DECOLORIZATION OF REMAZOL BRILLIANT BLUE R BY FLUIDIZED-BED DRIED *LENTINUS POLYCHROUS* LÉV.

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Abstract

Decolorization of 100 mg/l Remazol Brilliant Blue R (RBBR), a model of xenobiotics, by *Lentinus polychrous* Lév. was compared with *Pleurotus ostreatus* in fresh and dried forms with various drying times (5, 10, and 15 min). The result showed that *L. polychrous* Lév., in particular dried forms, had higher decolorization efficiency (91%) than *P. ostreatus* (6-15%). Then, the factors influencing decolorization of *L. polychrous* Lév. including mycelial ages, initial pHs, shaking speeds, and activation media were investigated. The 17-day-old dried fungi showed the highest decolorization efficiency; however, due to the long growth period, the 5-day-old cultures were selected. The optimal decolorization condition was initial pH 4 and the shaking speed 250 rpm, providing the shortest decolorization time (96 h) with 91% decolorization. The activation with Mushroom Complete Medium (MCM) and Minimal Medium showed that MCM caused more activation, providing 60 h of decolorization time and the highest laccase activity (3.76 U/ml) and manganese peroxidase activity (2.71 U/ml). After 6 month storage, the time required to complete activation increased from 36 h to 6 days. FTIR analysis proved that the biodegradation was the main mechanism of dye removal. The phytotoxicity tests on seed germination of *Zea mays* L. and *Ipomoea aquatic* Forsk. revealed that the toxicity of RBBR was reduced to a moderate level.

Keywords: Decolorization, Remazol Brillant Blue R, Fluidized-bed drying, *Lentinus polychrous* Lév., *Pleurotus ostreatus*

Introduction

Over 7×10^5 tons of synthetic dyes, used extensively for textiles, paper, leather, color photography etc., are produced annually worldwide. Due to the inefficiencies of dye production and application, 15% of the dyes are lost and can cause a serious environmental problem (Lewinsky, 2007). These dyes are recalcitrant compounds and a very small amount of them (10-50 mg/l) in water is highly visible. Moreover, the discharge of the dyes into water bodies leads to the reduction of sunlight penetration, photosynthetic activity, and dissolved oxygen concentration. These problems have become a major concern in aquatic organisms and the environment.

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Currently, wastewater contaminated with synthetic dyes has been treated by physico-chemical methods, e.g. adsorption (Ravikumar *et al*., 2006), oxidation (Tekbas *et al*., 2008), coagulation–flocculation (Golob *et al*., 2005), and photo-degradation (Mozia *et al*., 2009), whose overall disadvantages are high capital costs, operational problems, and the huge amounts of pollutants created. Therefore, there is a great need to develop an economical, effective, and environmentally friendly way for dye decolorization.

White-rot fungi can effectively degrade a wide variety of recalcitrant compounds, including polycyclic aromatic hydrocarbons (Ding *et al*., 2008), polychlorinated bi-phenyls (Kuem and Li, 2004), lignins (Wu *et al*., 2005), and synthetic dyes (Srinivasan and Murthy, 2009), by their extracellular ligninolytic enzymes, mainly of laccase, lignin peroxidase, and manganese peroxidase (Hatakka, 1994). *Lentinus polychrous* Lév., a basidiomyceteous white-rot fungus, is one of the most popular edible mushroom varieties in Thailand, besides *Lentinus edodes* and *Pleurotus ostreatus*. It has promising applications in both medicine and biotechnology. Biodegradation of many classes of synthetic dyes was carried out by crude enzymes of *L. polychrous* Lév. (Phetsom *et al*., 2009; Sarnthima *et al*., 2009). The fungi grown on different agro-industrial wastes under solid-state fermentation had high laccase and manganese peroxidase production levels (Sarnthima *et al*., 2009).

However, the procedure for the preparation of fungi used as dye degraders is inconvenient and considered complicated for inexperienced or non-educated users e.g. small and medium textile enterprises in Thailand. Moreover, the fungi sold as fresh cultures in the commercial sense have several disadvantages, including difficulty of transportation, requiring cool temperature during distributing processes, and short shelf life. Therefore, there is still a need for research on potential white rot fungi prepared as ready-to-use dye degraders.

