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Decolorization of Reactive Dye by White-Rot Fungus *Datronia* sp. KAPI0039

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ABSTRACT

This study focused on decolorization of two reactive dyes, Remazol Brilliant Blue R (RBBR) and Reactive Black 5 (RB5), by selected white-rot fungus *Datronia* sp. KAPI0039. The effects of reactive dye concentration, fungal inoculum size and pH were studied. Samples were collected periodically for the measurement of color, laccase (Lac), manganese peroxidase (MnP) and lignin peroxidase (LiP) activity. A level of 86% decolorization of 1,000 mgL^{-1} RBBR was achieved by 2% (w/v) *Datronia* sp. KAPI0039 at pH 5. The highest Lac activity (759.81 UL^{-1}) was detected under optimal conditions. For RB5, *Datronia* sp. KAPI0039 efficiently performed (88.01% decolorization) at 2% (w/v) fungal inoculum size for the reduction of 600 mgL^{-1} RB5 under pH 5. The highest Lac activity detected was 178.57 UL^{-1}, whereas there was no detected activity of MnP and LiP during this time. Therefore, the result indicated that *Datronia* sp. KAPI0039 was able clearly, to breakdown both reactive dyes and Lac was considered as a major lignin-degradation enzyme in this reaction.

Keywords: *Datronia* sp., oxidation, reactive dye, white-rot fungus

INTRODUCTION

Large amounts of chemical dyes (approximately 10,000 different dyes and pigments annually), are used for various industrial applications, such as in the textile and printing industries. It is estimated that about 10% of the dyes is lost in industrial effluents (Rodríguez *et al*., 1999). As a result, a significant proportion of these dyes are released to the environment in wastewater. Moreover, these dyes are designed to be resistant to light, water and oxidizing agents and therefore are difficult to degrade naturally once released into aquatic systems (Robinson *et al*., 2001). Thus, this can cause the obstruction of sunlight passing through the waters contaminated by synthetic dyes, which can lead to decreases in the level of oxygen dissolved in the water, the photosynthesis of water plants and the biodegradation of organic matter. At present, there are no biotechnological approaches that have proven potential to be effective in the treatment of this pollution source in an eco-efficient manner (Robinson *et al*., 2001). The possibility of using ligninolytic fungi to remove synthetic dyes is one approach that has attracted considerable attention.

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This is due to their production of ligninolytic enzymes (most frequently laccase and manganese peroxidase) that enable these microorganisms to oxidize a broad range of substrates, including synthetic dyes (Baldrian and Snajdr, 2006).

To date, many reports have demonstrated that white-rot fungi in the Basidiomycete class, such as *Phanerochaete chrysosporium, Trametes versicolor*, *Pleurotus ostreatus, Ganoderma* spp., *Irpex lacteus*, *Dichomitus squalens* and *Ischnoderma resinosum*, were capable of the efficient decolorization of pulping effluent and dye solutions by producing lignin-degrading enzymes, such as lignin peroxidase (LiP), laccase (Lac) and manganese peroxidase (MnP), through the oxidation of the phenolic group in the dyes (Jeffries *et al*., 1981; Hardin *et al*., 2000; Eicherová *et al*., 2005; Lopez *et al*., 2007; Svobodová *et al*., 2007). They have been widely researched also for their ability to degrade and adsorb dyes and some toxic chemicals, such as polycyclic aromatic hydrocarbons (PAHs) or chlorophenol compounds (Shim and Kawamoto, 2002; Hiratsuka *et al*., 2005). It was assumed that the color disappeared only after the chromophore structure of the dye molecule was destroyed by the many attacks of the lignin-degrading enzymes (Young and Yu, 1997). Attempts have been made to screen for new strains with these capabilties. Apiwattanapiwat *et al*. (2006) reported the efficiency of *Datronia* sp. KAPI0039 and *Trichaptum* sp. KAPI0025, isolated from rotten wood in Thailand, to achieve 54.9 and 54.4%, respectively, in the decolorization of pulp and paper mill effluent. Chedchant *et al*. (2009) showed that *Datronia* sp. KAPI0039 that had been cultivated on solid agar containing sawdust or rice straw, released extracellular Lac and MnP. However, no research has been conducted to determine whether the decolorization capability of this strain is related to lignin-degrading enzymes. The knowledge obtained from such research is not only important in determining the use of enzymes or microorganisms to control either synthetic dye removal or lignin degradation, but also to the understanding of alternatives to the conventional treatments.

