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# Decolourization of remazol brilliant blue R by enzymatic extract and submerged cultures of a newly isolated *Pleurotus ostreatus* MR3

**Fengxue Xin[1], Yumeng Sun[2], Siyao Hu[2], Karlai Cheong[2] and Anli Geng[1]***

[1]School of Life Sciences and Chemical Technology, Ngee Ann Polytechnic, Singapore 599489.
[2]Hwa Chong Junior College, Singapore.

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**A local white-rot fungus basidiomycete *Pleurotus ostreatus* MR3 was isolated from MacRitchie Reservoir Park, Singapore. Among all the ligninolytic activities, laccase was the only enzyme detected in the supernatant when the fungus was grown in liquid culture. This newly isolated white rot fungus was able to completely decolourise remazol brilliant blue R (RBBR) *in-vivo* on agar plates within five days and in the liquid culture (in the presence of inducers) within three days. The addition of inducers was able to enhance laccase production and therefore enhanced *in-vivo* RBBR decolourisation. Veratryl alcohol was shown to be the best inducer for laccase production with the maximum laccase activity reaching about 5.99 U/mL. $Cu^{2+}$ also had a positive effect on laccase production, the laccase activity being enhanced to 5.24 U/mL. *In-vitro* RBBR decolourisation using the laccase from *P. ostreatus* MR3 was much comparable to that using the commercial laccase from *Trameters versicolor*.**

**Key words:** Dyes, remazol brilliant blue R, *Pleurotus ostreatus* MR3, decolourisation, inducers, laccase activity.

## INTRODUCTION

Among industrial wastewaters, the treatment of dye wastewater from textile and dyestuff industries is one of the most challenging. Over 10,000 dyes with an annual production in excess of $7 \times 10^5$ metric tonnes worldwide are commercially available, and typically 5 to 10% of this amount is discharged in industrial effluents (Fu and Viraraghavan, 2001). As a wide range of structurally diverse dyes may be used within a short duration in a typical textile manufacturing process, the effluent can be highly variable in composition. Moreover, the discharge of those colored wastewaters into rivers leads to a reduction of sunlight penetration in natural water bodies, which in turn decreases both photosynthetic activity and dissolved oxygen concentration. Several of these dyes are very stable to light, temperature, and microbial attack, making them recalcitrant compounds (Rodriguez et al., 1999). This highlights the need for a non-specific dye treatment process for textile dye effluent.

The dye used in the biodegradation studies reported in the present study is one of the most importantly industrial dyes, Remazol brilliant blue R (RBBR), which is frequently used as a starting material in the production of polymeric dyes. It is an anthracene derivative and represents an important class of often toxic and recalcitrant organo-pollutants. The various treatment technologies currently available for dye removal primarily depend on physical and/or chemical principles. Most of these technologies suffer several shortcomings, including high

*Corresponding author. Email: gan2@np.edu.sg. Tel: (65) 64608617; Fax: (65) 64679109.

**Abbreviations: RBBR,** Remazol brilliant blue R; **WRF,** white rot fungi; **PCR,** polymerase chain reaction; **SmF,** submerged fermentation; **ABTS,** 2,2′-azino-di-[3-ethyl-benzothiazoline-(6)-sulfonic acid]; **LiP,** lignin peroxidase; **MnP,** manganese peroxidase.

amounts of chemical usage and/or sludge generation, costly infrastructure requirements and/or high operating expenses (Nigam et al., 2000). Therefore, new methods must be developed, in particular, by using biotechnological approaches for the degradation of the recalcitrant compounds. Specific microorganisms were proven to be potentially effective in treatment of this pollution source in an eco-efficient manner (Willmott et al., 1998; McMullan et al., 2001; Zissi and Lyberatos, 2001). The white rot fungi (WRF) are, so far, the most efficient microorganisms in degrading synthetic dyes, with basidiomycetous fungi that are able to depolymerize and mineralize lignin. This WRF's property is due to the production of extracellular lignin-modifying enzymes. Due to their low substrate specificity, lignin-modifying enzymes are also able to degrade a wide range of xenobiotic compounds (Barr and Aust, 1994; Pointing, 2001) including dyes (Glenn and Gold, 1983; Spadaro et al., 1992). Most of the previous studies reported, focused on the lignin-degrading enzymes of *Phanerochaete chrysosporium* and *Trametes versicolor*. Lately, there has been a growing interest in studying the lignin-modifying enzymes of a wide array of WRF searching for better lignin-degrading systems (Munari et al., 2007).