Drying fungi can overcome the problems mentioned above. It is known that there are many different drying methods for fungi such as vacuum drying (Hughes and Macer, 1964), freeze drying, spray drying, and fluidized-bed drying (Larena *et al*., 2003). However, the disadvantage of freeze drying is high energy demand, and that of spray drying and vacuum drying is the low cell viability after drying. Fluidized-bed drying is more advantageous in practice since air is forced through the product particles at a sufficiently high velocity providing better heat transfer and dehydration with a short time of drying period and low energy requirement. The fluidized-bed drying of conidia of *Penicillium oxalicum* at a temperature range of 30-40^{o}C for 10 min maintained 100% viability (Larena *et al*., 2003). However, previous studies of fluidized-bed drying of fungal mycelium have been limited.

In this study, *L. polychrous* Lév. was evaluated for its ability to decolorize an antraquinone dye Remazol Brilliant Blue R (RBBR) classified as a toxic and recalcitrant organopollutant, and was compared with an efficient dye degrader *P. ostreatus*, (Novotný *et al*., 2001; Palmieri *et al*., 2005). The fluidized-bed drying experiments were conducted to investigate the effects of the drying time on the decolorization efficiency. The dried fungal strain with higher remaining decolorization efficiency was selected to study the effects of mycelial ages, physicochemical conditions, activation media, and storage times. In addition, the biodegradation and phytotoxicity of the end products were investigated.

Materials and Methods

Dyes and Chemicals

The antraquinone dye Remazol Brillant Blue R (1-amino-9,10-dihydro-9,10-dioxo-4-[(3-{[2-(sulfooxy)ethyl]sulfonyl}phenyl)amino] 2-anthracenesulfonic acid; CI name Reactive Blue 19; CI number 61200), was a generous gift from DyStar Thai Co., Ltd. Veratryl alcohol, 2, 2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 3-dimethylaminobenzoic acid (DMAB) were obtained from Sigma-

Aldrich (USA). All chemicals were of an analytical grade.

Organisms

L. polychrous Lév. and *P. ostreatus* were purchased from the Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand. The stock cultures were grown on Potato Dextrose Agar (PDA) for 7 days at their optimum growth temperatures of 30 and 28°C, respectively. The cultures were maintained at 4°C for subsequent use as inoculums and refreshed every month.

Culture Conditions

L. polychrous Lév. and *P. ostreatus* were cultivated for 5 and 7 days, respectively, at their optimum growth temperatures on Mushroom Complete Medium (MCM) agar containing 20 g/l glucose, 2.0 g/l yeast extract, 2.0 g/l peptone, 1.0 g/l K_2HPO_4, 0.46 g/l, KH_2HPO_4, and 0.5 g/l $MgSO_4.7H_2O$. The pH of the MCM was adjusted to 6.0 for *L. polychrous* Lév. and 5.5 for *P. ostreatus*. Eight mycelial plugs with diameters of 10 mm cut from the margin of active mycelia from the cultural PDA plates were transferred into 250-ml Erlenmeyer flasks containing 10 g of para rubber sawdust in the size range of 0.250-0.425 mm. The initial moisture content of the sawdust was adjusted to 60% by Minimal Medium (MM) containing 0.68 g/l asparagine, 0.5 g/l yeast extract, 1.0 g/l K_2HPO_4, 0.5 g/l $MgSO_4.7H_2O$, 0.5 g/l KCl, 0.1 g/l $FeSO_4.7H_2O$, 0.008 g/l $MnSO_4.4H_2O$, 0.003 g/l $Zn(CH_3COO)_2$, 0.006 g/l $Ca(NO_3)_2.4H_2O$, and 0.003 g/l $CuSO_4.5H_2O$. In addition, the sawdust was enriched with 0.14 g/l glucose and 0.012 g/l ammonium tartrate. Prior to use, the sawdust medium was autoclaved at 121°C for 30 min. The mycelial plugs were mixed with the sawdust medium using a sterile spatula every day. The time required to complete the solid state fermentation of *L. polychrous* Lév. was 5 days and that of *P. ostreatus* was 7 days.

Effects of Fluidized-bed Drying Times

10 g of 2 different fungal strains, *L. polychrous* Lév. and *P. ostreatus*, grown on the sawdust medium was dried in a fluidized-bed dryer (Model 6 MAS 1009, Sherwood Scientific, England) supported with a 0.1 μm air filter membrane. Experiments were conducted for 3 different times of 5, 10, and 15 min at 50°C with the air flow rate of 1.85 m/s. The moisture content of the dried cultures was measured using the air-oven drying method (AOAC, 2000). All measurements were repeated 3 times. The investigation of the decolorization ability of the fluidized-bed dried fungi was also performed. A 0.2 g dry weight of the dried fungi was transferred to 500-ml Erlenmeyer flasks containing 250 ml MM, enriched with 5 g/l glucose. Highly visible RBBR at 100 mg/l filtered with a 0.45 μm cellulose nitrate membrane (Whatman) was also added to the MM. During the decolorization, the temperature was controlled at the optimal growth temperature and the shaking speed was 150 rpm. The decolorization time and percentage of decolorization was monitored. To measure the percentage of decolorization, 3 ml of the aliquots was withdrawn over a period of time and then centrifuged at 10000 rpm for 5 min. The supernatant was analyzed spectrophotometrically at 592 nm for residual dye concentration. The percentage of decolorization was calculated using the following formula:

