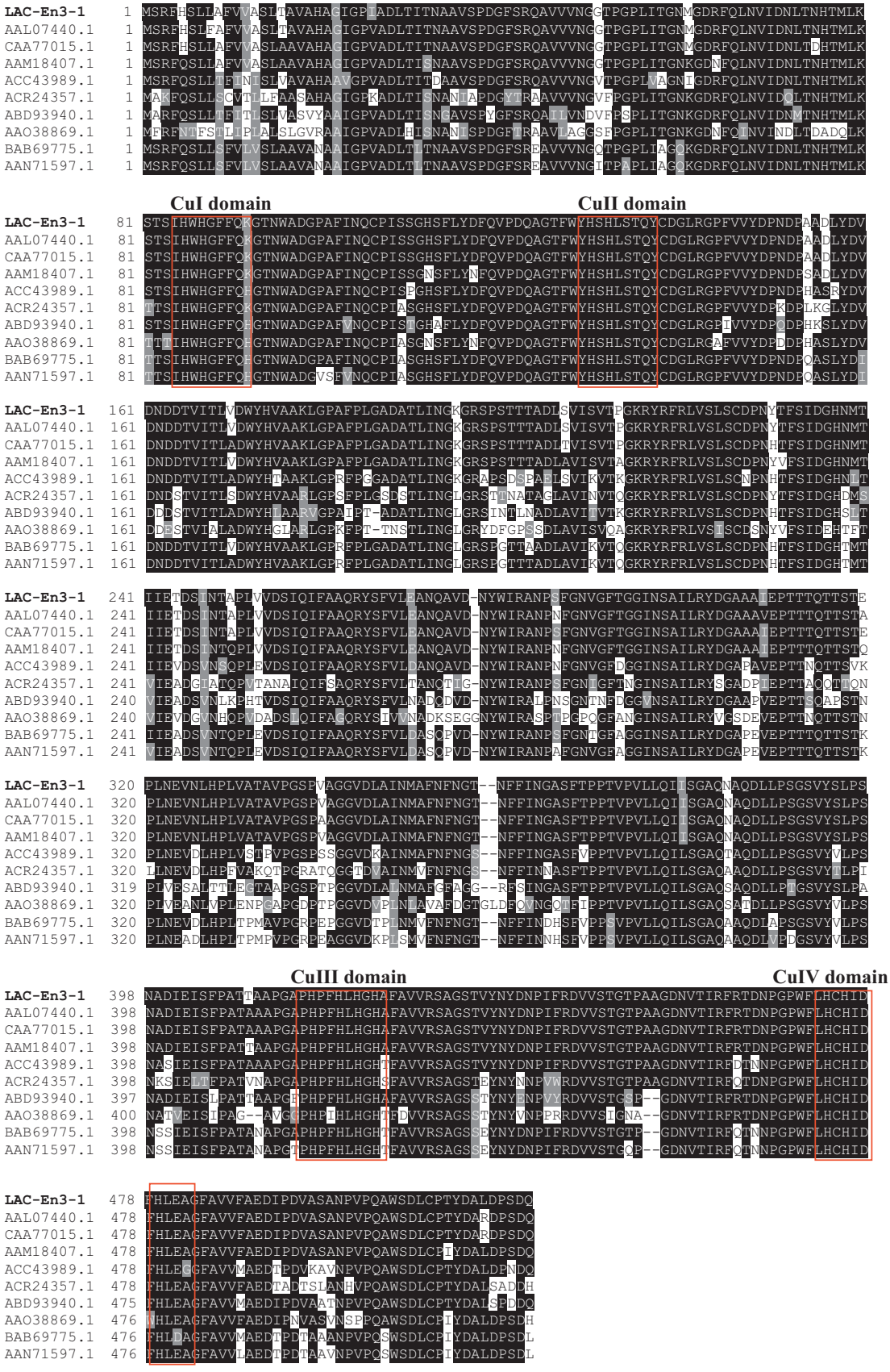


Fig. 9. Alignments of multiple amino acid sequences of LAC-En3-1 and other laccases protein (indicated as the GenBank accession number of each laccase protein). It showed that LAC-En3-1 protein contained four copper-binding conserved domains of typical laccase: CuI (HWHGFFQ), CuII (HSHLSTQ), CuIII (HPFHLHG) and CuIV (HCHIDFHL), which were indicted with box in the figure.

was designated as *lac-En3-1* (GenBank accession no. HM569745). The coding region of *lac-En3-1* consisted of a 1563bp ORF encoding 521 aa with a 21 aa as the signal peptide sequence. The product of *lac-En3-1* was predicted to be a mature protein of 500 aa residues with a calculated molecular mass of 57.1 kDa and isoelectric point of 5.54. LAC-En3-1 contained seven potential N-glycosylation sites (Asn-X-Ser/Thr). LAC-En3-1 protein was closest to laccase from *Ganoderma tsugae* (GenBank accession no. ABK59825), which had 78% identity with the amino acid sequence of *Ganoderma tsugae* laccase. The conserved domains of LAC-En3-1 protein were predicted and analyzed with Conserved Domain Database (CDD). It revealed that LAC-En3-1 contained typical conserved domains of multicopper oxidases. Alignments of multiple amino acid sequences of LAC-En3-1 and other laccase protein were generated with ClustalW2. It showed that LAC-En3-1 protein contained four copper-binding conserved domains of typical laccase: CuI (HWHGFFQ), CuII (HSHLSTQ), CuIII (HPFHLHG) and CuIV (HCHIDFHL) (Fig. 9). Ten conserved histidine residues and one cysteine residue were located in the copper-binding centers of LAC-En3-1 protein. Phylogenetic relationship between LAC-En3-1 and other laccases from different fungal sources was evaluated by constructing a neighbor joining phylogenetic tree. The result demonstrated that LAC-En3-1 from *Ganoderma* sp.En3 was closest to laccases from *Ganoderma tsugae* and *Ganoderma fornicatum* in phylogeny relationship.

3.5. The functionality of lac-En3-1 gene encoding active laccase was verified by expressing this gene in the yeast Pichia pastoris successfully

The plasmid pPIC3.5K-lac-En3-1 in which the full-length cDNA of *lac-En3-1* was cloned into pPIC3.5K (Invitrogen) and the control vector pPIC3.5K were transformed into *Pichia pastoris* GS115 respectively. His^+ transformants were proved to be correct transformants by PCR (data not shown). The transformants were designated as GS115(pPIC3.5K-lac-En3-1) and GS115(pPIC3.5K) respectively.

