World J Microbiol Biotechnol (2011) 27:535–545
DOI 10.1007/s11274-010-0487-3

ORIGINAL PAPER

Enzymes from spent mushroom substrate of *Pleurotus sajor-caju* for the decolourisation and detoxification of textile dyes

Avneesh D. Singh · Sabaratnam Vikineswary · Noorlidah Abdullah · Muniandy Sekaran

Received: 4 March 2010 / Accepted: 17 June 2010 / Published online: 1 July 2010

Abstract The potential of ligninolytic enzymes, including lignin peroxidase (LiP) as the main enzyme from the spent mushroom substrate of *Pleurotus sajor-caju* was evaluated for the decolourisation of five dyes from azo and anthraquinone dye groups. Among the azo dyes, reactive black 5 and reactive orange 16 were 84.0 and 80.9% decolourised respectively, after 4 h of incubation with 45 U of LiP as compared to 32.1% decolourisation of disperse blue 79. Among the anthraquinone dyes, disperse red 60 was decolourised to 47.2% after 4 h of incubation with 45 U of LiP as compared to 5.9% decolourisation of disperse blue 56. Increasing the LiP concentration and incubation time had a positive effect on the decolourisation of anthraquinone dyes as compared to azo dyes. A 67.9% decolourisation of synthetic textile waste-water was achieved after 4 h of incubation with 25 U of LiP. Increasing the incubation time significantly increased ($P < 0.05$) the decolourisation of synthetic textile waste-water. Further, there was a 52.4% reduction in the toxicity of synthetic textile waste-water treated with 55 U of LiP for 4 h. However, only 35.7% reduction in toxicity was achieved when the synthetic textile waste-water was treated with 55 U of LiP for 24 h. In this study, it was shown that the spent mushroom substrate of *P. sajor-caju* could be a cheap source of ligninolytic enzymes for the decolourisation of dyes in textile industry wastewaters.

Keywords Textile dye decolourisation · Spent mushroom substrate · *Pleurotus sajor-caju* · Toxicity reduction · *Artemia salina*

A. D. Singh
Institute of Post-Graduate Studies, University of Malaya, 50603 Kuala Lumpur, Malaysia

S. Vikineswary (✉) · N. Abdullah
Institute of Biological Sciences, University of Malaya, 50603 Kuala Lumpur, Malaysia
e-mail: viki@um.edu.my

M. Sekaran
Department of Molecular Medicine, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

Introduction

Textile dyes are classified as azo, anthraquinone, phthalocyanine and triarylmethane groups based on their chemical and dyeing properties. Azo dyes, which constitute 70% by weight of the estimated 10^9 kg of dyes that are produced annually in the world, are characterized by reactive groups that form covalent bonds with OH−, NH− or SH− groups in fibers, and are mostly used for yellow, orange and red colors (Christie 2001). Anthraquinone dyes which constitute the second most important class of textile dyes are used for violet, blue and green colors (Christie 2001). It has been estimated that 10% of the dyes used during the dyeing process does not bind to the fibers and are released into sewage treatment system or the environment (Clarke and Anliker 1980). The discharge of the dye effluents into rivers leads to a reduction of sunlight penetration in natural water bodies, which in turn decreases both photosynthetic activity and dissolved oxygen concentration (Banat et al. 1996). These dyes can be carcinogenic or mutagenic and under anaerobic conditions can be converted to aryl amines which are potentially more toxic than the parent compound (Chung and Stevens 1993). These dyes are stable and can remain in the environment for an extended period of time (Hao et al. 2000). As these dyes are designed to be recalcitrant, the degradation of textile dyes with conventional

processes occurs to only a limited extent (Wesenberg et al. 2003). Wastewaters from the textile industries that contain high concentrations of organic and inorganic chemicals as well as color constitute a threat to the environment in many parts of the world (Slokar et al. 1999).

Biological treatment with white-rot fungi may successfully decolourise textile dyes due to their lignin modifying enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP), laccase and H_2O_2 producing oxidases. The white rot fungi that have been explored for the decolourisation of various industrial dyes include *Bjerkandera adusta* (Heinfling et al. 1997), *Trametes versicolor* (Ramsay and Nguyen 2002), *Irpex lacteus* (Maximo and Costa-Ferreira 2004), *Pleurotus ostreatus* (Zhao et al. 2006) and *P. sajor-caju* (Munari et al. 2008). Many of the decolourisation studies with white-rot fungi had been conducted using fungal mycelia in the live or dead form. However, the direct application of fungus for the bioremediation has some disadvantages. One of the major disadvantage of using fungal cultures is the accumulation of biomass, which would cost the wastewater treatment on industrial scale. To overcome this disadvantage, the application of isolated enzymes for dye decolourisation in place of fungal mycelia has gained popularity in the recent years. The decolourisation of textile dyes using crude or purified enzymes such as laccase (Abadulla et al. 2000), LiP (Yu et al. 2006) and MnP (Heinfling et al. 1998) has been reported. However, the high cost involved in procuring isolated enzymes for the bioremediation purposes limits their application to a certain extent.

One of the readily available and cheap source of enzymes is the spent residue of commercially grown edible white-rot fungi. The spent mushroom substrate (SMS) consists of mycelia, extracellular enzymes produced by fungus during growth and unutilized lignocellulosic substrate. In Malaysia, *P. sajor caju* is grown commercially on supplemented rubber-wood sawdust. For every 200 g of mushroom produced, about 600–800 g of SMS is available. An average farm discards about 24 tons of SMS per month. The disposal of SMS is a major problem faced by the farmers. This SMS is normally disposed by spreading on land, burying, composting with household refuse, dumping in landfill sites or incineration. Earlier we had reported that the SMS of *P. sajor-caju* is an excellent source of ligninolytic and cellulolytic enzymes (Avneesh et al. 2003). The enzymes of SMS have potential in the decolourisation of chemically different dyes (Avneesh et al. 2002; Avneesh et al. 2010). In this paper, the potential of enzymes from the SMS of *P. sajor-caju* was further explored for the decolourisation of reactive and disperse textile dyes. In Malaysia, reactive and disperse dyes are extensively used as compared to dyes from other dye groups. The annual consumption of reactive and disperse dyes is 1,300–1,800 tons (Yong K.S., Sunny-Chem Color (M) Sdn. Bhd., personnel communication). Reactive group azo dyes are often used in textile dyeing due to their superior fastness to the applied fabric, high photolytic stability and resistance to microbial degradation (Gouvea et al. 2000). Disperse azo dyes are used for dyeing polyester, nylon, diacetate and triacetate of cellulose as well as in acrylic fibers (Zhao and Hardin 2007). Reactive dyes which exhibit low levels of fixation with fiber are particularly problematic as 10–20% of total dye and accessory chemicals used remain in the spent dye bath (Gouvea et al. 2000). Treatment of the textile effluents containing reactive dyes by conventional biological processes is difficult and ineffective (Peralta-Zamora et al. 1999; Arslan et al. 2000). Similarly, formation of toxic and mutagenic metabolites after reductive cleavage of disperse dyes has also been reported (Weber and Adams 1995). Therefore, the aims of this study were to investigate the potential of the enzymes from SMS of *P. sajor-caju* to decolourise reactive and disperse textile dyes and to detoxify the synthetic textile waste-water.