$$\text{Decolorization (\%)} = \frac{\text{Initial absorbance - Observed absorbance}}{\text{Initial absorbance}} \times 100 \quad (1)$$

All measurements were performed 5 times. The controls were carried out without dried fungi. The fungal strain with higher remaining decolorization efficiency after drying was selected for further decolorization study.

Effects of Mycelial Ages and Physicochemical Conditions

The effects of the mycelial ages, initial pHs of dye solution, and shaking speeds on the decolorization efficiency of fluidized-bed dried fungus selected from the previous experiment were investigated using a one-factor-at-a-time experiment. The optimal value of each factor was used for subsequent experiments. In the first set

of experiments with the initial pH 6 and shaking speed 150 rpm, the studied mycelial ages were 5, 7, 9, 13, and 17 days. In the second set of experiments with the mycelial age of 5 days and shaking speed 150 rpm, the studied initial pHs were 4, 6, and 7. In the third set of experiments with the mycelial age of 5 days and initial pH 4, the studied shaking speeds were 150, 200, 250, and 300 rpm. The RBBR decolorization procedures used in these experiments were as described previously.

Effects of Activation Media and Storage Times

The fluidized-bed dried fungi were activated in 2 different media (MM and MCM) for 36 h at 250 rpm prior to the decolorization step. The suspension was filtered through 2 layers of sterile muslin cloth and a 0.2 g dry weight of activated fungi was used in the decolorization study. To evaluate the effects of storage times on the decolorization efficiency of the dried fungi, they were stored in an amber glass bottle with a tight lid at ambient temperature. The decolorization efficiency of the cultures was reinvestigated for 3 and 6-month periods.

Enzyme Assay

The activities of ligninolytic enzymes including laccase, manganese peroxidase, and lignin peroxidase produced by the activated dried fungi were monitored during decolorization. Laccase activity was measured spectrophotometrically by an increase in the absorbance at 420 nm (ε_{420} = 36000 $M^{-1}.cm^{-1}$) from the oxidation of 0.5 mM ABTS to form ABTS+ in 0.1 M sodium acetate buffer (pH 4.5) according to Rodriguez *et al*. (1999). Lignin peroxidase activity was measured as described by Tien and Kirk (1984). The assay mixture contained 2.0 mM 3,4-dimethoxybenzylalcohol and 0.05 mM H_2O_2 in 0.1 M sodium acetate buffer as the substrate. The formation was followed at 310 nm ($\varepsilon_{310} = 9.3 \times 10^3$ $M^{-1}cm^{-1}$). Manganese peroxidase activity was measured as described by Castillo *et al*. (1994). The assay mixture contained 0.07 mM 3-methyl-2-benzothiazolinone hydrazone (MBTH), 0.99 mM 3-(dimethy- lamino) benzoic acid (DMAB), 0.3 mM $MnSO_4$, and 0.05 mM H_2O_2 in 0.1 M sodium acetate buffer as the substrate. The formation was followed at 590 nm ($\varepsilon_{590} = 5.3 \times 10^4$ $M^{-1}cm^{-1}$). 1 U of activity was defined as the amount of enzyme required to oxidize 1 μmol substrate in a minute.

Decolorization and Biodegradation Analysis

To confirm the decolorization and biodegradation of the dye, the supernatants of decolorized samples prepared as described above were subjected to decolorization and biodegradation analysis, while those without fungal cultures were used as abiotic controls. The decolorization was followed by monitoring changes in the UV-vis absorption spectrum between 400-800 nm using a UVG 111607 double beam spectrophotometer (HeLios, England). The biodegradation was followed by monitoring a change in the percentage of transmission at different wavelengths using Fourier transform infrared spectroscopy (FTIR, TENSOR 27, Bruker Optics GmbH, Ettlingen, Germany).