The expression of *lac-En3-1* gene in transformants was then detected. The BMM agar plates containing $CuSO_4$ and ABTS were used to screen the positive transformants which could produce active laccase. Under the induction of methanol, dark green zones were present around the colonies of GS115(pPIC3.5K-lac-En3-1) after three days growth. On the contrary, no dark green zones appeared around the colonies of the negative control-GS115(pPIC3.5K) (Fig. 10a). After selection of positive transformants which could produce active laccase on ABTS plates, the laccase-positive transformants as well as the negative control-GS115(pPIC3.5K) were then fermented with BMM liquid medium at 20 °C and induced by adding 0.5% (v/v) methanol daily. Laccase activities in cultures were measured daily (shown in Fig. 10b). The highest laccase activity of GS115(pPIC3.5K-lac-En3-1) was 39.34 U l^{-1} after 14-days growth. However, no extracellular laccase activity was detected in culture supernatants of the negative control-GS115(pPIC3.5K). Native-PAGE was also performed using the culture supernatants of yeast transformants to test the recombinant laccase protein. Protein band with the expected laccase activity was significantly detected in the culture supernatant of GS115(pPIC3.5K-lac-En3-1) by ABTS staining on the native PAGE gel, while no signal was detected in the culture supernatant of GS115(pPIC3.5K) (Fig. 10c).

All of above results demonstrated that *lac-En3-1* gene was successfully expressed in *Pichia pastoris*, the active laccase could be produced and secreted properly. The function of *lac-En3-1* gene encoding laccase was further confirmed by means of gene expression.

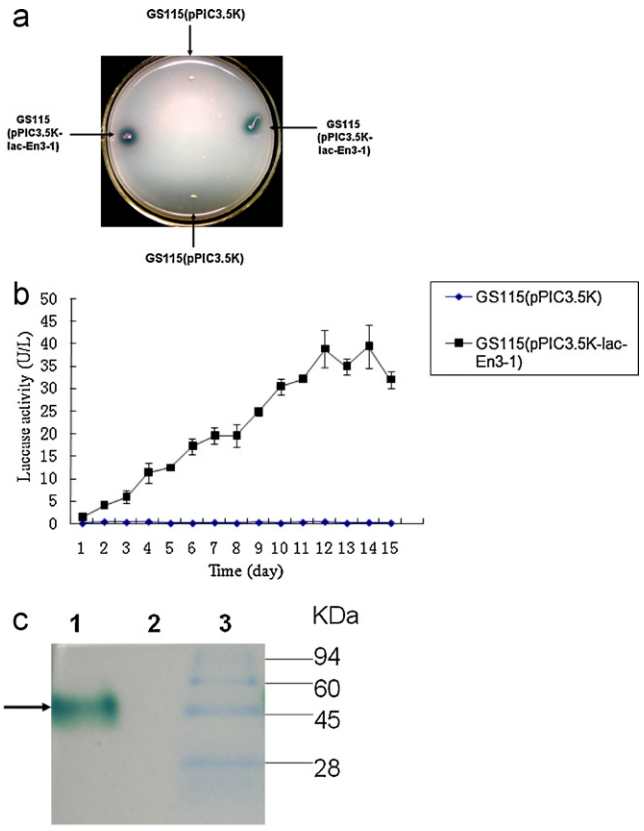


Fig. 10. Detection of the expression of *lac-En3-1* gene in yeast transformants. a: The BMM agar plates containing $CuSO_4$ and ABTS were used to screen the positive transformants which could produce active laccase. It showed that dark green zones were present around the colonies of GS115(pPIC3.5K-lac-En3-1) after three days growth. b: The laccase-positive transformant GS115(pPIC3.5K-lac-En3-1) as well as the negative control-GS115(pPIC3.5K) were fermented with BMM liquid medium at 20 °C, with 0.5% (v/v) methanol being added daily. Laccase activities in cultures were measured daily. All experiments were conducted in triplicates. Data are the mean ± SD of triplicate experiments. c: Native-PAGE was also performed for detecting the laccase activity in the culture supernatants of yeast transformants. Lane 1: GS115(pPIC3.5K-lac-En3-1); Lane2: GS115(pPIC3.5K); Lane 3: protein marker, 94 kDa: Taq enzyme, 60 kDa: BSA, 45 kDa: OVA.

3.6. Decolorization of different dyes by the Pichia pastoris transformants in which the laccase gene was successfully expressed

3.6.1. Decolorization of four synthetic dyes by the Pichia pastoris transformants

The *Pichia pastoris* transformants which could produce active laccase were used to decolorize four synthetic dyes: methyl orange, malachite green, bromophenol blue and crystal violet. As shown in Fig. 11, all of the four dyes could be decolorized by GS115(pPIC3.5K-lac-En3-1), while GS115(pPIC3.5K) not producing active laccase could not decolorize these four dyes. It suggested that the recombinant laccase produced by the transformant in which the *lac-En3-1* gene was successfully expressed could confer the ability to decolorize different dyes. However, different efficiency of decolorization was observed among these dyes. The highest decolorization efficiency for 24 h of incubation was detected to be 90.96% for malachite green, 61.4% for methyl orange, 52.22% for crystal violet, and 80.27% for bromophenol blue (Fig. 11), with malachite green and bromophenol blue being favored. Previous research have found that laccase from the white-rot fungus *Ganoderma lucidum* showed 40.7% decolorization of 25 mg l^{-1} malachite green after 24 h of incubation. The addition of natural phenolic compounds as the redox mediator resulted in several fold enhanced decoloriza-