*Pleurotus ostreatus* is the third most important cultivated mushroom for food purposes and it is also a well studied WRF. The genus *Pleurotus* is often associated with the bioconversion of agricultural wastes into valuable food products through the use of their ligninolytic enzymes for biodegradation of organo-pollutants, xenobiotics and industrial contaminants (Cohen et al., 2002). The ligninolytic system of *Pleurotus* sp. has been extensively studied, and it appears to be an effective alternative for the bioremediation of resistant pollutants (Barr and Aust, 1994; Cohen et al., 2002). Submerged cultures of *Pleurotus sajorcaju* has been shown to bring about effective degradation of total polyphenols from paper mill effluents (Munari et al., 2007). Laccases were identified as the main agents of wastewater decolourization by *P. ostreatus* (Faraco et al., 2009). Laccases are multicopper enzymes, which catalyse the oxidation of phenolic compounds including a range of dyes with concomitant reduction of oxygen (Eggert et al., 1996; Chivukula and Renganathan 1995; Muñoz et al., 1997). The findings that the substrate range of laccase can be expanded to non-phenolic dyes, even in the presence of suitable mediators (Bourbonnais and Paice, 1990), boosted recent interest in laccase. *P. ostreatus* laccases have been extensively studied; one of these, POXC, is the most abundantly produced under all the growth conditions examined (Giardina et al., 1996). Moreover, three other laccase isoenzymes secreted by the mycelium have been purified and characterised (POXA1w, POXA1b and POXA3) (Palmieri et al., 1997; Giardina et al., 1999; Palmieri et al., 2003).

In this study, *P. ostreatus* MR 3 was primarily screened for RBBR decolorization in agar dye plates. The fungus was also cultivated in media formulated with inducers to increase laccase production. The capability of the extracellular enzyme in decolorization of RBBR solution was assessed. Moreover, decolourization of industrial dyes was performed in liquid cultures of *P. ostreatus* MR3.

## MATERIALS AND METHODS

### Isolation of microorganism and its maintenance

Wild strains *P. ostreatus* MR3 and *Lentinula edodes* MR6 were isolated from MacRitchie Reservoir Park, Singapore using potato agar agar plates containing 200 mg $L^{-1}$ RBBR. Identification of the isolated fungi was carried out by 18S rDNA sequencing using a polymerase chain reaction (PCR) primers of NS1 (5′-GTAGTCATATGCTTGTCTC-3′) and NS-4 (5′-CTTCCGTCAATTCCTTTAAG-3′). The PCR fragments were sequenced and the results were compared with the database retrieved from GenBank. It was identified that the isolated microbes were closely related to *P. ostreatus* (99%) and *L. edodes* (98%), so they were designated the name of *P. ostreatus* MR3 and *L. edodes* MR6. *T. versicolor* ATCC 20869 was obtained from the American Type Culture Collection (ATCC). The above fungal strains were maintained in potato dextrose agar (Merck, Germany) plates at room temperature for 10 days before inoculation.

### Medium and cultural conditions for submerged fermentation (SmF)

The basal medium used for laccase production had the following composition (g/L): 2 g of glucose, 2 g of ammonium tartrate, 2 g of malt extract, 0.26 g of $KH_2PO_4$, 0.26 g of $Na_2HPO_4$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CuSO_4 \cdot 5H_2O$, 0.0066 mg of $CaCl_2 \cdot 2H_2O$, 0.005 mg of $FeSO_4$, 0.5 mg of $ZnSO_4 \cdot 7H_2O$, 0.02 mg of $Na_2MoO_4$, 0.09 mg of $MnCl_2 \cdot 4H_2O$, 0.07 mg of $H_3BO_3$. The pH was adjusted to 5.5 before autoclaving at 121°C for 20 min. Adequate aeration was provided by agitation at 150 rev/min at 30°C for four days. The culture grown under the same conditions for 48 h was used as the inoculum (10%) for enzyme production.