Phytotoxicity Study

The phytotoxicity study was performed in order to assess the effects of the RBBR solution before and after degradation on the growth of 2 kinds of seeds widely planted for consumption in Thailand: corn (*Zea mays* L.) and water spinach (*Ipomoea aquatic* Forsk.). Four replicates of 10 seeds of each were grown in a 9-cm diameter petri dish lined with a filter paper (Whatman #1) and wetted with 5 ml samples along with sterile distilled water as controls. After incubating the plates in the dark at room temperature for 72 h, the seed germination and root elongation were recorded. The germination index (GI) was calculated as follows:

$$GI\ \% = (RSG\ \% \times RRE\ \%) / 100\ \% \qquad (2)$$

where RSG is the relative seed germination and RRE is the relative root elongation calculated as a percentage of control values. The results was interpreted as described by Zucconi *et al*. (1985) and Tiquia and Tam (1998). GI values less than 50% were high phytotoxicity, 50-80% were

moderate phytotoxicity, 80-100% were no phytotoxicity, and greater than 100% were phytostimulant.

Results and Discussion

Effects of Fluidized-bed Drying Times

Due to the drawbacks of the utilization of fungal mycelia in fresh forms as stated in the introduction, the possibility of the development of fluidized-bed dried mycelia as a substitute for the decolorization of synthetic dyes was investigated. A comparison of the RBBR decolorization of a well-known dye degrader *P. ostreatus* with that of *L. polychrous* Lév. in both fresh and dried forms was performed. The results in Table 1 show that in the case of fresh fungal mycelia, *P. ostreatus* had much lower efficiency (lower decolorization percentage with longer decolorization time) than *L. polychrous* Lév. This could be the result of the toxicity of RBBR at a concentration range of 20-100 mg/l for *P. ostreatus* growth (Erkurt *et al*., 2007). Its toxicity was also reported to cause the substantial reduction in biomass production, growth rates, and morphological changes in the mycelia of white-rot fungus *Dichomitus squalens* during the decolorization in Kirk medium supplemented with RBBR (Eichleorova *et al*., 2007).

When the mycelia were dried in the fluidized-bed dryers provided with an effective air flow, the moisture contents were sharply reduced within 5 min, but gradually reduced afterwards. This result shows the advantages of fluidized-bed dryers for drying fungal mycelia with a short drying time and low drying temperature, but high thermal efficiency. As moisture contents decreased over the drying time, the decolorization percentage of *P. ostreatus* was significantly decreased, while that of *L. polychrous* Lév. remained constant. This is possibly due to the thermotolerant characteristics of *L. polychrous* Lév. (Pukahuta *et al*., 2004), which cannot be found in *P. ostreatus*. However, the heat damage might also possibly occur to *L. polychrous* Lév. and its deleterious effects increased with an increasing drying time, as evidenced by an increase in the decolorization time. Thus, 5 min drying time in fluidized-bed dryers was sufficiently long to produce dried fungal mycelia with maximum remaining decolorization ability compared to other longer drying times. It was also observed that no decolorization occurred in the controls without fungal mycelia throughout the experiment.

In order to determine whether dye removal of dried fungi involved the adsorption process, at the end of the decolorization process the mycelia were homogenized and extracted with methanol, filtered and centrifuged, and the supernatants were measured spectrophotometrically. The results showed that the adsorbed RBBR on the mycelia of *L. polychrous* Lév. and *P. ostreatus* was found to be 5% and 5-15%, respectively. This implies that the decolorization mechanism of dried *L. polychrous* Lév. is primarily due to biodegradation, but that of dried *P. ostreatus* is only adsorption.

Therefore, *P. ostreatus* is not suitable for a dried fungal formulation as ready-to-use dye

Table 1. Effects of fluidized-bed drying times on moisture contents and RBBR decolorization performance with initial pH6 and shaking speed 150 rpm of 7-day-old *P. ostreatus* and 5-day-old *L. polychrous* Lév.

Drying times (min)	Moisture (%)		Decolorization (%)		Decolorization time (h)	
	P. ostreatus	*L. polychrous* Lév	*P. ostreatus*	*L. polychrous* Lév	*P. ostreatus*	*L. polychrous* Lév
0	66.40±0.74	64.79±0.96	78.24±1.17	90.93±1.17	336.0±0.00	96.00±2.00
5	5.45±0.49	5.50±0.28	15.13±0.72	91.26±1.26	336.0±6.00	156.00±3.00
10	4.48±0.24	4.50±0.32	8.11±1.18	91.47±0.41	336.0±6.00	168.00±6.00
15	3.61±0.39	3.78±0.41	6.04±1.41	91.39±0.48	336.0±6.00	180.00±6.00

degraders. *L. polychrous* Lév. with 5 min drying was thus selected for further studies on the effects of mycelia ages and physicochemical conditions on decolorization.