The present study aimed to enhance knowledge of the white-rot fungus, *Datronia* sp. KAPI0039, regarding its involvement in the bio-oxidation of different reactive dyes. The relationship between ligninolytic enzyme production and the decolorization of a reactive dye solution by *Datronia* sp. KAPI0039 was assessed. Furthermore, the degradation efficiency of the fungus with regard to azo-based and anthraquinone-based reactions was compared.

MATERIALS AND METHODS

Microorganism and culture conditions

A culture of white-rot fungus, *Dratronia* sp. KAPI0039, obtained from Apiwattanapiwat *et al*. (2006), was used in the current study. The fungal stock culture was maintained through periodic transfer to potato dextrose agar (PDA) at 4°C until use. To prepare the inoculum, the fungus was transferred onto a fresh PDA plate and incubated at 30°C for 7 d, at which stage it was ready to be used in further experiments.

Dyes

The reactive dyes used in the study were Remazol Brilliant Blue R (RBBR) and Reactive Black 5 (RB5) that were obtained from DyStar Thai Company Limited, in Thailand. RBBR is a synthetic anthraquinone-based reactive dye. RB5 is a tetrasulphonated disazo reactive dye.

Bio-oxidation of the reactive dye solution by *Dratronia* sp.KAPI0039

Decolorization experiments were carried out in flasks. The dye solutions were prepared with a supplement of glucose, K_2HPO_4, $MgSO_4.7H_2O$, KCl, $FeSO_4.7H_2O$ and NH_4NO_3 in amounts of 10.0, 1.0, 0.5, 0.5, 0.01 and 1.75 gL^{-1}, respectively at pH 5.5. To prepare inocula for liquid cultures,

20 agar plugs (7 mm in diameter, from the edge of a 7-day-old agar culture) of *Dratronia* sp. KAPI0039 growing mycelia were inoculated into 250 mL glucose yeast extract (GYE) medium and then incubated at 30°C for 6 d, while being shaken at 150 rpm (Apiwattanapiwat *et al.*, 2006). Subsequently, they were filtered through cheese cloth to obtain fungal pellets. The bio-oxidation experiment was carried out in 500-mL flasks containing 300 mL dye solution. These were inoculated with 2.5% (w/v) wet *Datronia* sp. fungal pellets and incubated at 30°C for 7 d, while being shaken at 150 rpm. The color units and production of lignin-degrading enzymes were monitored periodically in order to evaluate the performance of the fungal cells in decolorization. All treatments were run in triplicate. Related parameters were studied, namely, the concentration of reactive dyes (200, 400, 600, 800 and 1,000 mgL^{-1}), fungal inoculum size (1 and 2% (w/v)) and pH (3, 5, 7 and 9).

Enzyme activities

Laccase (Lac) activity was measured by monitoring the oxidation of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) at 420 nm (molar extinction coefficient = 36000 $M^{-1}cm^{-1}$) according to Eggert *et al.* (1996). One unit of laccase activity was defined as the amount of enzyme that oxidizes 1 μmol ABTS in 1 min.

Lignin peroxidase (LiP) activity was measured by monitoring the oxidation of veratryl alcohol in the presence of H_2O_2 at 310 nm (molar extinction coefficient = 9300 $M^{-1}cm^{1}$) according to Tien and Kirk (1984). One unit of LiP activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of veratraldehyde in 1 min.

Determination of manganese peroxidase (MnP) activity using MBTH and DMAB was based on Castillo *et al.* (1994). MBTH and DMAB were coupled oxidatively by the action of the enzyme in the presence of added H_2O_2 and Mn^{2+} ions to give a purple indamine dye product. One unit of MnP activity was defined as an amount catalyzing the production of 1 μmol of green or purple product per ml in 1 min.

Color unit

The samples were filtered through 0.45 μm cellulose acetate membrane to remove suspended solids. The intensity of color, before and after treatment, was determined spectrophotometrically (HUCH DR/2010) at 592 nm (Baldrian and Snajdr, 2006).

