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Full Length Research Paper

Decolourisation of chemically different dyes by enzymes from spent compost of *Pleurotus sajor-caju* and their kinetics

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A total of eight dyes from the triphenylmethane, azo and polymeric/heterocyclic dye group were decolourized by enzyme cocktail extracted from five month old spent compost of *Pleurotus sajor-caju* with lignin peroxidase as the main enzyme. The percentage of decolourisation for tryphan blue, amido black, remazol brilliant blue R (RBBR) and bromophenol blue ranged between 80 - 90% after 4 h reaction. However, the percentage of decolourisation for crystal violet, methyl green and congo red was lower than the other dyes from the same dye group with only 60 - 65% after 12 h. Methylene blue exhibited the least decolourisation with only 43% after 24 h indicating that this dye is a poor substrate for the enzyme. The rate of decolourisation for crystal violet, tryphan blue, amido black, congo red and RBBR dyes by enzymes from spent mushroom compost (SMC) were also calculated. The rate of decolourisation for all the dyes was positively affected by the initial dye concentration, pH between 4.0 - 4.5 and temperature range of 30 - 35°C. The optimum concentration of veratryl alcohol as redox mediator was between 0 - 2 mM for all the dyes except for RBBR. The optimum veratryl alcohol concentration for RBBR was 4 mM. Based on the effect of hydrogen peroxide on the rate of decolourisation of each dye, the dyes could be divided into two groups. From the results of the present study, it could be concluded that the enzymes extracted from the spent compost of *P. sajor-caju* offers an economical advantage of obtaining industrially important enzymes, which have potential in the bioremediation of synthetic dyes. Furthermore, the utilization of spent compost for the extraction of enzymes can also offer a possible solution for the problem posed due to the disposal of large amounts of spent mushroom compost.

Key words: Synthetic dye decolourisation, spent mushroom compost, *Pleurotus sajor-caju*, dye kinetics.

INTRODUCTION

Synthetic dyes are used extensively for textile dyeing, paper printing, leather dyeing, colour photography and as additives in petroleum products. Pollution from the effluents has become increasingly alarming with the usage of a wide variety of dyes in industries (Zollinger, 1987; GonCalves et al., 2000). Based on the chemical structure of the chromophoric group, the dyes are classified as azo, anthraquinone, triarylmethane and phthalocyanine dyes (Liu et al., 2004). It is estimated that between 10 - 20% of dyestuff being used in the dyeing process could be found in wastewater. Several of these dyes are very stable to light, temperature and microbial attack, making them recalcitrant compounds (Banat et al., 1996). Conventional waste water treatments are not efficient in removing recalcitrant dyestuffs from effluents (Shaul et al., 1991). Physical and chemical methods used for removal of dyes, that is, adsorption, chemical transformation, incineration, photocatalysis or ozonation are effective but rather costly (DeMoraes et al., 2000). Furthermore, some anaerobic

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microorganisms degrade dyes by azoreductase activity and this leads to the formation of aromatic amines which pose a more serious biotoxic threat than the original azo dyes (Chung and Stevens, 1993). The biodegradation of dyes by white rot fungi and their ligninolytic enzyme system offers an advantage over other processes because of their ability to completely mineralize various dyes to CO_2 and H_2O. The ligninolytic enzymes of white rot fungi are lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase, laccase, cellobiose dehydrogenase and H_2O_2-producing enzymes (Christian et al., 2005). These enzymes are highly non-specific and are able to transform or mineralize organopollutants that have structural similarities to lignin as well as have been reported to decolourize various dyes (Harvey et al., 1986; Gold et al., 1989; Hammel et al., 1993).