Effects of Mycelial Ages

In order to find a suitable mycelial age of the fluidized-bed dried *L. polychrous* Lév. for the effective RBBR degradation, the experiment was conducted at 5 different mycelial ages i.e., 5, 7, 9, 13, and 17 days. The results in Table 2 show that the decolorization percentage of all dried fungi with different mycelia ages was approximately the same (89-91%), but the decolorization time of the 17-day-old fungi was the shortest. The best decolorization performance of the 17-day-old dried fungi is presumably due to the slightest injury of the mycelium from heat-induced damage. Among all the cultures, the 17-day-old fungi possessed the thickest cell walls, thereby causing the greatest heat resistance. Moreover, it is known that the initial moisture contents of materials have a great influence on heat-induced damage. Therefore, it was possible that the 17-day-old fungi with the lowest initial moisture contents of 50% had the highest survival compared to the other fungi with initial moisture contents of 65-68% for the same final moisture contents of 5%. Our findings on the effects of the mycelial ages on the decolorization time are in contrast with the results of Özsoy *et al*. (2005) showing that the RBBR decolorization time of fresh *Funalia trogii* ATCC 200800 was apparently shorter with 7-day-old cultures compared to 5-day-old cultures, although they both degraded the dyes to the same extent of 90%. This suggests that, possibly as a result of heat-induced damage occurring during fluidized-bed drying, the differences in the decolorization time of the 5 and 7-day-old dried cultures are not observed in our experiment. According to the results, the 5-day-old dried fungi were chosen for further experiments since the period of fungal growth under solid state fermentation was 12 days shorter, while the decolorization time was only 36 h longer than that of the 17-day-old dried fungi.

Effects of Initial pHs

In order to investigate the effects of the initial pHs of MM containing RBBR, which is one of the important environmental factors affecting decolorizarion, the study was carried out at 3 different pHs i.e. 4, 6, and 7. The optimal pH for RBBR decolorization by fluidized-bed dried *L. polychous* Lév. was found to be 4 at which the time required for maximum decolorization (91%) was shortest at 114 h (Table 2). Furthermore, it was apparently observed that a range of pH below 4 and beyond 7 resulted in no appreciable growth of the dried fungi and, therefore, the decolorization efficiency markedly decreased (data not shown). It was also found that the pH of each sample was almost constant during the decolorization period and there was no decolorization in abiotic controls at different pHs. The highest decolorization at pH 4 of the dye medium may be attributed to the highest ligninolytic enzyme activity of *L. polychrous* Lév. Our results are in agreement with the report of Murugesan *et al*. (2007) showing that the maximum RBBR decolorization (90%) by crude

Table 2. Effects of mycelial ages at initial pH6 and effects of initial pHs at mycelial age 5 day on RBBR decolorization performance with shaking speed 150 rpm of fluidized-bed dried *L. polychrous* Lév.

	Parameters							
	Mycelial ages (days)					pHs		
	5	7	9	13	17	4	6	7
Decolorization. (%)	91.26±1.26	90.43±1.03	91.19±0.78	90±0.44	89.07±0.72	91.48±0.62	91.26±1.26	89.29±1.71
Decolorization times (h)	156.0±3.0	156.0±6.0	156.0±3.0	156.0±3.0	120.00±3.0	114.00±3.0	156.0±3.0	168.0±6.0

laccase of *Ganoderma lucidum* was obtained after 60 min at pH 4 and the decolorization was reduced to 46% at pH 3, while no decolorization was observed in an alkaline pH 8 and 9. Nyanhongo *et al*. (2002) also found similar results on RBBR decolorization by *Tramates modesta*. The maximum decolorization (94-98%) was achieved after 6 h at pH 4-4.5, while they were only 42% and 32% at pH 3 and 6, respectively. Many investigators reported that decolorization of synthetic dyes by most white rot fungi preferred an acidic pH range of 4-6; however, it depended on the type of substrates as well (Kapdan *et al*., 2000; Radha *et al*., 2005; Asgher *et al*., 2008).