### Determination of enzyme activities

Enzyme activities were determined through colorimetric assays at 30°C in a total volume of 1 mL. Absorbance changes were measured through a spectrophotometer (UV-1601 OC, Shimadzu, Japan). Laccase activity was determined according to Xin and Geng (2010) using 2,2′-azino-di-[3-ethyl-benzothiazoline-(6)-sulfonic acid] (ABTS; Boehringer) as the substrate with some modifications. The reaction mixture contained 100 µL enzyme extract and 900 µL ABTS solution (1 mM) in sodium acetate buffer pH 5.0 (50 mM) for 10 min. Oxidation was followed via the increase in absorbance at 420 nm ($\varepsilon=3.6\times10^4$ $cm^{-1}M^{-1}$). Manganese peroxidase was determined using phenol red as a substrate. Concentration of the reaction product was measured at 610 nm ($\varepsilon=2.2\times10^4$ $cm^{-1}M^{-1}$) (Glenn and Gold, 1985). The reaction mixture contained 50 mM succinate buffer, pH 4.5, 0.01% phenol red and the appropriate volume of enzyme solution. The addition of $H_2O_2$ (0.1 mM final concentration) initiated the reaction. Lignin peroxidase (LiP) activity was determined by oxidation of veratryl alcohol to veratryl aldehyde in 0.1 sodium tartrate buffer, pH 3.0, 2 mM veratryl alcohol, 0.4 mM $H_2O_2$ Oxidation reaction was followed measuring optical absorbance at 310 nm ($\varepsilon=9.3\times10^6$ $cm^{-1}M^{-1}$). One enzyme unit (U) was defined as 1.0 µmol of product formed per