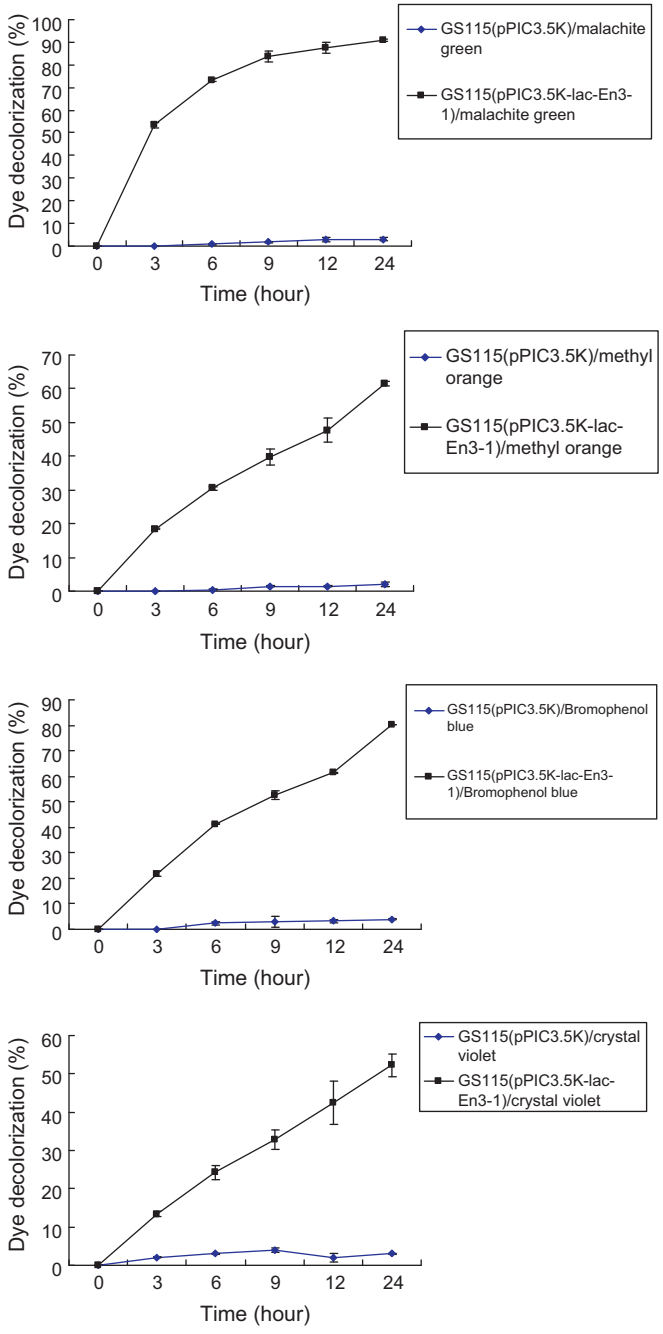


Fig. 11. Decolorization of four synthetic dyes by the *Pichia pastoris* transformants in which the laccase gene from *Ganoderma* sp.En3 was successfully expressed. GS115(pPIC3.5K-lac-En3-1) which could produce active laccase was used to decolorize four dyes: methyl orange, malachite green, bromophenol blue and crystal violet. GS115(pPIC3.5K) which could not produce active laccase was also used to decolorize these four dyes (as the negative control). All experiments were conducted in triplicates. Data are the mean ± SD of triplicate experiments.

tion rates. 50 mg l^{-1} malachite green was respectively decolorized up to 89.7%, 84.7%, 79.0% and 47.0% in the presence of catechol, phenol, guiacol, and 2,4-dimethoxy phenol within 24 h. If no mediator was added, 50 mg l^{-1} malachite green was decolorized up to only 12% within 24 h [63]. Our work revealed that the recombinant laccase from *Ganoderma* sp.En3 could produce 90.96% decolorization of 50 mg l^{-1} malachite green within 24 h in the absence of any mediator (Fig. 11), which suggested that the recombinant laccase derived from *Ganoderma* sp.En3 had stronger capability for decolorizing malachite green compared with some other known laccase [63]. Tong et al. have used the laccase from the white-rot fungus *Trametes* sp. 420 to decolorize bromophenol blue. The decolorization efficiency for bromophenol blue (50 mg l^{-1}) only reached 20% within 8 h without adding any mediator. If a redox mediator, ABTS was added, the decolorization efficiency for bromophenol blue (50 mg l^{-1}) slowly increased to 45% within 8 h [64]. Our work demonstrated that the recombinant laccase derived from *Ganoderma* sp.En3 could produce 52.51% decolorization of bromophenol blue (50 mg l^{-1}) within 9 h without any mediator. Bromophenol blue (50 mg l^{-1}) could be decolorized up to 80.27% within 24 h without any mediator (Fig. 11). It suggested that the recombinant laccase derived from *Ganoderma* sp.En3 could decolorize bromophenol blue more efficiently than the laccase from *Trametes* sp. 420 [64]. In summary, the recombinant laccase derived from *Ganoderma* sp.En3 possess stronger capacity for decolorizing malachite green and bromophenol blue compared with some other known laccase [63,64]. In addition, the high decolorization efficiency could be achieved by the recombinant laccase derived from *Ganoderma* sp.En3 without addition of any mediator, which was more economical for practical application.

Kojic acid was found to be a specific inhibitor of fungal laccase [57,58]. Therefore, it was used to further detect whether the decolorization of dyes was dependent on laccase. Kojic acid was added into the culture supernatants of GS115(pPIC3.5K-lac-En3-1) at various concentration of 0, 5, 10, 20 and 50 mM respectively. Then the laccase activity and decolorization of four dyes were detected at the same time. It was found that the laccase activity decreased gradually when the concentration of kojic acid increased. When the concentration of kojic acid was increased to be 50 mM, the laccase activity was inhibited to be only 8.02% of the control without adding kojic acid (Fig. 12). Following the decrease of laccase activity, the decolorization efficiency of four dyes (12 h of incubation) were also inhibited significantly. After addition of kojic acid (50 mM), the decolorization efficiency of malachite green, methyl orange, bromophenol blue and crystal violet were reduced to be 7.84%, 2.96%, 5.69% and 17.78% of the control without adding kojic acid respectively (Fig. 12). Thus, above results further demonstrated that the capability of decolorizing different dyes was positively related to the laccase activity. Laccase could play a very important role in the decolorization of different dyes. In summary, by using kojic acid as the specific inhibitor of fungal laccase, our work further prove that laccase is responsible for the capability of decolorization of different dyes.

3.6.2. Decolorization of simulated dye bath effluent by the purified recombinant laccase produced by Pichia pastoris transformant

The recombinant laccase produced by *Pichia pastoris* transformant in which *lac-En3-1* gene was successfully expressed was purified as described in Section 2. The purified recombinant laccase, which was designated as rLAC-EN3-1, had a specific laccase activity of 8.81 U mg^{-1}. Then the purified rLAC-EN3-1 was used to decolorize simulated dye effluent-I and simulated dye effluent-II respectively. As shown in Fig. 13, both of the simulated dye effluent could be decolorized by purified rLAC-EN3-1. Simulated dye effluent-I and simulated dye effluent-II were respectively decolorized up to 81.54% and 85.48% by the purified recombinant laccase within 36 h (Fig. 13). Thus this result demonstrated that the recombinant laccase produced by *Pichia pastoris* transformant, in which the laccase gene from *Ganoderma* sp.En3 was successfully expressed, was able to decolorize these two simulated dye effluents. We further compared the capability of recombinant laccase derived from *Ganoderma* sp.En3 for decolorizing simulated dye effluent with that of other known laccases reported previously. Murugesan et al. [46] studied the decolorization of simulated dye effluent-I by laccase obtained from *Ganoderma lucidum*. Laccase