Effects of Shaking Speed

The effect of the shaking speed on RBBR decolorization was determined at different shaking speeds i.e. 150, 200, 250, and 300 rpm. As shown in Figure 1, the decolorization efficiency was increased as the shaking speed increased from 150 to 250 rpm and was almost constant afterwards. For example, within 86 h, the percentage decolorization 36%, 44%, 79%, and 80% was achieved with 150, 200, 250, and 300 rpm, respectively. However, at the end of the experiment, 91% decolorization was obtained with all shaking speeds. The increase in decolorization efficiency might be attributed to an increase in nutrient and oxygen transfer between the media and the fungi, thus enhancing the cell growth and oxidative ligninolytic enzyme activity (Birhanli and Yesilada, 2006; Revankar and Lele, 2007). It was also observed that at higher shaking speeds, the smaller and more uniform fungal pellets were formed due to higher shear stress. A negligible difference in decolorization efficiency between 250 and 300 rpm indicates that at such a high shaking speed of 300 rpm, the integrity of cell walls is stably maintained. On the other hand, during decolorization under static conditions, most of the fluidized-bed dried fungi grown on the sawdust medium rested at the bottom of the flasks and a few formed mats at the surfaces. So these cultures obtained a small amount of dissolved oxygen, resulting in low decolorization extent. In contrast, Sukumar *et al*. (2009) reported that under static conditions, the highest decolorization (85.5%) of Acid Red 66 by *Trametes versicolor* was found and an increase in agitation speeds resulted in a decrease in decolorization. According to the result presented here, the optimal shaking speed for RBBR decolorization of fluidized-bed dried *L. polychous* Lév. was found to be 250 rpm and it was adopted for further experiments.

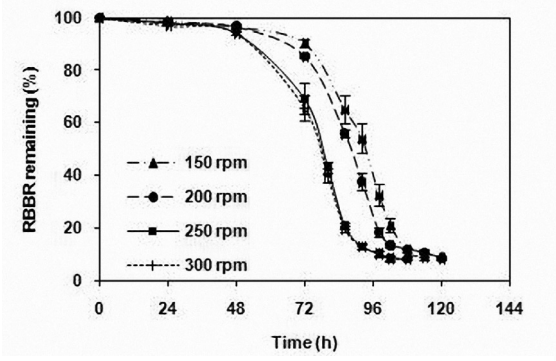

Figure 1. Effects of shaking speed on RBBR decolorization performance with initial pH 4 and shaking speed 150 rpm of fluidized-bed dried *L. polychrous* Lév at mycelial age of 5 days

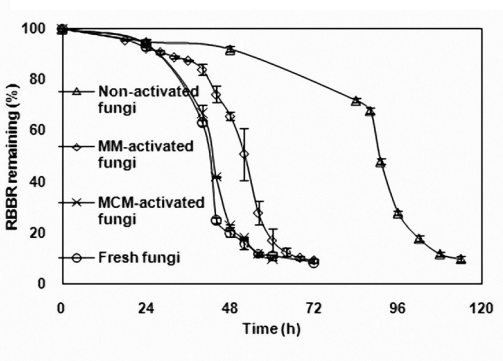

Figure 2. Effects of activation media on RBBR decolorization performance of fluid ized-bed dried *L. polychrous* Lév. under optimum decolorization conditions

Effects of Activation Media and Storage Times

The effect of activation media for fluidized-bed dried *L. polychous* Lév. on its decolorization efficiency was studied using 2 different activation media, MM and MCM, also used as decolorization media and growth media, respectively. As clearly demonstrated in Figure 2, the decolorization efficiency of MCM-activated fungi was comparable to that of fresh fungi and higher than that of MM-activated, and especially nonactivated fungi. Rejuvenation of the dried cultures by incubating in MCM for 36 h allowed the fungi to be ready for use and it can be seen that the lag phase duration was dramatically shortened and the decolorization time was reduced from 114 h to 60 h. Although, compared to MM, MCM is less enriched with trace elements, the concentration of glucose as its carbon sources and yeast extract as one of its nitrogen sources are 4 times greater. Moreover, peptone as the other kind of nitrogen sources in MCM is more complex than asparagine in MM. This suggests that enriched and complex activation media is necessary for enhancing the number of metabolically active cells and repairing damaged cells, which results in restoring the decolorization ability of fluidized-bed dried fungi. Thanh and Nout (2004) reported similar findings with activation of air-dried *Rhizopus oligosporus* sporangiospores by showing that malt extract broth containing malt extract and mycological peptone was more of an influence on growth activation than Czapek-Dox liquid medium mainly containing sucrose and sodium nitrate.

After 3 and 6 month storage of the dried cultures at ambient temperature, the decolorization time was increased from 5 days to 12 days, while that of the non-preserved dried cultures was only 60 h. However, after activation in MCM for 3 days for 3-month-old dried cultures and 6 days for 6-month-old dried cultures under conditions of 30°C and 250 rpm, the decolorization ability was restored. This indicates that an increase in storage times leads to the increase in time required for activation and the reduction of the decolorization efficiency.