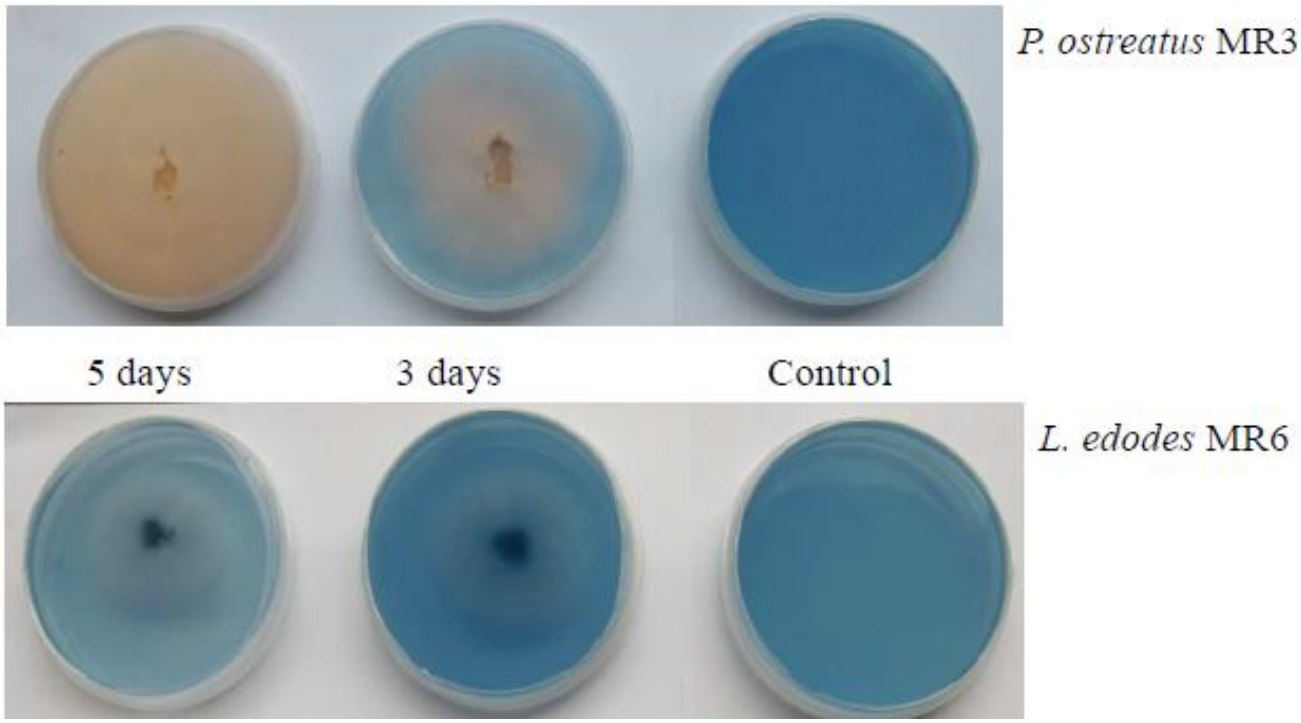


**Figure 1.** Decolourization of media containing 200 mg/L RBBR by local isolates *P. ostreatus* MR3 and *L.edodes* MR6. Controls without inoculums.

minute under the assay conditions. All activity assays were carried out in duplicates.

### *In vitro* decolorization of RBBR by the enzyme extract from *P. ostreatus* MR3

Unless otherwise indicated, all the experiments were performed using 3 mL-disposable cuvettes with 2 mL final reaction volume. The reaction mixture was composed of 100 mM acetate buffer pH 5.0, 200 mg $L^{-1}$ RBBR and 0.2 U $mL^{-1}$ laccase. The reaction was initiated with addition of laccase and further incubated in the dark at 30°C. The decolourization of the RBBR was followed by recording the spectra of the reaction mixture every 15 min with OD at 595 nm. All experiments were performed in duplicate and controls were performed by using heat inactivated enzymes after incubation at 100°C for 10 min. Decolourization of RBBR by the enzyme broth was also investigated using the same enzyme dosage. Dyes contents were monitored at 595 nm, which is the maximum visible absorbance for RBBR. The assays were done in duplicates. The decolourization degree was calculated according to Xin and Geng (2010) by means of the formula:

$$D = 100(A_{ini} - A_{obs}) / A_{ini}$$

Where, $D$ is the decolourization (in %), $A_{ini}$, the initial absorbance, and $A_{obs}$, the observed absorbance.

## RESULTS AND DISCUSSION

Laccases were firstly described in 1883 from the Japanese lacquer tree *Rhus vernicifera* (Fu and Viraraghavan, 2001). Since then, several laccases have been studied with respect to their biological function, substrate specificity, copper binding structure, and Industrial applications (Barr and Aust, 1994; Bourbonnais and Paice, 1990; Giardina et al., 1999). *Pleurotus* sp. is a wood rotting basidiomycetes and laccase is the dominant ligninolytic enzyme synthesized by this species. The newly isolated *P. ostreatus* MR3 could completely decolorize RBBR at day 5 on potatoes dextrose agar (PDA) agar plates containing RBBR (Figure 1). When grown in liquid culture, only laccase activity was detectable in the supernatant. The other two ligninolytic activities (manganese peroxidase (MnP) and lignin peroxidase (LiP) could not be detected.