Materials and methods

Extraction and concentration of enzymes from spent substrate of *P. sajor caju*

Five-month old *P. sajor-caju* bags were collected from Highland and Lowland Mushroom Industry, Semenyih, Selangor, Malaysia. The contents of six bags were thoroughly mixed and 100 ml of tap water pH 4.0 was added to 10 g of the SMS. The resultant mixture was then homogenized for eight min at 8,000 rpm at 28°C using IKA Ultra-Turrax T25 homogenizer (Avneesh et al. 2003). The solids were separated by centrifugation at 9,000 rpm (15,000 g) for 20 min and the supernatant containing the fungal enzymes was henceforth called crude enzymes. The crude enzymes were concentrated with Millipore stirred cells fitted with 10,000 daltons cut off membrane. Lignin peroxidase activity (U/g) in concentrated enzymes was analyzed (Tien and Kirk 1983) and the activity was expressed as international units (U). The enzyme activity was defined as the amount of enzyme required to produce 1 μmol product/min and was reported in terms of productivity as U/g of the substrate.

Preparation of textile dyes and synthetic textile waste-water stocks

The textile dyes studied were reactive black 5, reactive orange 16 and disperse blue 79 from azo dye group, disperse red 60 and disperse blue 56 from the anthraquinone dye group (Fig. 1). Reactive black 5 and reactive orange 16

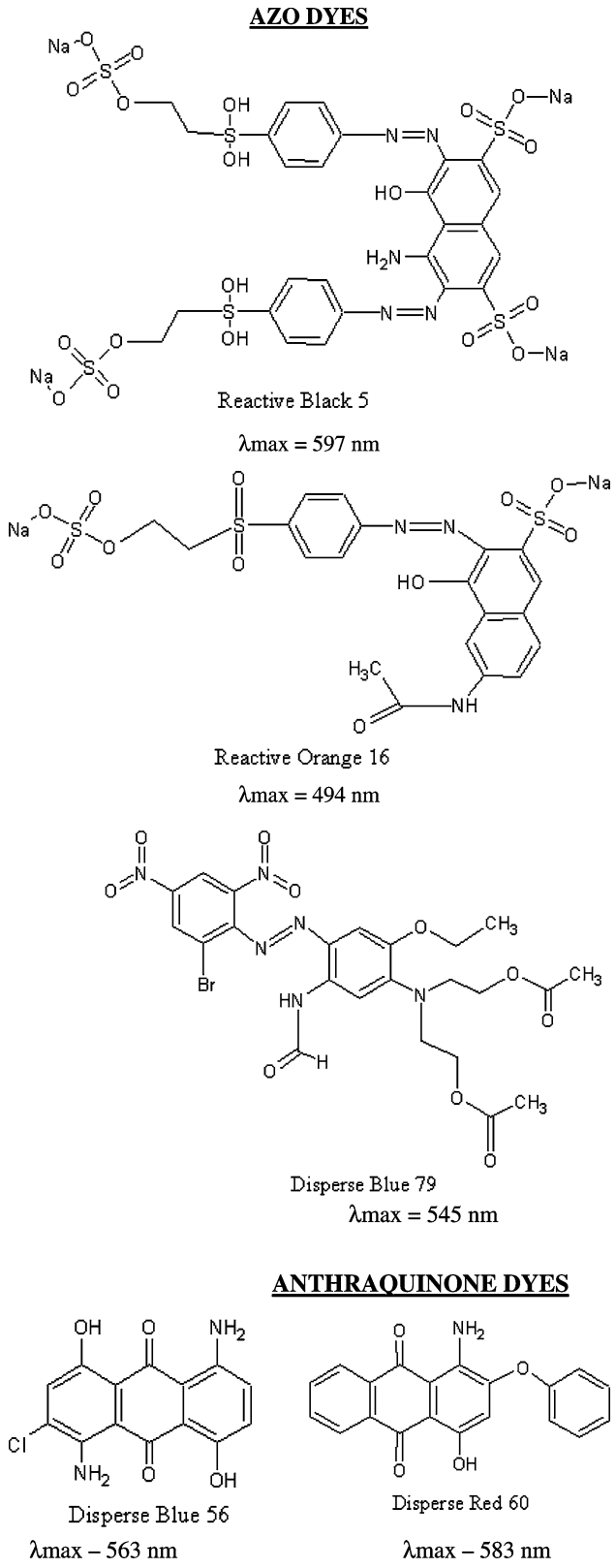

Fig. 1 Molecular structure and the absorption maxima of the dyes from azo and anthraquinone dye groups

were purchased from Sigma–Aldrich Chemical Co. (St Louis, MO, USA) whereas, disperse dyes were kindly provided by Sunny-Chem Color (M) Sdn. Bhd. (Cheras, Kuala Lumpur, Malaysia). The textile dyes stock (25 mg dye/100 ml of 50 mM sodium tartrate buffer pH 4.5 for all dyes, 10 mg dye/100 ml 50 mM sodium tartrate buffer pH 4.5 for reactive black 5) could be stored without any biochemical change in dark bottles at 4°C for one month. A synthetic textile waste-water was prepared by dissolving 5 mg of each of the five reactive and disperse dyes in 100 ml of 50 mM sodium tartrate buffer at pH 4.5 (Kirby et al. 1995). The final concentration of the dyes in the synthetic textile waste-water was 250 mg/l. The synthetic textile waste-water stock was also stored in dark bottles at 4°C.

Decolourisation of textile dyes and synthetic textile waste-water with concentrated enzymes from spent substrate of *P. sajor caju*

The decolourisation of reactive and disperse dyes except for disperse blue 56 was studied by varying the concentration of LiP from 25 U to 45 U with an increment of 10 U. The LiP concentrations for the decolourisation of disperse blue 56 and synthetic textile waste-water, however, was varied from 25 U to 65 U and 25 U to 55 U respectively, with an increment of 10 U. This variation in the LiP concentration was based on the trial runs whereby a low decolourisation of disperse blue 56 was achieved at LiP concentration of 25 U till 45 U. Therefore, in subsequent experiments, the LiP concentration was increased to 65 U for disperse blue 56. Similarly, in order to achieve a higher percentage decolourisation of synthetic textile waste-water, LiP concentration was increased to 55 U. The reaction mixtures for all the dyes in a final volume of 3.0 ml contained various LiP concentrations mixed with individual dyes in 50 mM sodium tartrate buffer at pH 4.5, 1 mM veratryl alcohol as a mediator and 0.2 mM H_2O_2. The final concentration of all the dyes and synthetic textile waste-water in the reaction mixture was 75 μg except for reactive black 5 which was 30 μg. All the reaction mixtures were incubated at 30°C for 4, 8, 12 and 24 h. After regular intervals, the decrease in the absorbance was measured using a UV/VIS Shimadzu spectrophotometer (model 160A, Shimadzu Corporation, Kyoto, Japan) at the absorption maxima (λmax) of each dye (Fig. 1). The decrease in the absorbance of synthetic textile waste-water was measured at 594 nm. All the experiments were run in triplicates. Decolourisation of each dye and synthetic textile waste-water was expressed in terms of percentage which was calculated according to the following equation:

$$\% \text{ decolourisation} = \frac{A_0 - A_t}{A_0} \times 100$$

where A_0 is an absorbance at λmax of each dye immediately measured after enzyme addition and A_t is an absorbance at λmax of each dye at a given time.