Pleurotus spp. are edible white rot fungi and have been used for the degradation of several pollutants including dyes (Chagas and Durrant, 2001; Rodriguez et al., 2004; Palmieri et al., 2005; Nilsson et al., 2006). In Malaysia, *Pleurotus sajor caju* is grown commercially on supplemented rubber-wood sawdust. For every 200 g of mushroom produced, about 800 g of spent mushroom compost (SMC) is available. An average farm discards about 24 tons of SMC per month. The SMC consists of mycelium, extracellular enzymes produced by fungus during growth and unutilized lignocellulosic substrate. The disposal of SMC is a major problem to farmers. Of the total spent compost produced in Malaysia, 69% is dumped in landfill sites, 28% is applied to agricultural land and remaining 3% is incinerated (Vigneswaran et al., 1997). The traditional method of disposal is to spread the compost on agricultural land where it is beneficial as it could add organic matter, balance pH, add beneficial microorganisms, fertilize with micro and macronutrients and improve moisture retention in soil (Wang et al., 1984). However, most spent compost is produced in large urban areas and it can be extremely expensive to transport it to farming areas where it is given free to farmers. A further difficulty is that farmers only require compost at certain times of the year whereas spent compost is produced all year round. In addition, the extent of agricultural land that is to take compost is also limited (Lohr et al., 1984; Wang et al., 1984). On the other hand, the incineration of compost is an alternative method of disposal whereby it offers a large reduction in the volume of the solid waste to be disposed of, along with the elimination of nuisance from biological decomposition. The method of incineration requires smaller space, relatively to land application but has the disadvantage of generation of ash (25 - 40% of the incoming load on a dry basis) which can have high polluting qualities (Vigneswaran et al., 1997). As both the main methods currently employed for the disposal of compost has some drawbacks, it becomes necessary to explore new applications of the spent mushroom compost.

So far spent compost of *Agaricus bisporus* has been employed for the recovery of laccase (Ball and Jackson, 1995; Mayolo-Deloisa et al., 2009). The decolourisation of Remazol brilliant blue R by laccase from the spent compost of *Lentinus polychrous* Lev. has also been reported (Khammuang and Sarnthima, 2007). Spent mushroom compost of *Pleurotus* spp. which represent the second largest group of cultivated basidiomycetes, has only been implicated in the bioremediation of polyaromatic hydrocarbons (Chiu et al., 1998; Eggen, 1999; Lau et al., 2003). However, the potential of enzymes from the spent compost of *Pleurotus* spp. for the decolourisation of dyes has not been explored. However, there has been an earlier report on the extraction of high titers of lignin peroxidase (LiP) from the spent compost of *P. sajor-caju* (Avneesh et al., 2003) and its potential in the decolourisation of selected synthetic dyes (Avneesh et al., 2002). In this paper, the potential of enzymes extracted from the spent compost of *P. sajor caju* for the decolourisation of chemically different dyes from triphenylmethane, azo and heterocyclic/polymeric groups is being reported. Furthermore, the effect of pH, time, temperature, initial dye concentration, veratryl alcohol concentration and H_2O_2 concentration on the rate of decolourisation of selected dyes were also evaluated.

MATERIALS AND METHODS

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

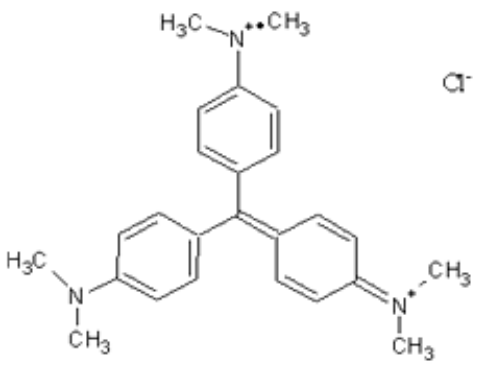
Five-month old *P. sajor-caju* bags were collected from a mushroom farm in Semenyih, Selangor, Malaysia. The contents of six bags were mixed thoroughly and the enzymes were extracted from the resultant mixture according to Avneesh et al. (2003). The crude enzyme was concentrated with Millipore stirred cells fitted with 10,000 daltons cut off membrane. Lignin peroxidase activity was analyzed after concentration of the enzymes.

Enzyme assay

Lignin peroxidase activity was measured by recording the increase in the absorbance at 310 nm due to the oxidation of 2 mM veratryl alcohol to veratryl aldehyde in the presence of 0.5 mM H_2O_2 (Have et al., 1998). The standard was 3, 4-dimethoxybenzaldehyde (veratryl aldehyde). The enzyme activity was expressed as international units (U) and 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.