Data reported in Figure 2A and B show that decolorizing ability of RBBR was strongly correlated to laccase production. *P. ostreatus* MR3 produced laccase activity of 1.56 IU/mL on day 3, while the maximum acivity of 2.82 IU/mL was obtained on day 5. Pattern of enzymatic activity production in control cultures (without any added dye) is similar to that monitored in the presence of RBBR (data not shown). Dye decolourization at 200 mg/L RBBR concentration (Figure 2B) occurred at a rapid rate and only three days are needed to obtain the complete transformation of the dye to colourless compounds (95%). Moreover, the blue colour disappearance was not due to any protonation/deprotonation of the dye, since the pH of cultures was not altered during fungal growth, remaining constant at about 5.0. Absorbance spectra of samples withdrawn from fungal culture at different growth times

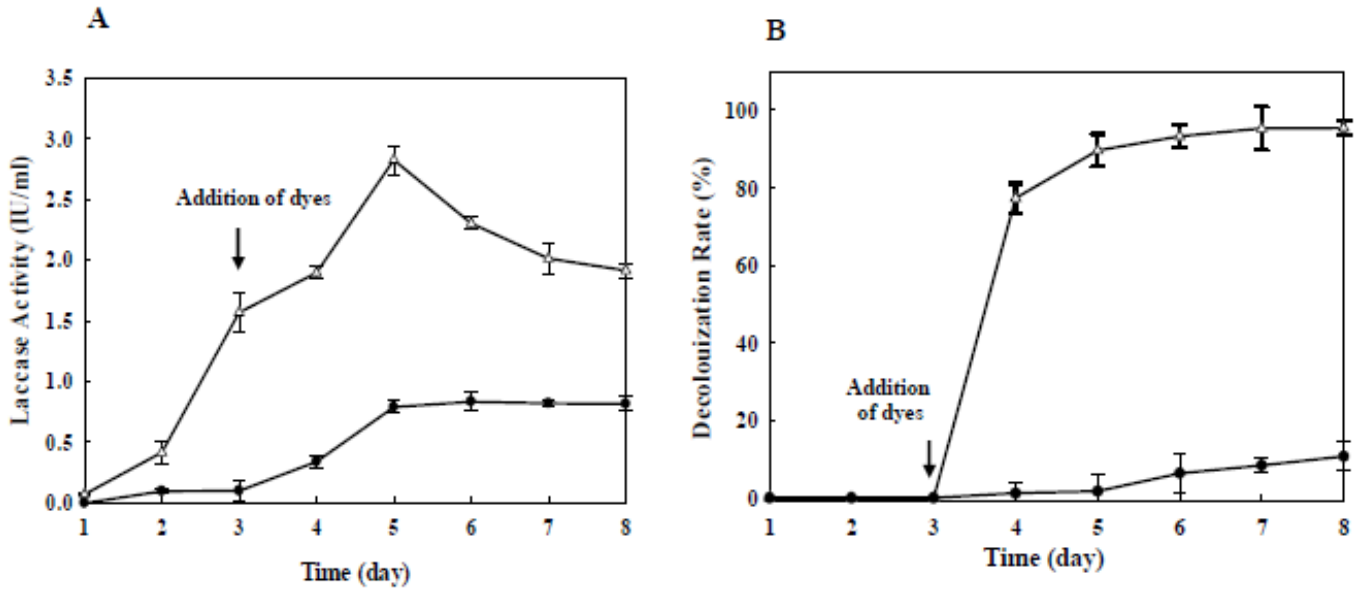


**Figure 2.** Time course of laccase production (A) and RBBR decolourization percentage (B). ∆, *P. ostreatus* MR3; ●, *L.edodes* MR6.