Toxicity bioassay

The toxicity of the synthetic textile waste-water before and after enzymatic treatment on *Artemia salina* was assayed (Rice and Mannes 2004). *Artemia salina* cysts were hatched in artificial sea salt solution (salinity—1.020 to 1.023) for 3 days to nauplii stage. The *Artemia* nauplii were then used in toxicity bioassay. Synthetic textile waste-water at a final dye concentration of 75 μg was treated with 55 U of concentrated LiP from spent substrate of *P. sajor-caju* at different time intervals of 4, 8, 12 and 24 h. After each time interval, 0.3 ml aliquots were added into 24-multiwell plate containing fifteen *Artemia salina* nauplii in each well. The final volume in each well was topped up to 2.5 ml with artificial sea salt solution. Two controls, one containing *Artemia* nauplii in sea salt solution only and the other containing 0.3 ml of synthetic textile waste-water without any enzyme treatment was also run parallel to the experiment. The multiwell plate was incubated at room temperature (27 ± 2°C) for 24 h. After incubation, the number of dead and live nauplii in each well was counted using binocular microscope Nikon SMZ-10. All the samples were run in triplicates. The percentage toxicity of untreated and enzyme treated synthetic textile waste-water was calculated by dividing the number of dead *Artemia* by initial number of live *Artemia* in each well. The percentage reduction of toxicity was calculated by subtracting the toxicity of treated samples from the toxicity of untreated sample (synthetic textile waste-water only).

Statistical analysis

The means of triplicate data were subjected to a two way analysis of variance (ANOVA) and the significance of the difference between means was determined by the Duncan's multiple range tests at 95% least significant difference ($P < 0.05$).

Results and discussion

Decolourisation of reactive and disperse textile dyes with concentrated enzymes from spent substrate of *P. sajor caju*

During the decolourisation of reactive and disperse textile dyes with varying concentrations of LiP from spent substrate of *P. sajor-caju*, it was observed that reactive dyes showed a higher percentage decolourisation as compared to disperse dyes. Incubation of reactive dyes with concentrated enzymes from spent substrate for 4 h resulted in above 60% decolourisation whereas, incubation of disperse dyes with concentrated enzyme for the same time interval of 4 h resulted in less than 50% decolourisation. An increase in the incubation time increased the percentage decolourisation of reactive dyes. However, an increase in both the incubation time and enzyme concentration was necessary for a higher percentage decolourisation of disperse dyes.

Among the reactive azo dyes tested, a lower concentration of reactive black 5 was employed for decolourisation studies. A preliminary study showed that at a concentration of 75 μg in the reaction mixture, there was no decolourisation of reactive black 5. Therefore, in subsequent experiments, the concentration of reactive black 5 was reduced to 30 μg. Reactive black 5 showed 80% decolourisation with 25 U of LiP after 4 h, which increased to 87% after 8 h (Fig. 2). A higher percentage decolourisation of reactive black 5 as compared to reactive orange 16 was also observed although reactive black 5 had a more complex structure as compared to reactive orange 16. This might be due to the lower concentration of reactive black 5 (30 μg) employed for decolourisation studies as compared to reactive orange 16 (75 μg). Increasing the LiP concentration from 35 U to 45 U had significant effect ($P < 0.05$) on the decolourisation of reactive black 5 after 4 h as the percentage decolourisation of reactive black 5 increased to 83.3 and 84.0% respectively, (Fig. 2). There was no significant difference between the percentage decolourisation of reactive black 5 after 8 h and 12 h when the LiP concentration was increased from 25 U to 35 U. However, a significantly high ($P < 0.05$) reactive black 5 decolourisation of 95.3% was achieved after 12 h when the LiP concentration was increased to 45 U (Fig. 2). Similarly, increasing the LiP concentration from 25 U to 35 U had a significant effect ($P < 0.05$) on the percentage decolourisation of reactive black 5 after 24 h (Fig. 2). Increasing the incubation time also had a significant effect ($P < 0.05$) on the decolourisation of reactive black 5 as above 90% decolourisation after 12 h was achieved with varying concentrations of LiP (Fig. 2). Reactive black 5 has been employed as a model azo dye in decolourisation experiments and has been reported to be decolourised by a number of white rot fungi such as *B. adusta*, *P. eryngii* (Heinfling et al. 1998), *T. versicolor* (Champagne and Ramsay 2005) and *Funalia trogii* (Mazmanci and Unyayar 2005). In many of the studies, decolourisation was achieved by growing the fungal cultures. Further, reactive black 5 has also been reported to be decolourised by purified laccases of *P. sajor-caju* (Murugesan et al. 2007),

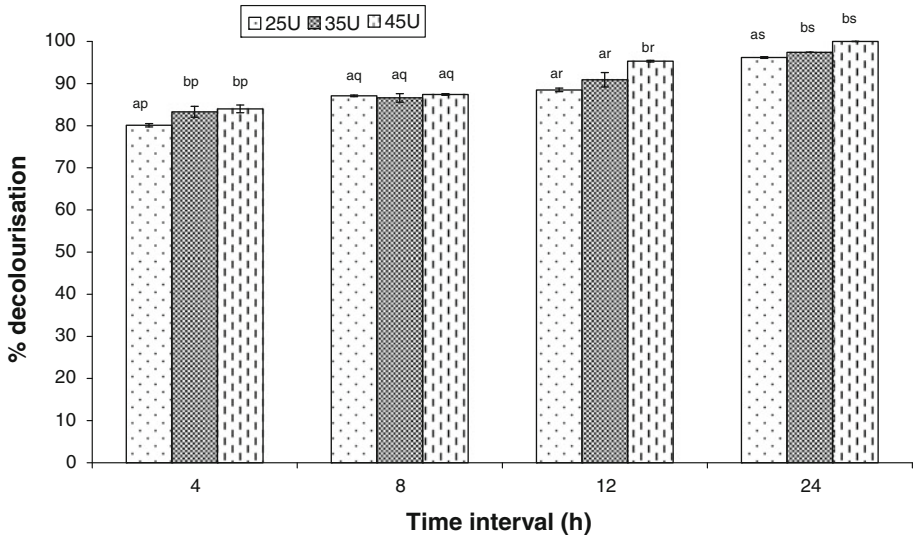

Fig. 2 Decolourisation of reactive black 5 by concentrated enzymes from spent substrate of *P. sajor-caju.* The reaction mixture (3.0 ml) contained LiP, 30 µg of reactive black 5, 1 mM veratryl alcohol, 0.2 mM H_2O_2 and 50 mM sodium tartrate buffer pH 4.5. The reaction mixture was incubated at 30°C. Values for different LiP concentration at same time interval with different letters (*a-b*) were significantly different ($P < 0.05$). Values for same LiP concentration at different time interval with different letters (*p-s*) were significantly different ($P < 0.05$). $n = 3$

T. villosa (Zille et al. 2003) and MnP of *T. versicolor* (Champagne and Ramsay 2005). Azo dyes that have a hydroxyl group in an ortho postion and a sulfonate group in the meta position or a para or ortho hydroxyl group and at least one or two electron-releasing substituents relative to azo bonds have been rapidly decolourised and degraded by MnP of *T. versicolor* (Champagne and Ramsay 2005) or LiP of *Streptomyces* sp. (Pasti-Grigsby et al. 1992). A similar preference for dyes with such structures during decolourisation by LiP from the spent mushroom substrate of *P. sajor-caju* might also be possible in the present study. Both reactive black 5 and reactive orange 16, that contained a hydroxyl group in an ortho position and a sulfonate group in the meta position relative to azo bonds were decolourised at a higher percentage.