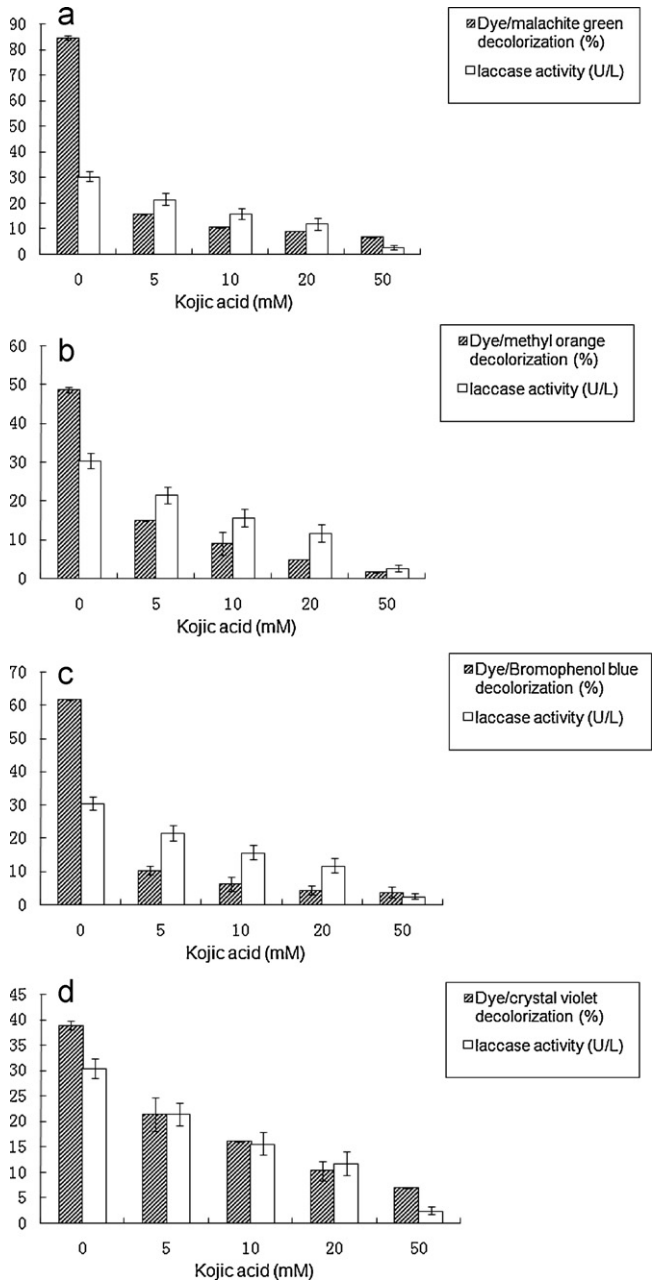


Fig. 12. The ability for decolorizing four synthetic dyes by the *Pichia pastoris* transformant GS115(pPIC3.5K-lac-En3-1) could be inhibited by adding kojic acid which decreased the laccase activity significantly. In this figure, the ability for decolorizing four dyes was indicated as dye decolorization (%) for 12 h of incubation. Kojic acid was added into the culture supernatants of GS115(pPIC3.5K-lac-En3-1) at various concentrations of 0, 5, 10, 20 and 50 mM respectively. Then the laccase activity and decolorization of four dyes were detected. a: malachite green; b: methyl orange; c: bromophenol blue; d: crystal violet. All experiments were conducted in triplicates. Data are the mean ± SD of triplicate experiments.

from *Ganoderma lucidum* alone failed to decolorize the effluent. Treatment of simulated dye effluent revealed that decolorization was observed only in the presence of redox mediator. During 24 h incubation, maximum of 82% and 80% decolorization of simulated dye effluent-I were observed in presence of HBT and syringaldehyde respectively [46]. Our work suggested that the recombinant laccase derived from *Ganoderma* sp.En3 could decolorize simulated dye effluent-I without addition of any redox mediator. Simulated dye effluent-I was decolorized up to 81.54% by the purified recombinant laccase within 36 h in the absence of any mediator (Fig. 13).

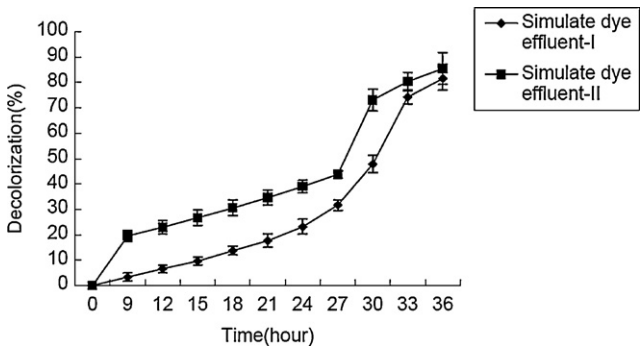


Fig. 13. Decolorization of simulated dye effluent by the purified recombinant laccase produced by *Pichia pastoris* transformant in which the laccase gene from *Ganoderma* sp.En3 was successfully expressed. All experiments were conducted in triplicates. Data are the mean ± SD of triplicate experiments.

Thus our work revealed that redox mediator was not required for efficient decolorization of simulated dye effluent by the recombinant laccase derived from *Ganoderma* sp.En3. This was the valuable advantage of laccase derived from *Ganoderma* sp.En3 for decolorizing dyeing effluent. Thus, the recombinant laccase derived from *Ganoderma* sp.En3 had stronger ability to decolorize simulated dye effluent-I than some other known laccase [46]. Osma et al. [48] have studied the biodegradation of simulated dye effluent-II by immobilised-coated laccase from *Trametes pubescens*. About 90% decolorization of the effluent was obtained within 36 h for the batch mode operation [48]. Our work revealed that the recombinant laccase derived from *Ganoderma* sp.En3 could produce 85.48% decolorization of simulated dye effluent-II within 36 h without addition of any redox mediator (Fig. 13). As for simulated dye effluent-II, the decolorization capability of recombinant laccase from *Ganoderma* sp.En3 was very similar to that of laccase from *Trametes pubescens* reported by Ref. [48].

3.6.3. Decolorization of the real textile dye effluent by the purified recombinant laccase produced by Pichia pastoris transformant

The purified rLAC-EN3-1 was also used to decolorize the real indigo effluent *in vitro*. As shown in Fig. 14, the real indigo effluent could be decolorized up to 83.72% by the purified recombinant laccase within 36 h (Fig. 14A). The laccase activity of rLAC-EN3-1 was inhibited greatly by adding kojic acid (Fig. 14B). Following the decrease of laccase activity, the decolorization efficiency of the real indigo effluent was also inhibited significantly (Fig. 14A). This result further proved that laccase played a very important role in the *in vitro* decolorization of the real textile dye effluent. Thus our research demonstrated that the recombinant laccase produced by *Pichia pastoris* transformant, in which the laccase gene from *Ganoderma* sp.En3 was successfully expressed, was able to decolorize the real indigo effluent. The laccase derived from *Ganoderma* sp.En3 had great potential for decolorizing the real textile effluent containing indigo dyes.

3.7. Cloning and analysis of the 5′-flanking sequence upstream of the start codon ATG of lac-En3-1 gene

The 2118bp complete structural gene of *lac-En3-1* was cloned and sequenced as described in Section 2. The *lac-En3-1* structural gene contained nine introns and ten exons. All of the splicing junctions and internal lariat formation sites of the introns adhered to the GT-AG rule of eukaryotic gene.