Ligninolytic Enzyme Activities

The relationship between RBBR remaining and ligninolytic enzyme activities present in the culture supernatant during the time course of decolorization is depicted in Figure 3. During the first 24 h, only 4% RBBR decolorization and extremely low activities of laccase (0.02 U/ml) and manganese peroxidase (0.02 U/ml) were observed. During this time period, the strong blue color of the fungal pellets was obviously monitored and 5% RBBR adsorption on the mycelium was detected. Therefore, it could be

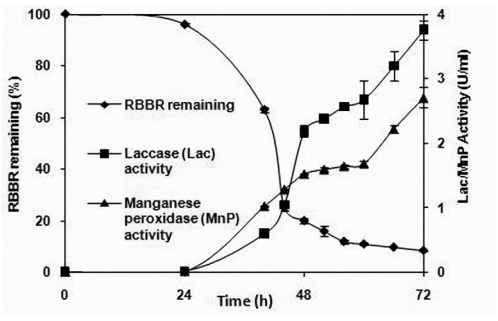

Figure 3. RBBR decolorization performance and laccase (Lac) and manganese peroxidase (MnP) activity of fluidized-bed dried *L. polychrous* Lév. under optimum decolorization conditions

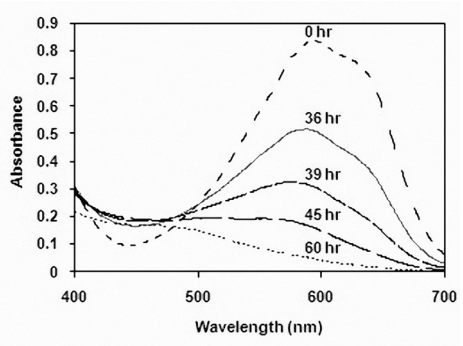

Figure 4. UV–visible spectral changes observed during RBBR decolorization by fluid ized-bed dried *L. polychrous* Lév. under optimum decolorization conditions

concluded that the initial period of dye removal was attributed to adsorption. As the decolorization proceeded, it was noticed that the fungal growth was dramatically increased and the strong blue color became faint within 60 h. In addition, the dye in the solution was significantly reduced coinciding with the continuous increase in laccase and manganese peroxidase activities. At the end of decolorization (within 72 h), the mycelium retained its natural color and the maximum decolorization (90%) was achieved with the maximum levels of laccase and manganese peroxidase activities of 3.76 and 2.71 U/ml, detectable respectively. However, lignin peroxidase activity could not be detected under the conditions employed in this study. According to the result, it is reasonably assumed that the decolorization mechanism of RBBR was oxidative biodegradation mediated by both laccase and manganese peroxidase. Similarly, these 2 enzymes were reported to be responsible for the decolorization of RBBR by other white rot fungi *Irpex lacteus* (Novotny *et al*., 2004) and *Dichomitus squalens* (Eichlerova *et al*., 2007). Our results are also in agreement with the literature data reported by Sarnthima *et al*. (2009) showing that *L. polychous* Lév. had the potent ability to produce and secrete high levels of both laccase and manganese peroxidase activities, but not lignin peroxidase activity under solid state fermentation of rice bran supplemented with rice husk. Furthermore, Khammuang and Sarnthima (2007) reported that partially purified laccase from spent *L. polychous* Lév. mushroom compost was able to decolorize 66% of 5 mg/l RBBR within 3.5 h.

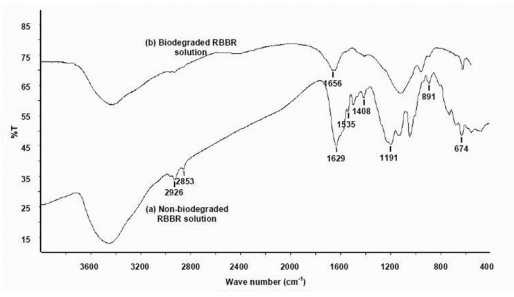

Figure 5. FTIR spectral analysis of (a) non-bio degraded RBBR solution; (b) biode graded RBBR solution obtained under optimum decolorization conditions by fluidized-bed dried *L. polychrous* Lév.