Increasing the concentration of LiP from 25 U to 45 U had a significant effect ($P < 0.05$) on the percentage decolourisation of reactive orange 16 after 4 h and 8 h. The decolourisation of reactive orange 16 increased from 67.4 to 80.9% after 4 h with 25 U and 45 U of LiP respectively, (Fig. 3). Similarly, the percentage decolourisation of reactive orange 16 increased from 81.8 to 88.4% after 8 h with 25 U and 45 U of LiP respectively, (Fig. 3). Increasing the LiP concentration from 25 U to 35 U had a significant effect ($P < 0.05$) on the decolourisation of reactive orange 16 after 12 h and 24 h. However, there was no significant difference between the percentage decolourisation with 35 U and 45 U of LiP (Fig. 3). Increasing the incubation time had a significant effect ($P < 0.05$) on the percentage decolourisation of reactive orange 16. A decolourisation ranging between 81.8–88.4% after 8 h was achieved with varying LiP concentrations which further increased after 12 h and ranged between 84.8–92.3% (Fig. 3). The increase in the percentage decolourisation of reactive orange 16 at higher enzyme concentration might be due to the higher reaction rate resulting from higher enzyme activity as well as better protection of the enzyme from unfavorable environmental conditions (Yu et al. 2006). It has been reported that degradation of reactive orange 16 follows different mechanisms based on the method employed. The degradation of reactive orange 16 by photo- oxidation results in the cleavage of azo bond of dye molecule followed by oxidation of naphthalene group of the dye molecule to N-(3,4-bis-hydroxymethyl-phenyl)-acetamide and phthalic acid (Bilgi and Demir 2005). However, during the degradation of reactive orange 16 by MnP of *I. lacteus*, degradation products identified by liquid-chromatography-mass spectroscopy were 6-acetamido-3,4-dioxo-3, 4-dihydronaphthalene-2—sulfonate, (E)-2-(4-acetamidophenyl)-1-carboxyethene sulfonate and 4-(2-hydroxyethylsulfonyl)phenolate (Svobodova et al. 2007). In the present study, the mechanism of degradation of reactive orange 16 with LiP from the spent substrate of *P. sajor-caju* was not followed but as the degradation of dyes by peroxidases is an oxidative process, the degradation products similar to that observed by the action of MnP on reactive orange 16 might also be possible in the present study.

There was a significant increase ($P < 0.05$) in the percentage decolourisation of disperse blue 79 at all the incubation times studied when the concentration of LiP was increased from 25 U to 45 U (Fig. 4). It was observed that the decolourisation of disperse blue 79 increased from 24.3 to 32.1% after 4 h when the concentration of LiP was increased from 25 U to 45 U (Fig. 4). Similarly, the decolourisation of disperse blue 79 also increased from 35.8 to 48.7% after 8 h and from 39.4 to 52.2% after 12 h when the LiP concentration was increased from 25 U to 45 U respectively, (Fig. 4). Increasing the concentration of LiP from 25 U to 45 U had a significant effect ($P < 0.05$) on the percentage decolourisation of disperse blue 79 after

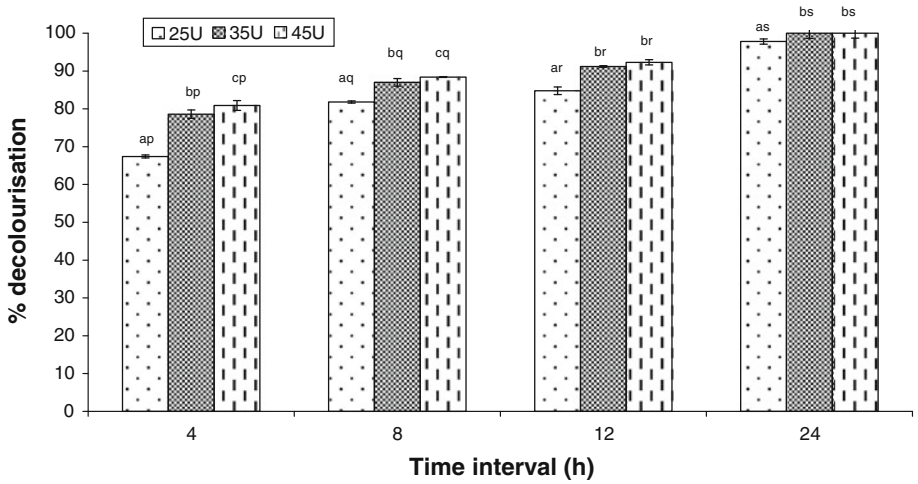

Fig. 3 Decolourisation of reactive orange 16 by concentrated enzymes from spent substrate of *P. sajor-caju*. The reaction mixture (3.0 ml) contained LiP, 75 μg of reactive orange 16, 1 mM veratryl alcohol, 0.2 mM H_2O_2 and 50 mM sodium tartrate buffer pH 4.5. The reaction mixture was incubated at 30°C. Values for different LiP concentration at same time interval with different letters (*a-c*) were significantly different ($P < 0.05$). Values for same LiP concentration at different time interval with different letters (*p-s*) were significantly different ($P < 0.05$). $n = 3$

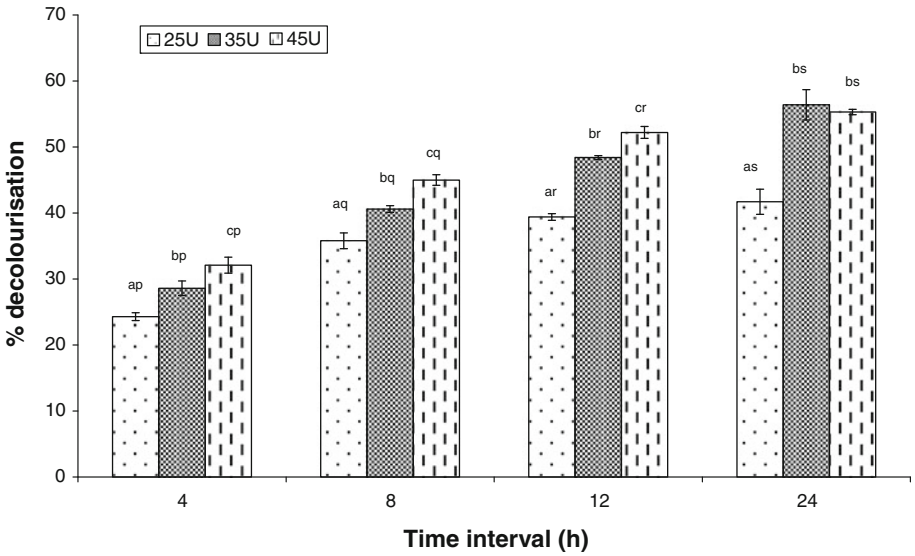

Fig. 4 Decolourisation of disperse blue 79 by concentrated enzymes from spent substrate of *P. sajor-caju*. The reaction mixture (3.0 ml) contained LiP, 75 μg of disperse blue 79, 1 mM veratryl alcohol, 0.2 mM H_2O_2 and 50 mM sodium tartrate buffer pH 4.5. The reaction mixture was incubated at 30°C. Values for different LiP concentration at same time interval with different letters (*a-c*) were significantly different ($P < 0.05$). Values for same LiP concentration at different time interval with different letters (*p-s*) were significantly different ($P < 0.05$). $n = 3$

24 h also. It was observed that the decolourisation of disperse blue 79 increased from 41.7 to 55.3% after 24 h when the LiP concentration was increased from 25 U to 45 U (Fig. 4). Increasing the incubation time from 4 h to 24 h had a significant effect ($P < 0.05$) on the percentage decolourisation of disperse blue 79 at varying LiP concentrations. It was observed that the decolourisation of disperse blue 79 increased from 24.3 to 41.7% when the incubation time was increased from 4 h to 24 h with 25 U of LiP (Fig. 4). Similarly, the decolourisation of disperse blue 79 increased from 32.1 to 55.3% when the incubation time was increased from 4 h to 24 h with 45 U of LiP (Fig. 4). In the present study, a lower percentage decolourisation of disperse blue 79 as compared to other azo dyes studied was observed which could be due to the difference in the dye structure. It has been reported that the dyes containing two nitro groups as well as electron withdrawing groups acted as a poor substrate for decolourisation by LiP of *Phanerochaete chrysosporium* (Podgornik et al. 1999). The molecular structure of disperse blue 79 lacks a hydroxyl group, contain two nitro groups and a bromine as an electron withdrawing group in its molecular structure which could have rendered this dye as a poor substrate also for LiP from spent substrate of *P. sajor-caju*.