The 1425bp 5′-flanking sequence upstream of the start codon ATG in *lac-En3-1* gene was obtained by Self-Formed Adaptor PCR (SEFA PCR) and then analyzed for the presence of putative *cis*-acting elements involved in transcriptional regulation. The putative

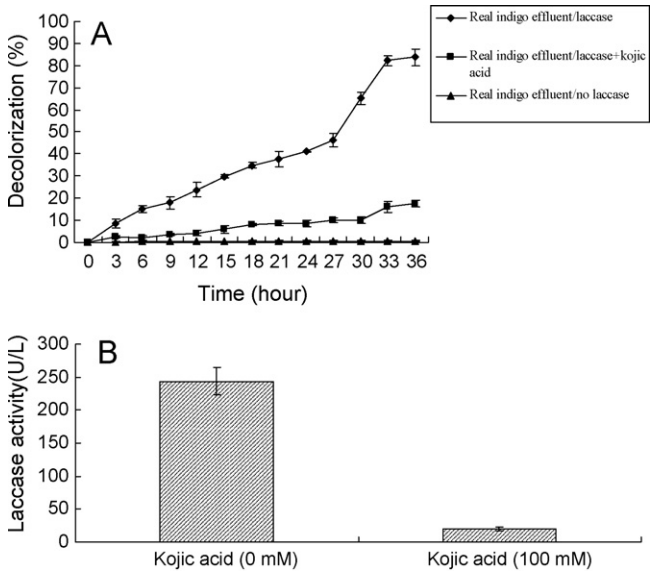


Fig. 14. Decolorization of the real textile dye effluent by the purified recombinant laccase produced by *Pichia pastoris* transformant in which the laccase gene from *Ganoderma* sp.En3 was successfully expressed. Real indigo effluent/laccase + kojic acid: kojic acid was added into the reaction mixture at a final concentration of 100 mM; Real indigo effluent/no laccase: no laccase was added into the reaction mixture. All experiments were conducted in triplicates. Data are the mean ± SD of triplicate experiments.

promoter region of *lac-En3-1* extending 1425bp upstream of the start codon was shown in Fig. 15. The TATA box was located at nt positions -63bp upstream from the start codon ATG. One CAAT box was located at -276 upstream from the start codon. Some putative response elements were also found in the promoter region (shown in Fig. 15). Five putative CreA-binding sites (SYGGRG) [65] were located at positions -261, -903, -1009, -1054, -1338. CreA was a DNA-binding protein belonging to Cys2-His2 zinc finger class. It was identified as a repressor involved in the glucose repression in *Aspergillus nidulans* [66]. The presence of five putative CreA-binding sites in the promoter region of the *lacEn3-1* implied that the expression of *lacEn3-1* gene may be repressed by glucose. Two putative metal-responsive elements (MREs) adhering to the consensus sequence TGCRCNC which conferred the ability to respond to heavy metal [67] were present at positions -536 and -1352 respectively. One putative xenobiotic-responsive element (XRE) matching the consensus sequence CACGCW [68] was also detected at position -906. XREs were important *cis*-acting elements which could mediate the transcriptional activation of eukaryotic genes by aromatic compounds [68]. One potential stress-responsive element (STRE) with the consensus sequence of CCCCT [69] was also detected at position -92. One ACE element adhering to the consensus sequence HWHNNGCTGD or NTNNHGCTGN was present at position -840. ACE element was the target sequence of the ACE1 copper-responsive transcription factor originally found in *Saccharomyces cerevisiae* [70,71]. Recent research have found that expression of genes encoding laccase and manganese-dependent

gtagacctgataggctcaatacttaccgtcttgtcgaacgcagtggtagagaaatctggt

cactttgcgatacgggcgcaaccgtaagtggagaatcttgtgccctcacatcgggagtcttgaatacattctgcttgcgtcac
NIT2 MRE CreA
tgtggtggttgtgtgggcgatggaggacagacgagaagtatacgcccttatgcaccgccatgattcaagttgcttggttcgg

ttttcaagggacatgcgcctgcttgtcaattaaagtcagatcacgacactctcaatccgaggagcgctcacatattggatatttt

atggcgcaatgcttgtgaaccgtagaggccggccgcaagggagcctcagcggcctcgtgccgcggggactcagataag
HSE CreA
tactccctggacgagtcgctcgtcaccgctggagagaaatggctcttcactgccctatcggacctagggtacctattcgcat
CreA
gcggccttttggattggtacgattttagtctttgaaatttgaatgggcgagcgtggagacatgatgtgatgtacagtgggagca
XRE CreA
cggtggttgaagacgatagacgcctgcaacaccagctcaagcgcttgattggcagaagagttaatattattattattaattcga
NIT2 ACE HSE
ctgacggactccgagtcggatatcataatcctaggcctcgcgcggactcggatgtgccggtggaccagtcaaggaccagt
NIT2
ccacatcgtcgttggcaccgtccacatcgtcgttggcaccgtccacatcgtcgttggcaccgtccacatcgtcgttggcgcc

gtccacatcgtcgttggcaccgtccacatcgtcgttggcacccttcaccgctcaacacgatccttttcatattcaggtcgtactt

acgtgagcgcaagtatccctgtcggacacccagcacctcaagttcgatcaaggccctaagttggccactggcatgggctg
MRE
caattcaagtacactgctggtacactgctcgccggccagactcgcggcgcatccatgctagcggccagtttatgagaggta

tggagtctagtaccacattgcatttctgtttctcatacgcaaggttgaagatcacccaagggtcgttggtccagttccccgttcg

atgatttcaactctgacaccaatgcctacgatgctccgcggatggcgccaacaaagttccgtttggggacgcgttctgtcctg
CAAT box CreA
tgtcaagcgaggctctcgcatatcccgtaggcttaggtagcatcagcatgcgtaactaaacctcgccatcgtcacgtacggc

ccccgtatggctatcggtctggcacacacagatatttccccctggcgggctgtcatcgctggctgggtataaagtactccta
STRE GC box TATA box
cctgatcctggttcatctcagactgggctcctctatctgttgcgccc***atggtcaga***
+1

Fig. 15. The 1425bp 5′-flanking sequence upstream of the start codon ATG in *lac-En3-1* gene. The putative *cis*-acting responsive elements in the promoter region were underlined and indicated as the following abbreviations. CreA: CreA-binding sites; MRE: metal-responsive elements; XRE: xenobiotic-responsive elements; ACE: ACE element; STRE: stress-responsive element; NIT2: consensus sequences for binding of NIT2 transcription factor; HSE: heat-shock element. CAAT box, TATA box and GC box were also underlined and boxed. The first nucleotide of the start codon ATG was designated as +1.

peroxidase in the fungus *Ceriporiopsis subvermispora* was mediated by an ACE1-like copper-fist transcription factor [72]. Three putative consensus sequences (TATCDH) for the binding of NIT2 transcription factor, which was the major positive regulatory protein involved in the nitrogen regulation of gene expression in fungi [73], were also present at positions -767, -859, and -1359 respectively. Two potential heat-shock elements (HSE) composed of the repeated 5bp NGAAN motif [74] were located at positions -818 and -1100 relative to start codon ATG. In summary, the presence of these putative *cis*-acting responsive elements in the promoter region of *lac-En3-1* suggested that these response elements may play a role in the regulation of *lac-En3-1* gene expression at the level of transcription.