RESULTS AND DISCUSSION

Activity of enzymes

Prior to the experiments, the production ability of Lac, MnP and LiP by *Datronia* sp. KAPI0039 was confirmed as a solid cultivation on a basal medium (made up of glucose, K_2HPO_4, $MgSO_4.7H_2O$, KCl, $FeSO_4.7H_2O$ and NH_4NO_3 in amounts of 10.0, 1.0, 0.5, 0.5, 0.01 and 1.75 g/L, respectively) containing rice straw, and as a liquid cultivation in the GYE medium. The results in a solid cultivation were consistent with Chedchant *et al.* (2009) and are shown in Figure 1. Lac was detectable in the early growth period and reached a maximum (4,502.2 Ug^{-1} substrate) after 4 d cultivation. MnP activity was minimal as expected with a maximum (471.7 Ug^{-1} substrate) after 8 d cultivation. No LiP activity was detected. The enzymes produced by the strain in the GYE medium are shown in Figure 2. Likewise, Lac and MnP activity was detected also in the early growth period, with maximum Lac and MnP activity at 1,130.0 UL^{-1} after 24 h and at 264 UL^{-1} after 48 h cultivation, respectively. Interestingly, maximum enzyme activity was observed much earlier in the liquid cultivation. This could have been due to the presence of more carbon and nitrogen sources in the GYE medium that stimulated the growth and enzyme production of *Datronia* sp. KAPI0039, as was suggested also by Hatvani and Mecs (2002).

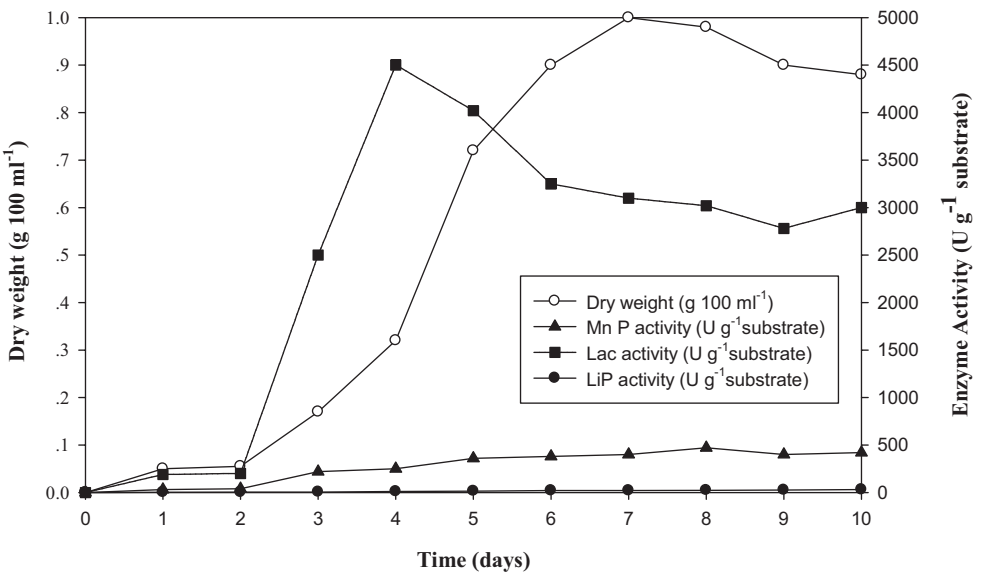


Figure 1 Production over time of Lac, MnP and LiP by *Datronia* sp. KAPI0039 in the basal medium containing rice straw (150 rpm) at 30°C for 10 d.

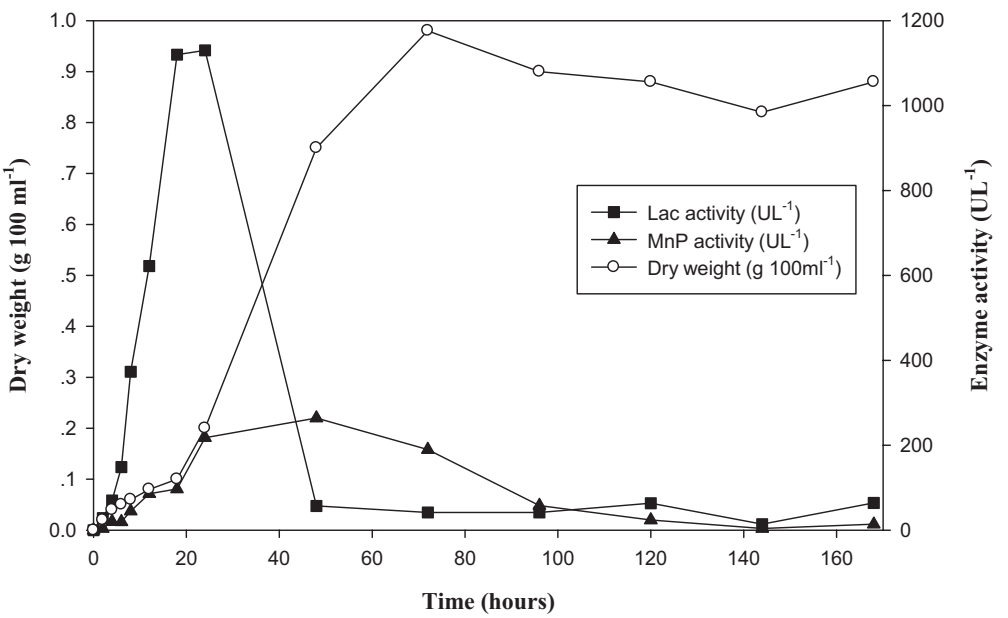


Figure 2 Production over time of Lac, MnP and LiP by *Datronia* sp. KAPI0039 in GYE medium (150 rpm) at 30°C for 7 d.