Among the disperse dyes tested, a high percentage decolourisation of disperse red 60 as compared to disperse blue 56 was observed (Figs. 5 and 6). The percentage

decolourisation of disperse red 60 and disperse blue 56 increased with increasing enzyme concentration (Figs. 5 and 6). There was a significant increase in the percentage decolourisation of disperse red 60 at all the incubation times studied when the LiP concentration was increased from 25 U to 45 U. It was observed that the decolourisation of disperse red 60 increased from 23.9 to 47.2% after 4 h when the LiP concentration was increased from 25 U to 45 U respectively, (Fig. 5). There was also a significant increase ($P < 0.05$) in the percentage decolourisation of disperse red 60 after 8, 12 and 24 h when the concentration of LiP was increased from 25 U to 45 U. A disperse red decolourisation of 80.9 and 82.9% was achieved after 12 and 24 h respectively, when the concentration of LiP was increased from 25 U to 45 U (Fig. 5). Increasing the incubation time from 4 h to 24 h also had a significant effect ($P < 0.05$) on the percentage decolourisation of disperse red 60 at all the LiP concentrations studied except for 45 U (Fig. 5). It was observed that the decolourisation of disperse red 60 increased significantly from 47.2 to 80.9% with 45 U of LiP when the incubation time was increased from 4 h to 12 h (Fig. 5). Further increasing the incubation time to 24 h had no significant effect on the decolourisation of disperse red 60 with 45 U of LiP (Fig. 5). However, the decolourisation of disperse blue 56 did not exceed from more than 25% employing LiP concentrations ranging between 25–55 U whereas, significantly high ($P < 0.05$) percentage decolourisation of disperse blue 56 was only obtained when the LiP concentration was increased to 65 U (Fig. 6). It was observed that a 23.9% decolourisation of disperse blue 56 with 65 U of LiP was obtained after 4 h which further increased to 33.5, 52.4 and 68.5% after 8, 12 and 24 h respectively, (Fig. 6). The increase in the percentage decolourisation of disperse red 60 and disperse blue 56 with increasing enzyme concentration might be due to the higher reaction rates as well as better protection of the enzyme from unfavorable environmental conditions (Yu et al. 2006). Although both disperse red 60 and disperse blue 56 belonged to the anthraquinone dye group, but a lower percentage decolourisation of disperse blue 56 by LiP from spent mushroom substrate might be due to the dye structure. It has been reported that LiP of *P. chrysosporium* is non-specific in decolourising dyes from different chromophoric system but the ability of LiP in dye decolourisation depends on the auxochromic group (Podgornik et al. 1999). The presence of an extra OH, Cl and NH_2 in the molecular structure of disperse blue 56 probably might have rendered this dye as a poor substrate as compared to disperse red 60.

Decolourisation of synthetic textile waste-water with concentrated enzymes from spent substrate of *P. sajor caju*

Industrial effluents generally contain a mixture of dyes. Thus successful decolourisation of a single dye sometimes does not fully indicate the suitability of a system for decolourisation (Yesilada et al. 2010). Therefore, in the present study, the simultaneously decolourisation of a synthetic textile waste-water prepared by mixing five dyes from different dye groups by concentrated enzyme from SMS of *P. sajor-caju* was evaluated. It was observed that there was no significant difference in the percentage decolourisation of synthetic textile waste-water after 4 h

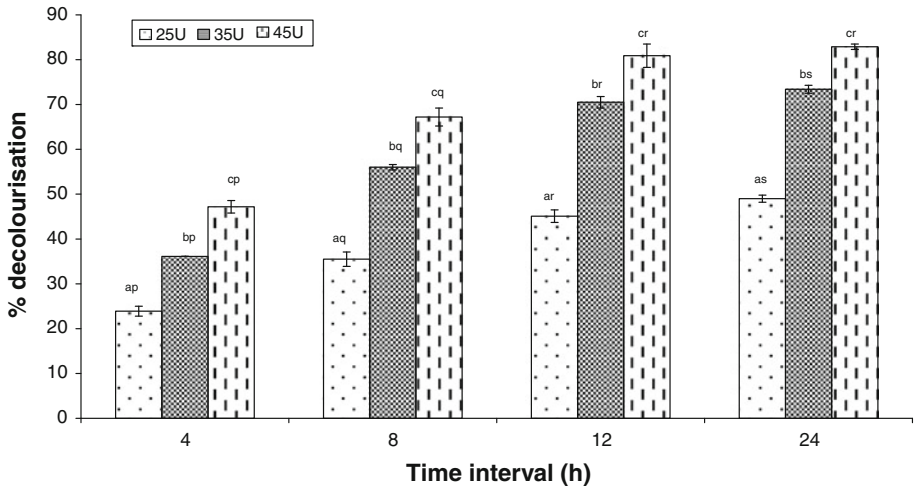

Fig. 5 Decolourisation of disperse red 60 by concentrated enzymes from spent substrate of *P. sajor-caju*. The reaction mixture (3.0 ml) contained LiP, 75 µg of disperse red 60, 1 mM veratryl alcohol, 0.2 mM H_2O_2 and 50 mM sodium tartrate buffer pH 4.5. The reaction mixture was incubated at 30°C. Values for different LiP concentration at same time interval with different letters (*a-c*) were significantly different ($P < 0.05$). Values for same LiP concentration at different time interval with different letters (*p-s*) were significantly different ($P < 0.05$). $n = 3$

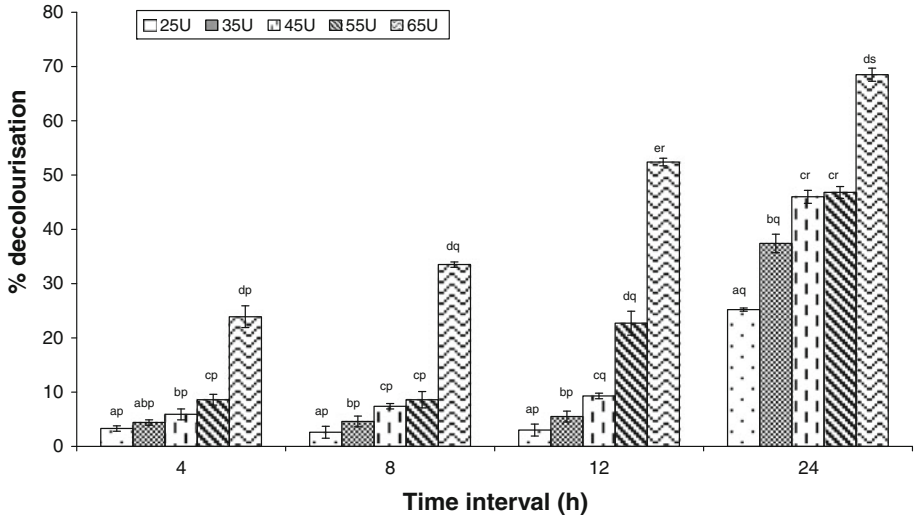

Fig. 6 Decolourisation of disperse blue 56 by concentrated enzymes from spent substrate of *P. sajor-caju*. The reaction mixture (3.0 ml) contained LiP, 75 µg of disperse blue 56, 1 mM veratryl alcohol, 0.2 mM H_2O_2 and 50 mM sodium tartrate buffer pH 4.5. The reaction mixture was incubated at 30°C. Values for different LiP concentration at same time interval with different letters (*a-e*) were significantly different ($P < 0.05$). Values for same LiP concentration at different time interval with different letters (*p-s*) were significantly different ($P < 0.05$). $n = 3$