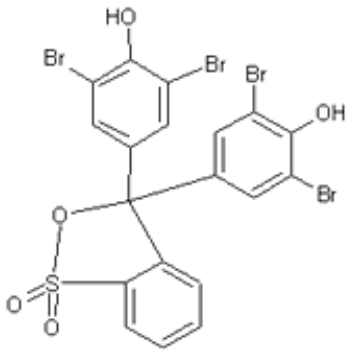
Decolourisation of dyes with concentrated enzymes from spent compost of *P. sajor caju*

The potential of enzymes from spent compost of *P. sajor caju* for the decolourisation of crystal violet, methyl green and bromophenol blue from triphenylmethane dye group, amido black, tryphan blue and congo red from azo dye group and methylene blue and remazol brilliant blue R (RBBR) from heterocyclic/polymeric dye group was studied (Figures 1a-1c). The reaction mixture contained 25 U of concentrated LiP from SMC, 12.3 µM of individual dyes and 1 mM of veratryl alcohol in 50 mM sodium tartrate buffer at pH 4.5



Crystal Violet

MW = 407.98, λmax = 591 nm



Bromophenol Blue

MW = 669.96, λmax = 590 nm

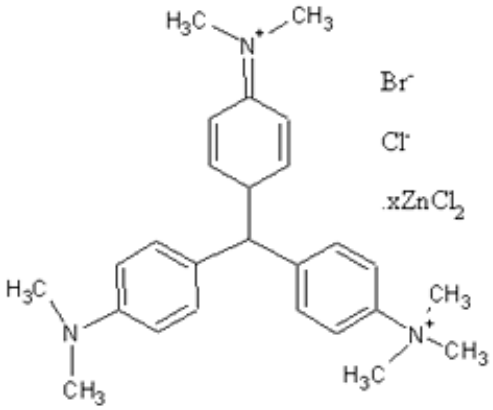


Figure 1a. Molecular structure, molecular weight and absorption maxima of dyes from triphenylmethane dye group.

(Yesilada, 1995). The reaction was initiated by adding 0.2 mM H_2O_2 and incubated at 30°C. The total reaction volume was 3 ml. The decrease in the absorbance was measured spectrophotometrically (UV-1601 Shimadzu spectrophotometer, Shimadzu Corp. Japan) at the absorption maxima of each dye at time intervals of 4, 8, 12 and 24 h. All the reactions were run in triplicate. Decolourisation of each dye was expressed in terms of percentage which was calculated according to the following equation:

Decolourisation = $[(A_0 – A_t)/A_0] \times 100$

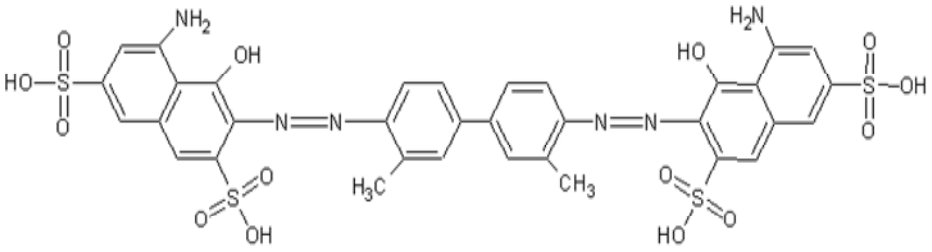
Where, A_0 = absorbance at λmax of each dye immediately measured after enzyme addition; A_t = absorbance at λmax of each dye at a given time.

Determination of rate of decolourisation

The rate of decolourisation was determined for five dyes from the representative dye group. The dyes were crystal violet, tryphan blue, amido black, congo red and RBBR. The reaction mixture contained 25 U of concentrated LiP from SMC mixed with individual dyes in 50 mM sodium tartrate buffer at optimum pH, 1 mM veratryl alcohol and 0.2 mM H_2O_2 in a total volume of 3.0 ml. A control with buffer replacing the enzyme in the reaction mixture was conducted in parallel. The reaction mixture was incubated at 30°C. The decrease in the absorbance of each dye was measured spectrophotometrically at the absorption maxima of each dye. All the reactions were run in triplicate. The rate of decolourisation for each dye was defined as a decrease in the initial concentration of dye per min at 25 U of enzyme from SMC and was reported as µmol/ L/ min.