Bio-degradation of the reactive dye solution by *Dratronia* sp. KAPI0039

The effect of reactive dye concentration

The effects of reactive RBBR and RB5 concentrations on %decolorization, Lac and MnP activities by *Datronia* sp. KAPI0039 were examined. Dye solutions were varied with concentrations of 200, 400, 600, 800 and 1000 mgL^{-1}. The results indicated a dramatic decrease (>90%) in color reduction of both RBBR and RB5 solutions at every concentration (Figures 3 and 4, respectively). The results (99.86% decolorization in 72 h) also indicated that the rate and extent of

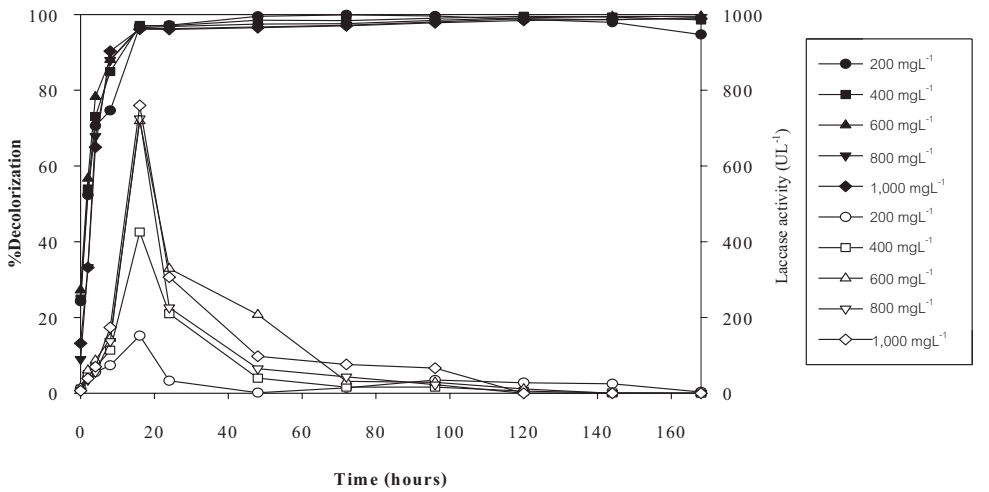


Figure 3 The effect of reactive RBBR dye concentration on %decolorization and Lac activity after cultivation with *Datronia* sp. KAPI0039 (150 rpm) at 30°C for 7 d. Solid symbols = %decolorization; open symbols = Laccase activity.

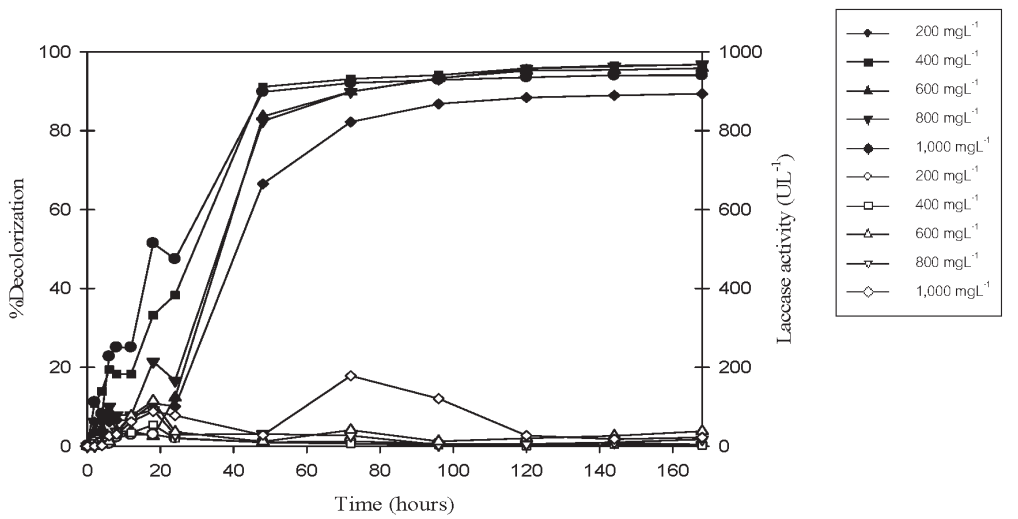
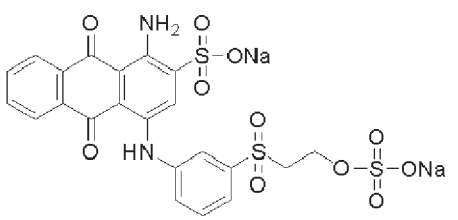