when the LiP concentration was increased from 25 U to 55 U (Fig. 7). However, increasing the concentration of LiP from 25 U to 55 U had a significant effect ($P < 0.05$) on the percentage decolourisation of synthetic textile waste-water after 8, 12 and 24 h. A 72.9, 75.1 and 79.1% decolourisation of synthetic textile waste-water was achieved after 8, 12 and 24 h respectively, with 25 U of LiP which increased to 85.0, 86.1 and 91.6% respectively, when the LiP concentration was increased to 55 U (Fig. 7). A significant increase ($P < 0.05$) in the percentage decolourisation of synthetic textile waste-water with 25 and 35 U of LiP was also observed when the incubation time was increased from 4 h to 24 h (Fig. 7). The decolourisation of synthetic textile waste-water with 25 U of LiP increased from 67.9 to 79% when the incubation time was increased from 4 h to 24 h. Similarly, the decolourisation of synthetic textile waste-water with 35 U of LiP also increased from 65.5 to 85.3% when the incubation time was increased from 4 h to 24 h (Fig. 7). In the present study, a 91.6% decolourisation of the synthetic textile waste-water with 55 U of LiP from SMS of *P. sajor-caju* after 24 h was achieved. However, Darah and Ibrahim (1998) reported a 50% decolourisation of a local batik (textile) factory effluent by 55 U/ml of LiP from *P. chrysosporium* after 14 days. In another report, 15% decolourisation of dye industry effluent by 15 U of LiP from *Thelephora* sp. after one h has been reported by Selvam et al. (2003). The decreased extent of decolourisation reported by Darah and Ibrahim (1998) and Selvam et al. (2003) as compared to the present study employing LiP might be either due to a higher concentration of dye present in the dye industry effluents or due to the presence of additives in the dye effluents. It has been reported that dye industry effluents usually contain a dye concentration ranging between 0.6–0.8 g/l (Vandevivere et al. 1998; O'Neill et al. 1999), which is higher than the dye concentration of the synthetic textile waste-water used in the present study. The wastewaters from the dye containing industries contain a number of salts like Na_2SO_4, Na_2CO_3, NaCl and NaOH in varying concentrations depending on the dyeing process employed (Abadulla et al. 2000; Mohorcic et al. 2006). Some of these salts e.g. NaCl have no effect on the LiP activity (Tuisel et al. 1990) while others e.g. Na_2SO_3 has been reported to be inhibitory to LiP activity (Fawer et al. 1991). In the present study, the synthetic textile waste-water was prepared without the addition of any salt. It remains unanswered at this point of time whether the ability of LiP to decolourise actual textile effluent would be similar to that observed during decolourisation of synthetic textile waste-water or would be reduced due to the presence of additives which in turn might effect enzyme activity. Moreover, as the composition of dye effluent from one dye house may vary from another dye house based on the dyeing process employed, a further study to evaluate the potential of LiP in decolourising the actual textile effluent and the effect of different additives or salts used in dyeing process on LiP activity from spent substrate of *P. sajor-caju* is needed.

The enzyme extract from the SMS of *P. sajor-caju* containing LiP as main enzyme thus could be used in the decolourisation of textile dyes. This could be a cost effective method as compared to the direct application of

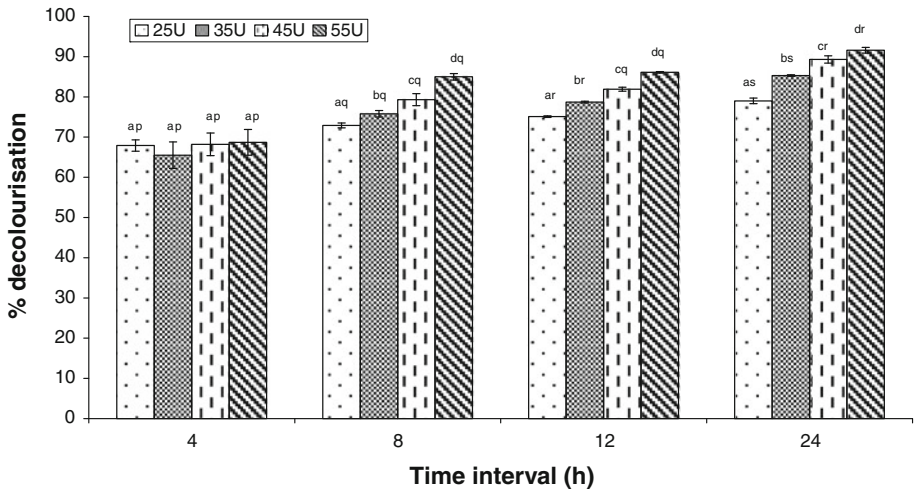

Fig. 7 Decolourisation of a synthetic textile waste-water (250 mg/l) by concentrated enzymes from spent substrate of *P. sajor-caju*. The reaction mixture (3.0 ml) contained LiP, 75 µg of synthetic textile waste-water, 1 mM veratryl alcohol, 0.2 mM H_2O_2 and 50 mM sodium tartrate buffer pH 4.5. The reaction mixture was incubated at 30°C. Values for different LiP concentration at same time interval with different letters (*a-d*) were significantly different ($P < 0.05$). Values for same LiP concentration at different time interval with different letters (*p-s*) were significantly different ($P < 0.05$). $n = 3$

the fungal cultures. So far, the decolourisation studies with white-rot fungi have been conducted employing living or dead fungal cultures in the waste-water for biodegradation or biosorption of dyes. These methods however has the disadvantage of disposal of fungal cultures after biosorption, requirement of pre growth of fungal cultures before effluent treatment, specialized fermentations and/or the requirement of a lag phase to induce the production of enzymes of interest. Further, use of commercial crude enzymes which are available for bioremediation purposes may not be cost effective. Thus the use of SMS offers an economical source of industrially important enzymes such as LiP. It has been estimated that 100 U of LiP can be recovered from 10 g of SMS of *P. sajor-caju* at a cost of 9.85 Ringgit Malaysia (RM) (unpublished data) as compared to commercially available partially purified LiP which is sold at 100 USD for 100 U (1 USD = 3.4 RM).