4. Conclusions

Ganoderma sp.En3 isolated from the forest of Tzu-chin Mountain in China had a strong ability of decolorizing and detoxifying synthetic dyes, simulated dye bath effluents and the real textile dye effluent. Laccase played an important role in the efficient decolorization of different dyes by this fungus. The laccase gene *lac-En3-1* and its corresponding full-length cDNA were cloned and characterized from *Ganoderma* sp.En3. The functionality of *lac-En3-1* gene encoding active laccase was verified by expressing this gene in the yeast *Pichia pastoris* successfully. The recombinant laccase produced by the yeast gene-engineering strain was able to decolorize different synthetic dyes, simulated dye effluents and real textile dye effluent. Many putative *cis*-acting responsive elements, which may play a role in the transcriptional regulation of *lac-En3-1* gene by different factors, were discovered in the promoter region of *lac-En3-1*. In summary, *Ganoderma* sp.En3 had great potential for decolorizing and detoxifying synthetic dyes and dye bath effluents.

Acknowledgments

This work was supported by the Open Fund of Key Laboratory of Oil Crops Biology of Ministry of Agriculture in China, National Natural Sciences Foundation of China (Nos. 30800007, 31070069), Doctoral Fund of the New Teacher Program of Ministry of Education of China (No. 200804871024), The Fundamental Research Funds for the Central Universities, HUST: M2009046, Natural Sciences Foundation of Hubei Province (No. 2009CDB009), Major S&T Projects on the Cultivation of New Varieties of Genetically Modified Organisms (Grant 2009ZX08009-120B), Major State Basic Research Development Program of China (2007CB210200).