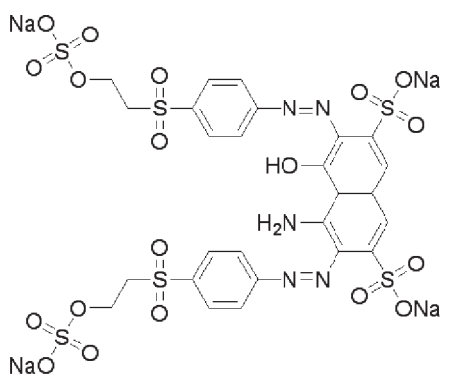
Figure 4 The effect of reactive RB5 dye concentration on %decolorization and Lac activity after cultivation with *Datronia* sp. KAPI0039 (150 rpm) at 30°C for 7 d. Solid symbols = %decolorization; open symbols = Laccase activity.

decolorization of RBBR compared favorably with those by other white-rot fungi, such as *P. chrysosporium* (83% decolorization in 264 h, Swamy and Ramsay, 1999), *Bjerkandera* sp. BOS55 (65% decolorization in 480 h, Swamy and Ramsay, 1999) and *Trametes trogii* (85% decolorization in 72 h, Mechichi *et al.*, 2006). Furthermore, the results demonstrated that dye concentration did affect the time period required to reach maximum decolorization for solutions of both RBBR and RB5. A general tendency was that higher concentrations of dye solution caused slower rates of and thus longer time periods for decolorization (Young and Yu, 1997). Pearce *et al.* (2003) suggested that the dye concentration influenced the efficiency of dye removal through a combination of factors, including the toxicity of the dye at higher concentrations and the ability of the enzymes to recognize the substrate efficiently at very low concentrations. For example, *Datronia* sp. KAPI0039 reached maximum color reduction (99.86%) from the solution containing 200 mgL^{-1} RBBR within only 72 h of treatment, whereas maximum decolorization (98.87%) from the solution containing 1,000 mgL^{-1} RBBR was achieved after 168 h of treatment (Figure 3). This was consistent with the study by Aksu *et al.* (2007) that reported the white-rot, *T. versicolor*, took 8 d to reach maximum color reduction (95%) from the starting solution with 58.4 mgL^{-1} RB5, whereas maximum color reduction (77%) from a starting solution with 358.6 mgL^{-1} RB5 was achieved within 14 d. Interestingly, RBBR seemed to have better degradation than RB5, as indicated by the higher %decolorization (Figures 3 and 4). Revankar and Lele (2007) reported azo dyes were recalcitrant to decolorization and could be decolorized to a limited extent. Sani and Banerjee (1999) suggested that dyes with simple structures and low molecular weights exhibited higher rates of color removal, whereas color removal was more difficult with highly substituted, high molecular weight dyes (Figure 5). However, Eichlerová *et al.* (2006) stated that the difference between decolorization of structurally different dyes was not easy to explain because this process required the destruction of the chromophore. Thus, the slow decolorization rate of some dyes could be attributed to the complexity of their chromophores, but the overall complexity alone was not an indicator of the difficulty of decolorization of a particular dye.

During the course of dye decolorization, maximum Lac activities at 759.81 UL^{-1} and 178.57 UL^{-1} were detected in the fungal-treatedRBBR and RB5 solutions (Figures 3 and 4, respectively). Only a little MnP and no LiP activity (data not shown) were detected. The results also indicated



Remazol Brilliant Blue R (RBBR)



Reactive Black 5 (RB5)

Figure 5 Chemical structures of the dyes studied.