Toxicity bioassay

The toxicity of the synthetic textile waste-water after treatment with 55 U of LiP was evaluated using *Artemia* nauplii (Fig. 8). The toxicity of dyes might increase or decrease after enzymatic treatment depending on the structure of dye (Ramsay and Nguyen 2002). Therefore, the evaluation of toxicity of dyes after enzymatic treatment for the formation of toxic compounds as a result of dye degradation should be performed. A number of in vitro techniques have been employed to evaluate the toxicity of dyes from different dye groups (Novotny et al. 2006). Among others, the reduction in the luminescence of *Vibrio fishcheri* has been the most commonly used method for the evaluation of the toxicity of dyes (Abadulla et al. 2000; Ramsay and Nguyen 2002). In the present study, the reduction in the toxicity of dyes after enzymatic treatment was evaluated using brine shrimps. So far, brine shrimps have been used to evaluate the toxicity of arsenic in Great Salt Lake (Brix et al. 2003) and to test the toxicity of natural compounds (Krishnaraju et al. 2005). However, the use of brine shrimp to evaluate the toxicity of dyes has not been reported. The brine shrimp lethality assay represents a rapid, inexpensive and simple bioassay for testing the toxicity levels of dyes during decolourisation. This assay allows the use of smaller quantities of extracts or pure compounds and permits examination of a larger number of samples within a short period of time (Kanegusuku et al. 2002).

During the evaluation of toxicity, synthetic textile waste-water at concentrations more than 42.5 µg was toxic to the *Artemia* nauplii. Fifty millimolar sodium tartrate buffer pH 4.5, veratryl alcohol, H_2O_2 and concentrated enzyme extract was not toxic to *Artemia* nauplii. The toxicity was therefore only due to the synthetic textile waste-water. Moreover, the treatment of synthetic textile waste-water with 55 U of LiP at different time intervals revealed that there was a 52.4% reduction in toxicity after 4 h (Fig. 8). There was a slight decrease in the percentage toxicity reduction after 8 h and 12 h which further decreased by 24 h. It was observed that although a 90% decolourisation of synthetic textile waste-water was achieved after 24 h but there was only 35% reduction in toxicity. One reason for the increase in toxicity of samples treated with concentrated enzymes for more than 8 h might be due to the re—polymerization of degradation products

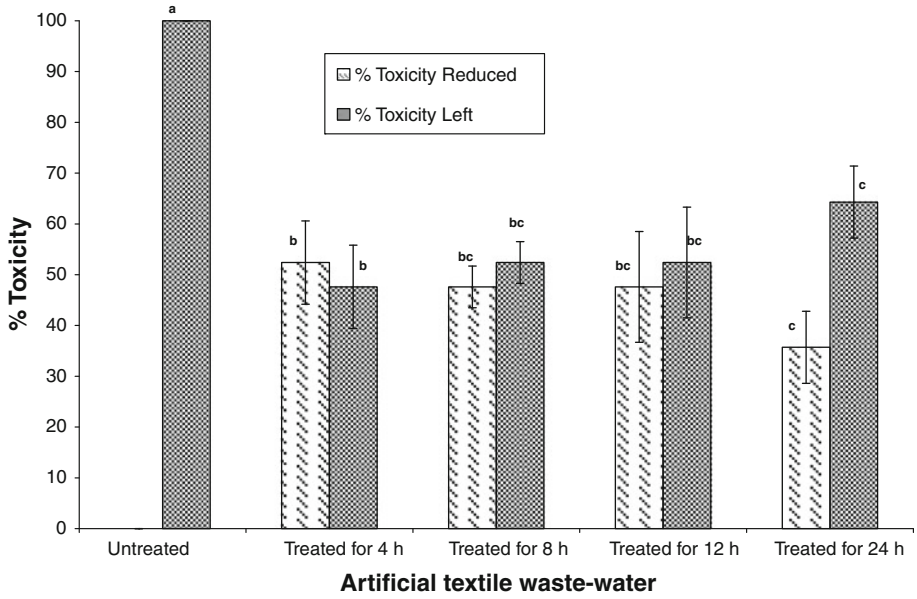

Fig. 8 Percentage toxicity of untreated (synthetic textile waste-water only) and synthetic textile waste-water treated with 55 U of LiP to *Artemia salina*. Means in the same column with different letters (*a-c*) were significantly different ($P < 0.05$). $n = 3$

of dye over a period of 24 h. This re—polymerization in turn might be due to the inactivation of certain enzymes such as veratryl alcohol oxidases (VAO) present in the enzyme extract, which have been reported to prevent re-polymerization (Marzullo et al. 1995). A similar prevention of polymerization of degradation products of dye by VAO might also be possible in the present study as significant titers of VAO were produced by *P. sajor-caju* during the solid-state fermentation of rubber wood sawdust (unpublished data).

Conclusion

In this study, the potential of enzymes from the spent substrate of *P. sajor-caju* in the decolourisation of reactive and disperse textile dyes is reported. The results of this study propose an efficient way of utilizing one waste to treat another waste i.e. the waste from the mushroom growing industry to treat textile effluents. The present study offers an economical alternative of procuring industrially important enzymes for bioremediation purposes. The results of the present study can be used to further expand the research scope in elucidating the degradation mechanisms of different dyes by the enzymes, various enzyme immobilization techniques as well as reactor designs for decolourisation of dyes.

Acknowledgments The authors thank Ministry of Science, Technology and Environment, Malaysia, for the IRPA (Intensive Research in Priority Areas) grants 09-02-03-0675, 01-02-03-1002, University of Malaya for the short-term research fund (Vot F) F0145/2003A and Mr. John Kuan from Highland and Lowland Mushroom Industry, Semenyih, Selangor, Malaysia for providing *Pleurotus sajor-caju* bags.

References

Abadulla E, Tzanov T, Costa S, Robra KH, Cavaco-Paulo A, Gubitz GM (2000) Decolorization and detoxification of textile dyes with a laccase from Trametes hirsuta. Appl Environ Microbiol 66:3357–3362

Arslan I, Balcioglu IA, Bahnemann DW (2000) Advanced chemical oxidation of reactive dyes in simulated dyehouse effluents by ferrioxalate-fenton/UV-A and TiO2/UV-A processes. Dyes Pigments 47:207–218

Avneesh DS, Abdullah N, Vikineswary S (2002) Extraction of enzymes from spent compost of *Pleurotus sajor-caju* and its potential use for decolourisation of synthetic dyes. Mal J Sci 21:9–16

Avneesh DS, Abdullah N, Vikineswary S (2003) Optimization of extraction of bulk enzymes from spent mushroom compost. J Chem Technol Biotechnol 78:743–752

Avneesh DS, Vikineswary S, Abdullah N, Annuar MSM, Ramachandran KB (2010) Decolourisation of chemically different dyes by enzymes from spent compost of *Pleurotus sajor-caju* and their kinetics. Afr J Biotechnol 9:041–054

Banat IM, Nigam P, Singh D, Marchant R (1996) Microbial decolorization of textile-dye containing effluents: a review. Biores Technol 58:217–227

Bilgi S, Demir C (2005) Identification of photooxidation degradation products of C. I. Reactive orange 16 dye by gas chromatography–mass spectrometry. Dyes Pigments 66:69–76

Brix KV, Cardwell RD, Adams WJ (2003) Chronic toxicity of arsenic to the Great Salt lake brine shrimp *Artemia franciscana*. Ecotox Environ Safety 54:169–175

Champagne PP, Ramsay JA (2005) Contribution of manganese peroxidase and laccase to dye decoloration by Trametes versicolor. Appl Environ Microbiol 69:276–285

Christie RM (2001) Colour chemistry. Royal Society of Chemistry, Cambridge

Chung KT, Stevens SEJ (1993) Degradation of azo dyes by environmental microorganisms and helminthes. Environ Toxicol Chem 12:2121–2132

Clarke A, Anliker R (1980) Organic dyes and pigments. In: Hutzinger O (ed) The handbook of environmental chemistry. Springer, Heidelberg, pp 181–215