References

[1] C.F. Thurston, The structure and function of fungal laccases, Microbiology 140 (1994) 19–26.
[2] P. Baldrian, Laccases-occurrence and properties, FEMS Microbiol. Rev. 30 (2006) 215–242.
[3] C. Eggert, U. Temp, K.E.L. Eriksson, Laccase is essential for lignin degradation by the white-rot fungus *Pycnoporus cinnabarinus*, FEBS Lett. 407 (1997) 89–92.
[4] J. Zhao, H.S. Kwan, Characterization, molecular cloning, and differential expression analysis of laccase genes from the edible mushroom *Lentinula edodes*, Appl. Environ. Microbiol. 65 (1999) 4908–4913.
[5] H.H. Suguimoto, A.M. Barbosa, R.F.H. Dekker, R.J.H. Castro-Gomez, Veratryl alcohol stimulates fruiting body formation in the oyster mushroom, *Pleurotus ostreatus*, FEMS Microbiol. Lett. 194 (2001) 235–238.
[6] S.C. Chen, W. Ge, J.A. Buswell, Molecular cloning of a new laccase from the edible straw mushroom *Volvariella volvacea*: possible involvement in fruit body development, FEMS Microbiol. Lett. 230 (2004) 171–176.
[7] G.H. Choi, T.G. Larson, D.L. Nuss, Molecular analysis of the laccase gene from the chestnut blight fungus and selective suppression of its expression in an isogenic hypovirulent strain, Mol. Plant Microbe Interact. 5 (1992) 119–128.
[8] P.R. Williamson, K. Wakamatsu, S. Ito, Melanin biosynthesis in *Cryptococcus neoformans*, J. Bacteriol. 180 (1998) 1570–1572.
[9] M. Nagai, M. Kawata, H. Watanabe, M. Ogawa, K. Saito, T. Takesawa, K. Kanda, T. Sato, Important role of fungal intracellular laccase for melanin synthesis, purification and characterization of an intracellular laccase from *Lentinula edodes* fruit bodies, Microbiology 149 (2003) 2455–2462.
[10] K. Shuttleworth, J.M. Bollag, Soluble and immobilized laccase as catalysts for the transformation of substituted phenols, Enzyme Microb. Technol. 8 (1986) 171–177.
[11] A.I. Yaropolov, O.V. Skorobogat'ko, S.S. Vartanov, S.D. Varfolomeyev, Laccase: properties, catalytic mechanism, and applicability, Appl. Biochem. Biotechnol. 49 (1994) 257–280.
[12] N. Duran, E. Esposito, Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment, a review, Appl. Catal. B: Environ. 28 (2000) 83–99.
[13] A.M. Mayer, R.C. Staples, Laccase, new functions for an old enzyme, Phytochemistry 60 (2002) 551–565.
[14] L.J. Jönsson, K. Sjöström, I. Häggström, P.O. Nyman, Characterization of a laccase gene from the white rot fungus *Trametes versicolor* and structural features of basdiomycete laccases, Biochim. Biophys. Acta 1251 (1995) 210–215.
[15] D.S. Yaver, F. Xu, E.J. Golightly, K.M. Brown, S.H. Brown, M.W. Rey, P. Schneider, T. Halkier, K. Mondorf, H. Dalboge, Purification, characterization, molecular cloning and expression of two laccase genes from the white rot basidimoycete *Trametes villosa*, Appl. Environ. Microbiol. 62 (1996) 834–841.
[16] E. Ong, W. Brent, R. Pollock, M. Smith, Cloning and sequence analysis of two laccase complementary DNAs from the ligninolytic basidiomycete *Trametes versicolor*, Gene 196 (1997) 113–119.
[17] C. Galhaup, S. Goller, C.K. 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.
[18] C. Pezzella, F. Autore, P. Giardina, A. Piscitelli, G. Sannia, V. Faraco, The *Pleurotus ostreatus* laccase multi-gene family, isolation and heterologous expression of new family members, Curr. Genet. 55 (2009) 45–57.
[19] L. Otterbein, E. Record, S. Longhi, M. Asther, S. Moukha, Molecular cloning of the cDNA encoding laccase from *Pycnoporus cinnabarinus* I-937 and expression in *Pichia pastoris*, Eur. J. Biochem. 267 (2000) 1619–1625.
[20] D.M. Soden, J. O'Callaghan, A.D.W. Dobson, Molecular cloning of a laccase isozyme gene from *Pleurotus sajor-caju* and expression in the heterologous *Pichia pastoris* host, Microbiology 148 (2002) 4003–4014.
[21] M. Guo, F.P. Lu, J. Pu, D.Q. Bai, L.X. Du, Molecular cloning of the cDNA encoding laccase from *Trametes versicolor* and heterologous expression in *Pichia methanolica*, Appl. Microbiol. Biotechnol. 69 (2005) 178–183.
[22] P. Cassland, L.J. Jönsson, Characterization of a gene encoding *Trametes versicolor* laccase A and improved heterologous expression in *Saccharomyces cerevisiae* by decreased cultivation temperature, Appl. Microbiol. Biotechnol. 52 (1999) 393–400.
[23] H. Hoshida, M. Nakao, H. Kanazawa, K. Kubo, T. Hakukawa, K. Morimasa, R. Akada, Y. Nishizawa, Isolation of five laccase gene sequences from the white-rot fungus *Trametes sanguinea* by PCR, and cloning, characterization and expression of the laccase cDNA in yeasts, J. Biosci. Bioeng. 92 (2001) 372–380.
[24] T. Bulter, M. Alcalde, V. Sieber, P. Meinhold, C. Schlachtbauer, F.H. Arnold, Functional expression of a fungal laccase in *Saccharomyces cerevisiae* by directed evolution, Appl. Environ. Microbiol. 69 (2003) 987–995.
[25] V. Faraco, C. Ercole, G. Festa, P. Giardina, A. Piscitelli, G. Sannia, Heterologous expression of heterodimeric laccases from *Pleurotus ostreatus* in *Kluyveromyces lactis*, Appl. Microbiol. Biotechnol. 77 (2008) 1329–1335.
[26] C. Jolivalt, C. Madzak, A. Brault, E. Caminade, C. Malosse, C. Mougin, Expression of laccase IIIb from the white-rot fungus *Trametes versicolor* in the yeast *Yarrowia lipolytica* for environmental applications, Appl. Microbiol. Biotechnol. 66 (2005) 450–456.
[27] L.F. Larrondo, M. Avila, L. Salas, D. Cullen, R. Vicuna, Heterologous expression of laccase cDNA from *Ceriporiopsis subvermispora* yields copper-activated apoprotein and complex isoform patterns, Microbiology 149 (2003) 1177–1182.
[28] E. Record, P.J. Punt, M. Chamkha, M. Labat, C.A.M.J.J. van Den Hondel, M. Asther, Expression of the *Pycnoporus cinnabarinus* laccase gene in *Aspergillus niger* and characterization of the recombinant enzyme, Eur. J. Biochem. 269 (2002) 602–609.
[29] H. Hoshida, T. Fujita, K. Murata, K. Kubo, R. Akada, Copper-dependent production of a *Pycnoporus coccineus* extracellular laccase in *Aspergillus oryzae* and *Saccharomyces cerevisiae*, Biosci. Biotechnol. Biochem. 69 (2005) 1090–1097.
[30] M. Saloheimo, M.L. Niku-Paavola, Heterologous production of a ligninolytic enzyme: expression of the *Phlebia radiata* laccase gene in *Trichoderma reesei*, Biotechnology 9 (1991) 987–990.
[31] L.L. Kiiskinen, K. Kruus, M. Bailey, E. Ylosmaki, M. Siika-aho, M. Saloheimo, Expression of *Melanocarpus albomyces* laccase in *Trichoderma reesei* and characterization of the purified enzyme, Microbiology 150 (2004) 3065–3074.
[32] P.J. Collins, A.D.W. Dobson, Regulation of laccase gene transcription in *Trametes versicolor*, Appl. Environ. Microbiol. 63 (1997) 3444–3450.
[33] D.M. Soden, A.D.W. Dobson, Differential regulation of laccase gene expression in *Pleurotus sajor-caju*, Microbiology 147 (2001) 1755–1763.
[34] M. Mansur, T. Suarez, A.E. Gonzalez, Differential expression in the laccase gene family from basidiomycete I-62 (CECT 20197), Appl. Environ. Microbiol. 64 (1998) 771–774.
[35] M.C. Terrón, T. González, J.M. Carbajo, S. Yagüe, A. Arana-Cuenca, A. Téllez, A.D. Dobson, A.E. González, Structural close-related aromatic compounds have different effects on laccase activity and on lcc gene expression in the ligninolytic fungus *Trametes* sp. I-62, Fungal Genet. Biol. 41 (2004) 954–962.
[36] Y.G. Liu, N. Mitsukawa, T. Oosumi, R.F. Whittier, Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR, Plant J. 8 (1995) 457–463.