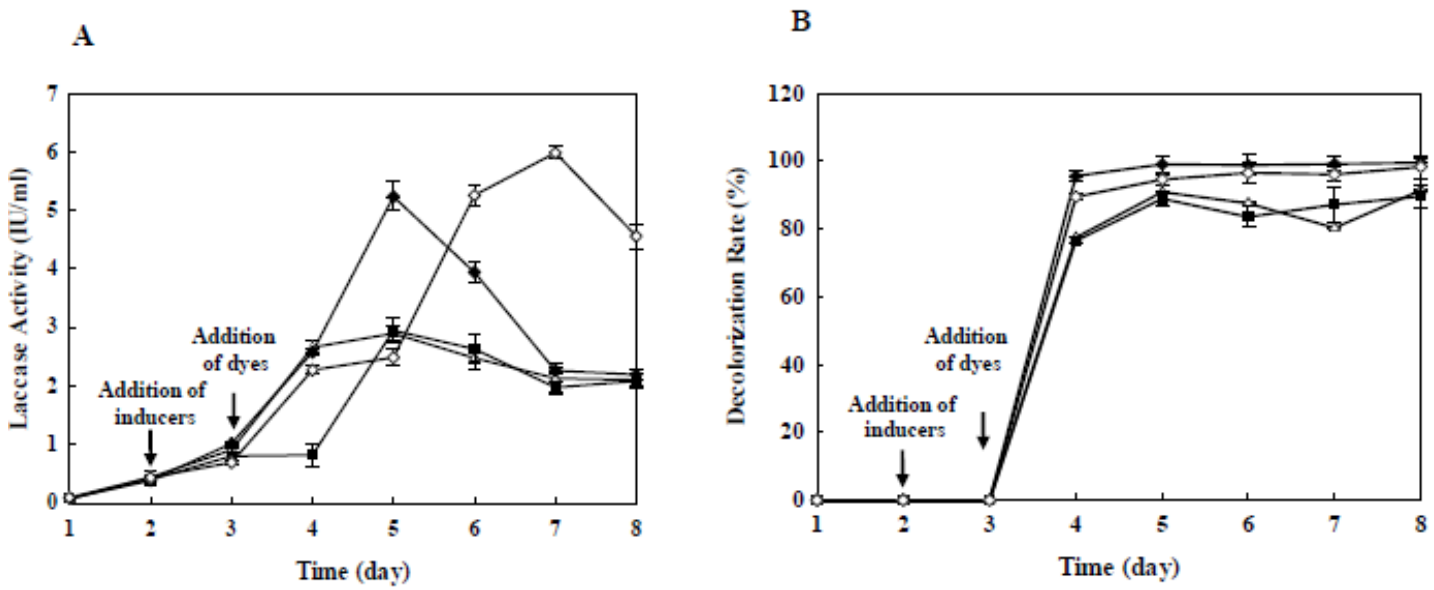


**Figure 3.** Time course of laccase production by *P. ostreatus* MR3 with different inducers (A) and decolouirization rate (B). ∆, Control; ♦, copper sulphate; ■, ABTS; ◊, veratryl alcohol.

showed a disappearance of the 592 nm absorbance peak concomitantly with changes in absorption in the 350 to 400 nm regions.

One of the most effective approaches to increase the yield of ligninolytic enzymes is the supplementation of the nutrient medium with an appropriate inducer. Aromatic and phenolic compounds have been widely used to elicit enhanced laccase production by different organisms (Chivukula and Renganathan, 1995) and the nature of the compound that induces laccase activity differs greatly with the species. Among the various laccase inducers reported in the literature, copper sulfate, veratryl alcohol, and ABTS were selected in this study and a control culture with no inducer addition was also performed (Chivukula and Renganathan, 1995; Xin and Geng, 2010; Giardina et al., 1996). The final inducer concentration in the basal medium was kept at 1 mM and added to the medium at day 2 (Figure 3). The results of our studies using different laccase inducers indicate that all the inducers included in the study were capable to enhance laccase production by *P. ostreatus* MR3. It is notable that the highest laccase activity obtained in the presence of