Darah I, Ibrahim CO (1998) Biotreatment of local batik (Textile) factories effluent by cultures of *Phanerochaete chrysosporium*. J Biosci 9:105–111

Fawer MS, Stierli J, Cliffe S, Fiechter A (1991) The characterization of immobilized lignin peroxidase by flow injection analysis. Biochim Biophys Acta 1076:15–22

Gouvea CAK, Wypych F, de Moraes SG, Duran N, Nagata N, Peralta-Zamora P (2000) Semiconductor—assisted photocatalytic degradation of reactive dyes in aqueous solution. Chemosphere 40:433–440

Hao OJ, Kim H, Chang PC (2000) Decolorization of wastewater. Crit Rev Environ Sci Technol 30:449–505

Heinfling A, Bergbauer M, Szewzyk U (1997) Biodegradation of azo and phthalocyanine dyes by *Trametes versicolour* and *Bjerkandera adusta*. Appl Environ Microbiol 48:261–266

Heinfling A, Martinez MJ, Martinez AT, Bergbauer M, Szewzyk U (1998) Transformation of industrial dyes by manganese peroxidases from *Bjerkandera adusta* and *Pleurotus eryngii* in a manganese—independent reaction. Appl Environ Microbiol 64:2788–2793

Kanegusuku M, Benassi JC, Perosa RC, Yunes RA, Filho VC, Maia AA, de-Souza MM, Monache FD, Niero R (2002) Cytotoxic, hypoglycaemic activity and photochemical analysis of *Rubus imperialis* (Rosaceae). Biol Study Rubus imperialis 57:272–276

Kirby N, Mc Mullan G, Marchant R (1995) Decolourisation of an artificial textile effluent by *Phanerochaete chrysosporium*. Biotechnol Letters 17:761–764

Krishnaraju AV, Rao TVN, Sundararaju D, Vanisree M, Tsay HS, Subbaraju V (2005) Assessment of bioactivity of Indian medicinal plants using brine shrimp (*Artemia salina*) lethality assay. Int J Appl Sci Eng 3:125–134

Marzullo L, Cannio R, Giardina P, Santini MT, Sannia G (1995) Veratryl alcohol oxidase from *Pleurotus ostreatus* participates in lignin biodegradation and prevents polymerization of laccase oxidized substrates. J Biol Chem 270:3823–3827

Maximo C, Costa-Ferreira M (2004) Decolourisation of reactive textile dyes by *Irpex lacteus* and lignin modifying enzymes. Process Biochem 39:1475–1479

Mazmanci MA, Unyayar A (2005) Decolourisation of reactive black 5 by *Funalia trogii* immobilized on *Luffa cylindrica* sponge. Process Biochem 40:337–342

Mohorcic M, Teodorovic S, Golob V, Friedrich J (2006) Fungal and enzymatic decolourisation of artificial textile dye baths. Chemosphere 63:1709–1717

Munari FM, Gaio TA, Calloni R, Dillon AJP (2008) Decolorization of textile dyes by enzymatic extract and submerged cultures of *Pleurotus sajor-caju*. W J Microbiol Biotechnol 24:1383–1392

Murugesan K, Dhamija A, Nam IH, Kim YM, Chang YS (2007) Decolourization of reactive black 5 by laccase: Optimization by response surface methodology. Dyes Pigments 75:176–194

Novotny C, Dias N, Kapanen A, Malachova K, Vandrovcova M, Itavaara M, Lima N (2006) Comparative use of bacterial, algal and protozoan tests to study toxicity of azo- and anthraquinone dyes. Chemosphere 63:1436–1442

O'Neill C, Hawkes FR, Hawkes DL, Lourenco ND, Pinheiro HM, Delée W (1999) Colour in textile effluents—sources, measurement, discharge consents and simulation: a review. J Chem Technol Biotechnol 74:1009–1018

Pasti-Grigsby MB, Paszczynski A, Goszczynski S, Crawford DL, Crawford RL (1992) Influence of aromatic substitution patterns on azo dye degradability by *Streptomyces* spp. and *Phanerochaete chrysosporium*. Appl Environ Microbiol 58:3605–3613

Peralta-Zamora P, Kunz A, de Moraes SG, Pelegrini R, Moleiro PD, Reyes J (1999) Degradation of reactive dyes—I. A comparative study of ozonation, enzymatic and photochemical processes. Chemosphere 38:835–852

Podgornik H, Grgic I, Perdih A (1999) Decolorization rate of dyes using lignin peroxidases of *Phanerochaete chrysosporium*. Chemosphere 38:1353–1359

Ramsay JA, Nguyen T (2002) Decoloration of textile dyes by *Trametes versicolor* and its effect on dye toxicity. Biotechnol Letters 24:1757–1761

Rice SA, Mannes IB (2004) Brine shrimp bioassays: a useful technique in biological investigation. Am Biol Teach 66:208–215

Selvam K, Swaminathan K, Chae KS (2003) Decolourization of azo dyes and a dye industry effluent by a white rot fungus *Thelephora* sp. Biores Technol 88:115–119

Slokar YM, Zupan J, Majcen Le Marechal A (1999) The use of artificial neural network (ANN) for modeling of the H2O2/UV decoloration process: part I. Dyes Pigments 42:123–135

Svobodova K, Senholdt M, Novotny C, Rehorek A (2007) Mechanism of reactive orange 16 degradation with white rot fungus *Irpex lacteus*. Process Biochem 42:1279–1284

Tien M, Kirk TK (1983) Lignin degrading enzymes from the hymenomycete, *Phanerochaete chrysosporium* Burds. Science 221:661–663

Tuisel H, Sinclair R, Bumpus JA, Ashbaugh W, Brock BJ, Aust SD (1990) Lignin peroxidase H2 from *Phanerochaete chrysosporium*: purification, characterization and stability to temperature and pH. Arch Biochem Biophys 279:158–166

Vandevivere PC, Bianchi R, Verstraete W (1998) Treatment and reuse of wastewater from the textile wet-processing industry: review of emerging technologies. J Chem Technol Biotechnol 72:289–302

Weber EJ, Adams RL (1995) Chemical and sediment mediated reduction of the azo dye Disperse Blue 79. Environ Sci Technol 29:1163–1170

Wesenberg D, Kyriakides I, Agathos SN (2003) White-rot fungi and their enzymes for the treatment of industrial dye effluents. Biotechnol Adv 22:161–187

Yesilada O, Yildirim SC, Birhanli E, Apohan E, Asma D, Kuru F (2010) The evaluation of pre-grown mycelial pellets in decolorization of textile dyes during repeated batch process. W J Microbiol Biotechnol 26:33–39

Yu G, Wen X, Li R, Qian Y (2006) In vitro degradation of a reactive azo dye by crude ligninolytic enzymes from nonimmersed liquid cultures of *Phanerochaete chrysosporium*. Process Biochem 41:1987–1993

Zhao X, Hardin IR (2007) HPLC and spectrophotometric analysis of biodegradation of azo dyes by *Pleurotus ostreatus*. Dyes Pigments 73:322–325

Zhao X, Hardin IR, Hwang HM (2006) Biodegradation of a model azo disperse dye by the white rot fungus *Pleurotus ostreatus*. Int Biodeter Biodegr 57:1–6

Zille A, Tzanov T, Gubitz GM, Cavaco-Paulo A (2003) Immobilized laccase for decolourization of reactive black 5 dyeing effluent. Biotechnol Letters 25:1473–1477