[37] S.M. Wang, J. He, Z.L. Cui, S.P. Li, Self-formed adaptor PCR, a simple and efficient method for chromosome walking, Appl. Environ. Microbiol. 73 (2007) 5048–5051.
[38] C. Eggert, U. Temp, K.E. Eriksson, The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*, purification and characterization of the laccase, Appl. Environ. Microbiol. 62 (1996) 1151–1158.
[39] G. Palmieri, P. Giardina, C. Bianco, B. Fontanella, G. Sannia, Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus*, Appl. Environ. Microbiol. 66 (2000) 920–924.
[40] M.S. Revankar, S.S. Lele, Synthetic dye decolorization by white rot fungus, *Ganoderma* sp. WR-1, Bioresour. Technol. 98 (2007) 775–780.
[41] K.T. Steffen, M. Hofrichter, A. Hatakka, Mineralisation of ^{14}C-labelled synthetic lignin and ligninolytic enzyme activities of litter-decomposing basidiomycetous fungi, Appl. Microbiol. Biotechnol. 54 (2000) 819–825.
[42] F.S. Archibald, A new assay for lignin-type peroxidases employing the dye Azure B, Appl. Environ. Microbiol. 58 (1992) 3110–3116.
[43] J.P. Jadhav, S.P. Govindwar, Biotransformation of malachite green by *Saccharomyces cerevisiae* MTCC 463, Yeast 23 (2006) 315–323.
[44] Y.G. Makas, N.A. Kalkan, S. Aksoy, H. Altinok, N. Hasirci, Immobilization of laccase in k-carrageenan based semi-interpenetrating polymer networks, J. Biotechnol. 148 (2010) 216–220.
[45] K. Vijayaraghavan, M.W. Lee, Y.S. Yun, A new approach to study the decolorization of complex reactive dye bath effluent by biosorption technique, Bioresour. Technol. 99 (2008) 5778–5785.
[46] K. Murugesan, Y.M. Kim, J.R. Jeon, Y.S. Chang, Effect of metal ions on reactive dye decolorization by laccase from *Ganoderma lucidum*, J. Hazard. Mater. 168 (2009) 523–529.
[47] M. Mohorcic, S. Teodorovic, V. Golob, J. Friedrich, Fungal and enzymatic decolourisation of artificial textile dye baths, Chemosphere 63 (2006) 1709–1717.
[48] J.F. Osma, J.L. Toca-Herrera, S. Rodriguez-Couto, Biodegradation of a simulated textile effluent by immobilised-coated laccase in laboratory-scale reactors, Appl. Catal. A: Gen. 373 (2010) 147–153.
[49] G. Parshetti, S. Kalme, G. Saratale, S. Govindwar, Biodegradation of Malachite green by *Kocuria rosea* MTCC 1532, Acta Chim. Slov. 53 (2006) 492–498.
[50] W.X. Liu, Y.P. Chao, X.Q. Yang, H.B. Bao, S.J. Qian, Biodecolorization of azo, anthraquinonic and triphenylmethane dyes by white-rot fungi and a laccase-secreting engineered strain, J. Ind. Microbiol. Biotechnol. 31 (2004) 127–132.
[51] L. Ayed, K. Chaieb, A. Cheref, A. Bakhrouf, Biodegradation of triphenylmethane dye malachite green by *Sphingomonas paucimobilis*, World J. Microbiol. Biotechnol. 25 (2009) 705–711.
[52] G.S. Nyanhongo, J. Gomes, G.M. Gübitz, R. Zvauya, J. Read, W. Steiner, Decolorization of textile dyes by laccases from a newly isolated strain of *Trametes modesta*, Water Res. 36 (2002) 1449–1456.
[53] D.T. D'Souza, R. Tiwari, A. Kumar Sah, C. Raghukumar, Enhanced production of laccase by a marine fungus during treatment of colored effluents and synthetic dyes, Enzyme Microb. Technol. 38 (2006) 504–511.
[54] U.U. Jadhav, V.V. Dawkar, G.S. Ghodake, S.P. Govindwar, Biodegradation of Direct Red 5B, a textile dye by newly isolated *Comamonas* sp. UVS, J. Hazard. Mater. 158 (2008) 507–516.
[55] V.L. Papinutti, F. Forchiassin, Modification of malachite green by *Fomes sclerodermeus* and reduction of toxicity to *Phanerochaete chrysosporium*, FEMS Microbiol. Lett. 231 (2004) 205–209.
[56] J.S. Knapp, P.S. Newby, L.P. Reece, Decolorization of dyes by wood-rotting basidiomecete fungi, Enzyme Microb. Technol. 17 (1995) 664–668.
[57] S. Murao, Y. Hinode, E. Matsumura, A. Numata, K. Kawai, H. Ohishi, H. Jin, H. Oyama, T. Shin, A novel laccase inhibitor, N-hydroxyglycine, produced by *Penicillium citrinum* YH-31, Biosci. Biotechnol. Biochem. 56 (1992) 987–988.
[58] Y. Kim, S. Yeo, H.G. Song, H.T. Choi, Enhanced expression of laccase during the degradation of endocrine disrupting chemicals in *Trametes versicolor*, J. Microbiol. 46 (2008) 402–407.
[59] V. Faraco, C. Pezzella, A. Miele, P. Giardina, G. Sannia, Bio-remediation of colored industrial wastewaters by the white-rot fungi *Phanerochaete chrysosporium* and *Pleurotus ostreatus* and their enzymes, Biodegradation 20 (2009) 209–220.
[60] A.K. Verma, C. Raghukumar, P. Verma, Y.S. Shouche, C.G. Naik, Four marine-derived fungi for bioremediation of raw textile mill effluents, Biodegradation 21 (2010) 217–233.
[61] D.S.L. Balan, R.T.R. Monteiro, Decolorization of textile indigo dye by ligninolytic fungi, J. Biotechnol. 89 (2001) 141–145.
[62] J.T. Spadaro, L. Isabelle, V. Renganathan, Hydroxyl radical mediate degradation of azo dyes: evidence for benzene generation, Environ. Sci. Technol. 28 (1994) 1389–1393.
[63] K. Murugesan, I.H. Yang, Y.M. Kim, J.R. Jeon, Y.S. Chang, Enhanced transformation of malachite green by laccase of *Ganoderma lucidum* in the presence of natural phenolic compounds, Appl. Microbiol. Biotechnol. 82 (2009) 341–350.
[64] P.G. Tong, Y.Z. Hong, Y.Z. Xiao, M. Zhang, X.M. Tu, T.G. Cui, High production of laccase by a new basidiomycete, *Trametes* sp, Biotechnol. Lett. 29 (2007) 295–301.
[65] P. Kulmberg, M. Mathieu, C.E.A. Dowzer, J.M. Kelly, B. Felenbok, Specific binding sites in the alcR and alcA promoters of the ethanol regulon for the CreA repressor mediating carbon catabolite repression in *Aspergillus nidulans*, Mol. Microbiol. 7 (1993) 847–857.
[66] J. Strauss, H.K. Horvath, B.M. Abdallah, J. Kindermann, R.L. Mach, C.P. Kubicek, The function of CreA, the carbon catabolite repressor of *Aspergillus nidulans*, is regulated at the transcriptional and post-transcriptional level, Mol. Microbiol. 32 (1999) 169–178.
[67] D.J. Thiele, Metal regulated transcription in eukaryotes, Nucleic Acids Res. 20 (1992) 1183–1191.
[68] T.H. Rushmore, R.G. King, K.E. Paulson, C.B. Pickett, Regulation of glutathione S-transferase Ya subunit gene expression, identification of a unique xenobiotic-responsive element controlling inducible expression by planar aromatic compounds, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 3826–3830.
[69] J.M. Treger, T.R. Magee, K. McEntee, Functional analysis of the stress response element and its role in the multistress response of *Saccharomyces cerevisiae*, Biochem. Biophys. Res. Commun. 243 (1998) 13–19.
[70] D.J. Thiele, ACE1 regulates expression of the *Saccharomyces cerevisiae* metallothionein gene, Mol. Cell. Biol. 8 (1988) 2745–2752.
[71] J.L. Thorvaldsen, A.K. Sewell, C.L. McGowen, D.R. Winge, Regulation of metallothionein genes by the ACE1 and AMT1 transcription factors, J. Biol. Chem. 268 (1993) 12512–12518.
[72] M.Á. José, C. Paulo, A.M. Rodrigo, P. Rubén, A.S. Paulina, V. Rafael, Expression of genes encoding laccase and manganese-dependent peroxidase in the fungus *Ceriporiopsis subvermispora* is mediated by an ACE1-like copper-fist transcription factor, Fungal Genet. Biol. 46 (2009) 104–111.
[73] Y.H. Fu, G.A. Marzluf, nit-2, the major positive-acting nitrogen regulatory gene of *Neurospora crassa*, encodes a sequence-specific DNA-binding protein, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 5331–5335.
[74] W.H. Mager, A.J. De Kruijff, Stress-induced transcriptional activation, Microbiol. Rev. 59 (1995) 506–531.