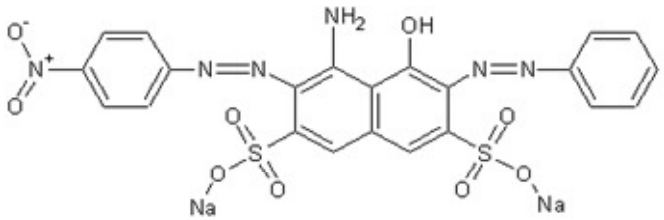
Determination of optimal incubation time for rate of decolourisation

Based on a preliminary study (data not shown), pH 4.0 and 4.5 were selected to study the effect of incubation time on the rate of decolourisation. The reaction mixture in a total volume of 3 ml contained 25 U of concentrated LiP from SMC and 12.3 µM of individual dyes in 50 mM sodium tartrate buffer at pH 4.0 and 4.5. Veratryl alcohol at a concentration of 1 mM was added to the reaction mixture. The reaction was initiated by adding 0.2 mM H_2O_2 and incubated at 30°C for 10, 20 and 30 min.



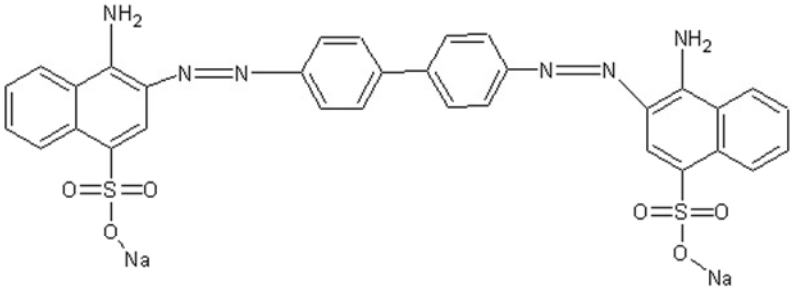
Tryphan Blue

MW = 960.81, λmax = 597 nm



Amido Black

MW = 616.49, λmax = 617 nm



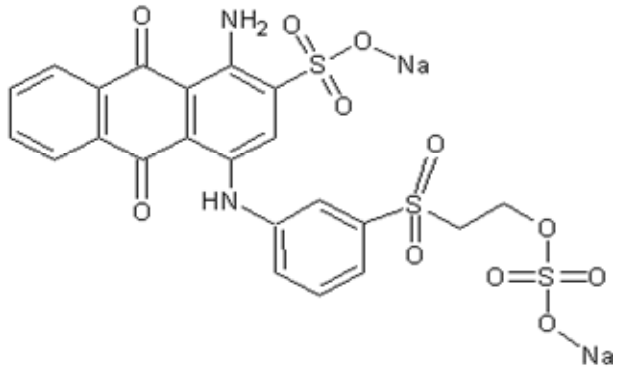
Congo Red

MW = 696.70, λmax = 506 nm

Figure 1b. Molecular structure, molecular weight and absorption maxima of dyes from azo dye group.

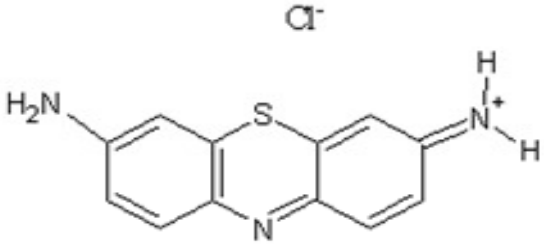
Effect of dye concentration on the rate of decolourisation

The effect of varying initial dye concentrations on the rate of decolourisation of each dye was determined separately. The reaction mixture for each dye contained different concentrations of dye in 50 mM sodium tartrate buffer pH 4.0, 25 U of LiP from SMC, 1 mM veratryl alcohol and 0.2 mM H_2O_2 in a total volume of 3.0 ml. The reaction was incubated at 30°C.