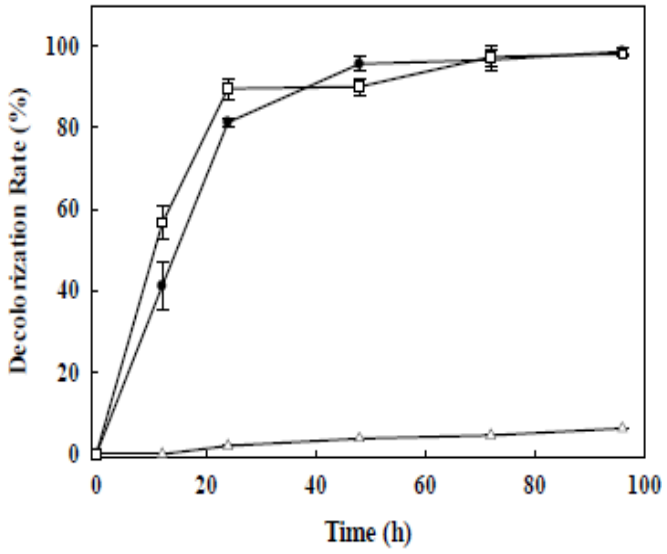


**Figure 4.** Decolorization potentials of the prepared laccase samples and the commercial laccase. ∆, Control; ●, samples; □, commercial laccase.

veratryl alcohol was 2.07-fold higher than that of the control followed by copper sulfate (1.81-fold) and ABTS (1.02-fold). Copper sulfate and veratryl alcohol have been reported to be strong laccase inducers for many fungal species (Giardina et al., 1996). According to Giardina et al. (1999), the presence of inducers, their chemical nature, the amount added, and the time of their addition influenced laccase production by many fungal species (Myrna et al., 2012).

Some fungal laccases as well as laccase mediator systems are efficient for dye decolorization. Different dyes are decolorized by different laccases at different rates. The decolorization rate depends on the structure and the redox potential of the enzyme as well as the structure of the dye. Some studies showed that the laccase alone could decolorize RBBR (Wong and Yu, 1999; Schliephake and Lonergan, 1996). However, Soares et al. (2001) found that the laccase alone did not decolorize RBBR and a small molecular weight redox mediator was necessary for decolorization to occur. The difference in fungal species from which the laccase was obtained might be a reason for this discrepancy. The redox potential of the laccase varied depending on the source of the laccase. In order to test the decolourization potentials of the laccase produced by *P. ostreatus* MR3, crude enzyme mixture was prepared. The fungus was grown in cultures which were supplemented with or without $Cu^{2+}$ as laccase inducer. After 12 days of incubation, the cultures were filtered through Whatman filter paper, RBBR was injected from a stock solution into the culture filtrates (final concentration 100 mg $L^{-1}$) and then the OD was measured at 431 nm. The decolourization results are displayed in Figure 4. No decolourization was observed in the control, indicating that the decolourization was biological and it was caused by laccase in the samples since no activities of other ligninolytic enzymes such as LiP and MnP were detected in the enzyme extract. *T. versicolor* is one of the best studied white rot fungi. The most active of the enzymes from *T. versicolor* is laccase. Laccase from *T. versicolor* can directly decolorize RBBR and the decolourization rate decreased with the increase of dye concentration (Guo et al., 2008). For both the prepared laccase sample and the laccase from *T. versicolor*, the decolourization degree increased with the increase of the prepared laccase samples and the com-mercial laccase. The initial decolourization rate for com-mercial laccase was higher, indicating that commercial laccase has better decolourization performance than our own enzyme samples. However, such difference became insignificant when the decolorization time reached 48 h. The highest decolorization degree was reached at 72 h for both enzyme samples, being 97.41% for the commercial laccase and 96.67% for our sample. This result suggests that laccase produced by *P. ostreatus* MR3 with the commercial laccase, having a high capability in decolourization.

## Conclusion

The indigenous strain of *P. ostreatus* MR3 isolated in Singapore was found to be effective in *in-vivo* treatment of RBBR. A significant increase in the *in-vivo* decolourization rate by *P. ostreatus* MR3 was observed on day 5, corresponding well to the high laccase production at that point of time. *In-vitro* dye decolourization by *P. ostreatus* MR3 compared favorably with the extensively studied laccase from *T. versicolor* strain, ATCC 20869. Veratryl alcohol was effective in inducing laccase production by *P. ostreatus* MR3 and therefore enhancing RBBR *in-vivo* decolourisation. Strain *P. ostreatus* MR3 is therefore a potential white rot fungus that can be used for laccase production and dye decolourization.

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