the corresponding increase in Lac activity with increased %decolorization, with the enzyme activity peaking at the time of maximum color reduction (16-h cultivation). Thus, only Lac seemed to be correlated with dye decolorization, which was supported also by Rodríguez *et al.* (1999). Moreover, the highest Lac activity and %decolorization were obtained when solutions of 1000 mg/L RBBR and RB5 were applied. Thus, the higher dye concentration induced more Lac production, which in turn resulted in more decolorization (Robinson *et al.*, 2001; Baldrian and Snajdr, 2006). This could imply also that decolorization of reactive dyes depended partially on Lac activity in the liquid cultures, but not on MnP and LiP activity. In addition, Lac activity in the liquid culture containing RBBR was much higher than in the culture containing RB5 (Figures 3 and 4), even though similar %decolorization levels were observed. This could have been associated with the specificity of ligninolytic enzymes on different dye structures (Rodríguez *et al.*, 1999); thus, different dye structures led to the induction of different ligninolytic enzymes. The important role of purified LiP in color reduction of several azo-, triphenyl methane-, heterocyclic- and polymeric-dyes has been clearly demonstrated (Ollikka *et al.*, 1993; Young and Yu, 1997; Rodríguez *et al.*, 1999). In the current experiment no LiP was detected, so, therefore, high %decolorization of the RB5 solution was thought to be involved in other mechanisms. One approach was attributed to the sorption of the dye on the fungal mycelium (Baldrian and Snajdr, 2006; Svobodová *et al.*, 2007). Thus, it could be assumed that the mechanism of synthetic dye degradation by *Datronia* sp. KAPI0039 was shared by the extracellular enzyme activity and biosorption on fungal cells. However, the relative contributions of ligninolytic enzymes to the decolorization of dyes might be different for each fungal strain and each dye (Park *et al.*, 2007).

The effect of fungal inoculum size

The effect of fungal inoculum size on %decolorization, and Lac and MnP activities by *Datronia* sp. KAPI0039 was investigated (Figures 6 and 7). The inoculum sizes used in the study were 1, 2 and 3% (w/v). The strain decolorized

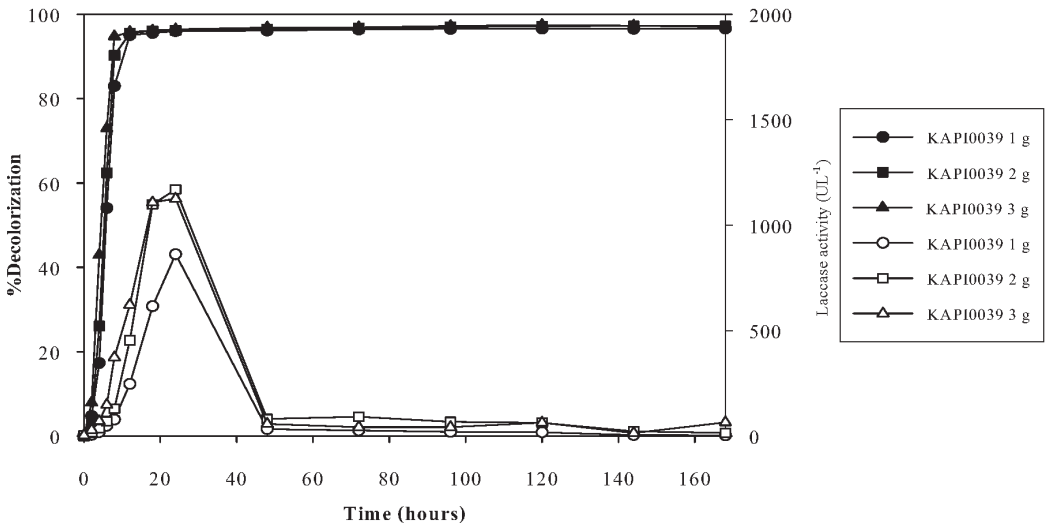


Figure 6 The effect of fungal inoculum size on %decolorization of RBBR dye solution and Lac activity after cultivation with *Datronia* sp. KAPI0039 (150 rpm) at 30°C for 7 d. Closed symbol = %decolorization; open symbol = Laccase activity.

both dyes tested, but RBBR decolorization was faster and started earlier than that of RB5. Over 90% of RBBR was decolorized as early as in the first 24 h cultivation, but only 20% of RB5 was removed within the same period, and then was continuously removed to a maximum of 90% after 100 h cultivation. The fungal inoculum size was found to have a slight effect on the decolorization of both dyes. The production of Lac and MnP was also studied under the same conditions as in the decolorization experiments. High activity of Lac was detected, whereas only a low amount of MnP was detected. The results showed that Lac activity was affected partially by the fungal inoculum size, as was observed by the similar Lac activity for every fungal inoculum size used in the study. Furthermore, the results demonstrated the direct relationship between Lac activity and dye decolorization, as shown by the highest Lac activity and dye decolorization occurring in the same period. The study of Baldrin and Snajdr (2006) was also consistent, showing that RBBR was more efficiently degraded by the litter-decomposing fungi than RB5, as well as that Lac was the major ligninolytic enzyme found in that condition.