RBBR

MW = 626.50, λmax = 590 nm



Methylene Blue

MW = 319.85, λmax = 665 nm

Figure 1c. Molecular structure, molecular weight and absorption maxima of dyes from polymeric/heterocyclic dye group.

Effect of pH on the rate of decolourisation

The effect of pH on the rate of decolourisation of individual dyes in 50 mM sodium tartrate buffer at pH 2.5, 3.0, 3.5, 4.0 and 4.5 was studied. The reaction mixture of individual dyes contained 25 U of concentrated LiP from SMC, 1 mM veratryl alcohol and 0.2 mM H_2O_2 in a total volume of 3.0 ml.

Effect of veratryl alcohol concentration on the rate of decolourisation

The effect of varying concentrations of veratryl alcohol on the rate of decolourisation of each dye was evaluated. The concentrations of the veratryl alcohol used were 1, 2, 4, 6, 8 and 10 mM. The reaction contained optimum concentration of individual dye mixed with 25 U of concentrated LiP from SMC in 50 mM sodium tartrate buffer at optimum pH. The reaction was initiated by adding 0.2 mM H_2O_2 and incubated at 30°C. The total volume of the reaction mixture was 3.0 ml.

Effect of H_2O_2 concentration on the rate of decolourisation

The effect of H_2O_2 on the rate of decolourisation of each dye was evaluated by varying the concentration of H_2O_2 to 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mM. The reaction contained optimum concentration of individual dye mixed with 25 U of LiP from SMC in 50 mM sodium tartrate buffer at optimum pH and optimum veratryl alcohol concentration in a total volume of 3.0 ml. The reaction mixture was incubated at 30°C.

Effect of temperature on the rate of decolourisation

The effect of temperature on the rate of decolourisation was evaluated at the incubation temperature of 25, 30, 35, 40 and 45°C. The reaction contained optimum concentration of individual dye mixed with 25 U of LiP from SMC in 50 mM sodium tartrate buffer at optimum pH, optimum veratryl alcohol and H_2O_2 concentration. The total volume of the reaction mixture was 3.0 ml.

RESULTS

Decolourisation of dyes with concentrated enzymes from spent compost of *P. sajor caju*

A significant decolourisation of all the dyes from the different dye groups was observed after 24 h. More than 50% decolourisation was observed for all the dyes studied after 4 h except for methyl green and methylene blue (Table 1). A 64.3 and 97.2% decolourisation was observed after 8 h for crystal violet and bromophenol, respectively. A 49.2% decolourisation after 8 h was observed for methyl green which increased to 64.7% after 24 h (Table 1). Among the azo dye group, more than 50% decolourisation after 8 h was observed for amido black, tryphan blue and congo red (Table 1). Decolourisation percentage of the azo dyes also increased with increasing incubation time. Among the dyes from the polymeric dye group, 89.1% decolourisation was observed for RBBR after 4 h which further increased to 95.2 % after 8 h. The percentage of decolourisation for methylene blue was low as compared to RBBR and did not increase from 43.9% even after 24 h (Table 1).

Rate of decolourisation and effect of different parameters on the rate of decolourisation

The rate of decolourisation for all the dyes except for congo red was optimum at 10 min at pH 4.0 and 4.5 (Table 2). Increasing the incubation time to 20 or 30 min decreased the rate of decolourisation. However, no rate of decolourisation was observed for congo red at 10 min whereas an increase in the rate of decolourisation was observed when samples were incubated for 20 or 30 min

Table 1. Decolourisation percentage of dyes from the triphenylmethane, azo and polymeric/heterocyclic dye group by enzymes from spent compost of *Pleurotus sajor-caju*.