Decolorization and Biodegradation of RBBR

The decolorization of RBBR was monitored by UV-vis spectroscopic analysis and the absorbance spectrum at various decolorization times is presented in Figure 4. As the decolorization proceeded, the absorbance at maximum wavelength of 592 nm, corresponding to the blue color, was reduced and it almost completely disappeared within 72 h. However, there was a small, but noticeable continuous shift in the maximum wavelength towards the lower values from 592 nm at the beginning to 548 nm at 72th h of decolorization indicating the presence of other metabolites produced by the fungi. The reduction and shift of the absorbance at the maximum wavelength confirms that the RBBR decolorization was caused by biodegradation, not adsorption. It was also observed that the absorbance of RBBR solution in the control experiments remained virtually unchanged during the whole incubation period.

The biodegradation of RBBR was monitored using FTIR spectroscopy and comparisons of the transmission spectra before and after decolorization are shown in Figure 5. It clearly indicated that both the chromophore and the functional group of RBBR were degraded. The cleavage of the chromophore having tricyclic anthraquinone structures was evidenced by the disappearance of several small absorption bands and shoulders in the range of 1535-1408 cm^{-1} and 891-674 cm^{-1}, which denote aromatic the C=C stretching vibration and aromatic C-H bending vibration, respectively (Fanchiang and Tseng, 2009; Cardoso *et al*., 2011). The degradation of anthraquinone was also confirmed by the decrease in the peak intensity of 1656 cm^{-1}, which represents C=C conjugated with C=0 stretching vibration from the initial strong and sharp peak at 1629 cm^{-1}. The C-N bonds connecting between the anthraquinone ring and monobenzene ring were completely destructed as indicated by the disappearance

Table 3. Germination responses of *Z. mays* L. and *I. aquatic* Forsk. grown in non-biodegraded and biodegraded RBBR solution obtained under optimum decolorization conditions by fluidized-bed dried *L. polychrous* Lév.

Media	RSG (%)		RRE (%)		GI (%)	
	Z. mays L.	*I. aquatic* Forsk.	*Z. mays* L.	*I. aquatic* Forsk.	*Z. mays* L.	*I. aquatic* Forsk.
Non-biodegraded RBBR solution	92.50±0.45	96.67±0.65	53.37±2.52	56.62±2.21	49.37±1.14	54.73±1.44
Biodegraded RBBR solution	93.33±0.49	97.50±0.45	76.04±2.23	75.85±3.58	70.97±1.10	73.96±1.62

of the peak at 1191 cm^{-1}. Furthermore, the vinylsulfonyl reactive group ($-SO_2CH_2CH_2O-$) was partially degraded as shown by the reduction in peak intensity of 2926 and 2853 cm^{-1}, which represent the C-H asymmetrical and C-H symmetrical stretching of CH_2, respectively.

Phytotoxicity Study

The phytotoxicity study was conducted to evaluate the toxicity levels of RBBR solution before and after biodegradation as well as the potential for reuse of the treated RBBR solution in agriculture. Two kinds of seeds, *Z. mays* L. and *I. aquatic* Forsk. were chosen as representatives of land plants and floating aquatic plants, respectively. Table 3 shows the relative seed germination (RSG), relative root elongation (RRE), and germination index (GI) of these seeds grown in untreated and treated RBBR solution. It was found that the RBBR solution at a concentration of 100 mg/l did not cause a significant toxic effect to the seed germination of both plants as compared to controls with 100% seed germination. On the other hand, it had a greater toxic effect on the root elongation of both plants. These results are in accordance with those by El Hammadi *et al*. (2007) showing that the inhibition of root elongation appeared as more sensitive phytotoxicity indicator than the seed germination. However, the biodegradation by *L. polychrous* Lév. reduced its toxicity as shown by an increase in root elongation. Considering GI, the factor of the relative seed germination and relative root elongation, the biodegraded RBBR solution was less toxic than the original RBBR for both plants. Similar results were observed by Eichleorova *et al*. (2007) and Osma *et al*. (2010) who found that the significant degrees of toxicity reduction of RBBR were accomplished along with color reduction by white-rot fungus *Dichomitus squalens* and by immobilized laccase from white-rot fungus *Tramates pubescens*, respectively.

Conclusions

This study demonstrated that fluidized-bed dried *L. polychrous* Lév. was capable of decolorizing RBBR. The decolorization efficiency of the dried cultures strongly depended on the mycelia age, initial pH of the dye solution, shaking speed, and activation medium. Biodegradation of RBBR by the cultures was attributed to the extracellular ligninolytic enzymes, laccase, and manganese peroxidase. According to the results, fluidized-bed dried mycelia of *L. polychrous* Lév. showed a great potential for the preparation of ready-to-use dye degraders; however, future work with these dried cultures should concentrate upon finding the methods of inducing desiccation tolerance during the growth of fungi, drying protection, storage, packaging and activation.

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