The effect of reaction pH

The effect of pH on dye decolorization was investigated at pH 3, 5, 7 and 9. The results are shown in Figures 8 and 9. Although the decolorization of individual dyes (RBBR and RB5) was affected by pH to different extents, better decolorization was observed for RBBR. In addition, the results indicated that better decolorization of RBBR was achieved under the neutral to basic conditions, whereas the decolorization of RB5 was better under acidic conditions. This was consistent with the study by Young and Yu (1997) that found an azo-based dye was more effectively degraded by white-rot fungi under acidic conditions. However, Pearce *et al*. (2003) reported that the optimum pH for color removal by white-rot fungi was often at a neutral or slightly alkaline pH, and the rate of color removal tended to decrease rapidly under strongly acid or strongly alkaline conditions, without any relationship to dye structure. Ciullini *et al*. (2008) showed that the decolorization efficiency of

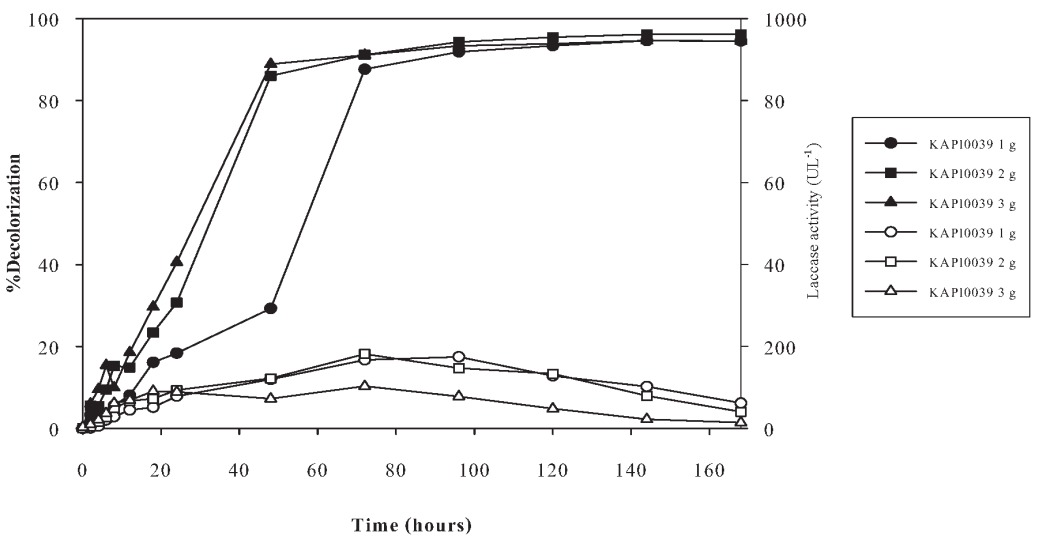


Figure 7 The effect of fungal inoculum size on %decolorization of RB5 dye solution and Lac activity after cultivation with *Datronia* sp. KAPI0039 (150 rpm) at 30°C for 7 d. Closed symbol = %decolorization; open symbol = Laccase activity.

different dye structures was not affected by pH, but was related to the Lac concentration. During the decolorization experiment, the production of Lac and MnP by the fungus was determined as a function of time (Figures 8 and 9). Of the two enzymes studied, Lac activity was greater in the crude extract of both RBBR and RB5. Furthermore, the results indicated the relationship of Lac production to time and the ability of *Datronia* sp. KAPI0039 to decolorize the two dyes.

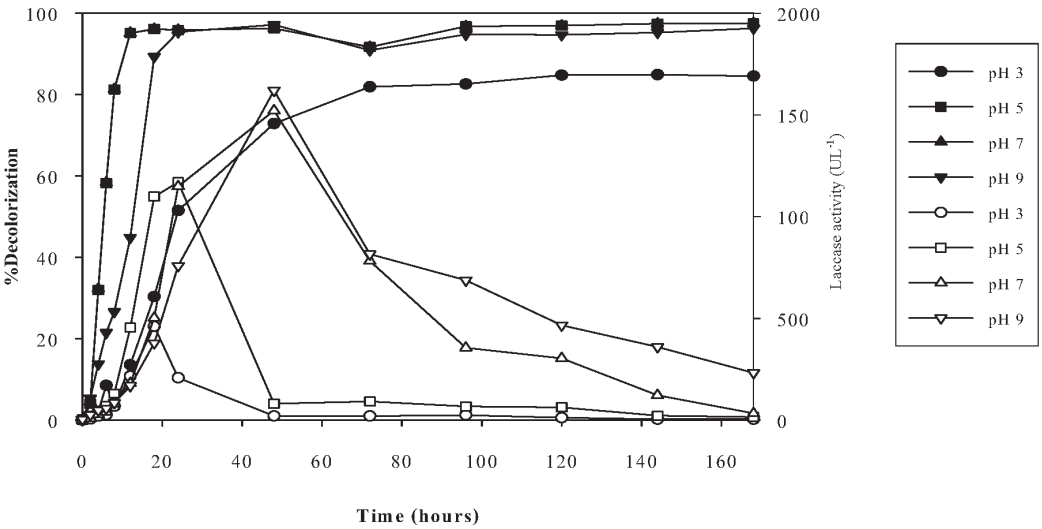


Figure 8 The effect of pH on %decolorization of RBBR dye solution and Lac activity after cultivation with *Datronia* sp. KAPI0039 (150 rpm) at 30°C for 7 d. Closed symbol = %decolorization; open symbol = Laccase activity.

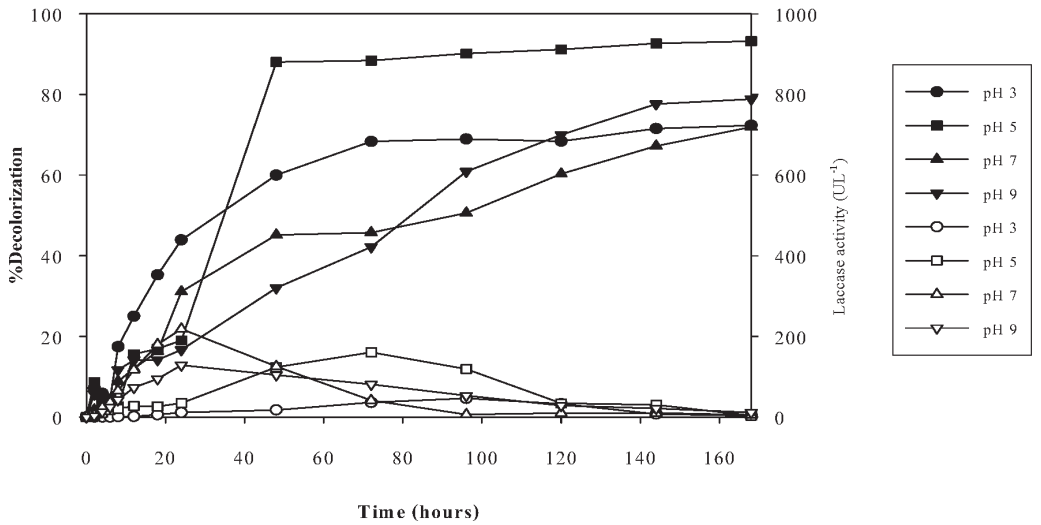


Figure 9 The effect of pH on %decolorization of RB5 dye solution and Lac activity after cultivation with *Datronia* sp. KAPI0039 (150 rpm) at 30°C for 7 d. Closed symbol = %decolorization; open symbol = Laccase activity.

CONCLUSION

Recently, there has been growing interest in studying lignin-degrading enzymes, with the expectation of finding systems that are more effective to apply in various biotechnological approaches. Previous studies demonstrated the presence of ligninolytic enzymes (Lac, MnP and LiP) in several species of white-rot fungi, especially in *P. chrysosporium* and *T. versicolor*, but there have been no reports of those enzymes in the genus *Datronia*. The current study provides the first evidence to report the decolorization capability and the production of ligninolytic enzymes, mainly Lac and MnP, by the genus *Datronia* in a reactive dye solution. This study supports the different extents to which fungi have the ability to degrade synthetic dyes of diverse structures. Although high dye concentrations might have a toxic effect on fungi, it was found that even a concentration of 1,000 mg/L of reactive dye was tolerated by the tested species. Interestingly, no one has reported on the decolorization of such high dye concentrations by any white-rot fungi, except the study by Eichlerová *et al*. (2006). The current study suggests the possibility to decolorize a high concentration of commercial dyes, which could be a great advance in the treatment of dye contained in wastewater, and the method may have a potential application for dye decolorization, especially in the textile industry. The results also seem to indicate that Lac is the major ligninolytic enzyme involved in the breakdown of the dye in the solution. The crude extract from *Datronia* sp. KAPI0039 cultures showed the highest Lac activity and %decolorization. Thus, Lac from *Datronia* sp. KAPI0039 should be purified and their kinetic constants determined, with ABTS, RBBR and RB5 as substrates, in order to elucidate the specificity of Lac on these reactive dye structures. Moreover, the performance of Lac on the decolorization reaction should be studied *in vitro*.

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