Dyes used	Reaction Time (h)			
	4	8	12	24
Triphenylmethane dyes				
Crystal violet	57.6 ± 0.4	64.3 ± 0.4	65.7 ± 0.4	65.0 ± 0.5
Bromophenol blue	94.7 ± 0.4	97.2 ± 0.7	95.6 ± 0.2	99.5 ± 0.7
Methyl green	29.7 ± 0.3	49.2 ± 0.6	59.4 ± 0.3	64.7 ± 0.8
Azo dyes				
Tryphan blue	79.9 ± 0.3	84.2 ± 0.3	84.8 ± 0.2	87.8 ± 0.3
Amido black	77.8 ± 0.7	83.2 ± 0.2	87.0 ± 0.5	87.2 ± 1.2
Congo red	47.9 ± 3.1	56.9 ± 1.5	60.7 ± 1.5	63.8 ± 2.2
Polymeric/heterocyclic dyes				
RBBR	89.1 ± 1.8	95.2 ± 1.2	95.2 ± 1.2	100.0 ± 6.7
Methylene blue	16.27 ± 0.6	27.6 ± 0.7	35.9 ± 1.3	43.9 ± 1.7

Table 2. Effect of incubation time on the rate of decolourisation of tryphan blue, crystal violet, congo red, amido black and RBBR at pH 4.0 and pH 4.5.

Dyes used	Rate of reaction at different time interval		
	10 min	20 min	30 min
Tryphan blue (pH 4.0)	0.62 ± 0.01	0.34 ± 0.01	0.25 ± 0.00
Tryphan blue (pH 4.5)	0.54 ± 0.00	0.33 ± 0.00	0.26 ± 0.00
Amido black (pH 4.0)	0.24 ± 0.01	0.17 ± 0.01	0.13 ± 0.01
Amido black (pH 4.5)	0.19 ± 0.01	0.15 ± 0.01	0.13 ± 0.00
Congo red (pH 4.0)	0.0 ± 0.0	0.20 ± 0.01	0.19 ± 0.01
Congo red (pH 4.5)	0.0 ± 0.0	0.19 ± 0.00	0.17 ± 0.00
Crystal violet (pH 4.0)	0.19 ± 0.02	0.10 ± 0.01	0.08 ± 0.00
Crystal violet (pH 4.5)	0.18 ± 0.01	0.11 ± 0.01	0.10 ± 0.00
RBBR (pH 4.0)	0.57 ± 0.01	0.33 ± 0.01	0.28 ± 0.00
RBBR (pH 4.5)	0.46 ± 0.02	0.31 ± 0.01	0.27 ± 0.01

at pH 4.0 and 4.5 (Table 2). Based on the results, tryphan blue, crystal violet, amido black and RBBR were incubated for 10 min, and congo red was incubated for 20 min in subsequent experiments. Similarly, a decrease in the rate of decolourisation of all the dyes was also observed at pH 4.5 as compared to pH 4.0 at all the incubation times (Table 2). All further experiments were performed at pH 4.0.

The rate of decolourisation of each dye increased with the increase in dye concentration (Figure 2). The rate of decolourisation for congo red, crystal violet and tryphan blue ranged between 3.95 - 6.62 µmol/ L/min whereas for amido black and RBBR, it was only 0.90 and 0.60 µmol/ L/min, respectively. Based on the highest rate of decolourisation, the maximum concentration of the dyes observed was 160 - 200 µM for tryphan blue, congo red and crystal violet, whereas it was only 60 and 100 µM for amido black and RBBR respectively (Figures 2 and 3, Table 3).

The rate of reaction for all the dyes was high in the pH range of 4.0-4.5 (Figure 4). A high rate of decolourisation was observed for crystal violet at pH 2.5 also indicating that an acidic environment is required for a better rate of decolourisation.

Veratryl alcohol had a different effect on the rate of decolourisation of each dye. For amido black and RBBR, the optimum concentration of veratryl alcohol was 1 mM and 4 mM respectively (Figure 5). No significant rate of reaction was observed in the absence of veratryl alcohol for these two dyes (Figure 5). However, for crystal violet, tryphan blue and congo red, although the optimum concentration of veratryl alcohol was approximately 2 mM, a significant rate of decolourisation was also observed in the absence of veratryl alcohol (Figure 5), indicating that these dyes can probably act as direct substrates for LiP. In general, the optimum concentration of veratryl alcohol was between 0 - 2 mM for all the dyes except for RBBR. There was a decrease in the rate of

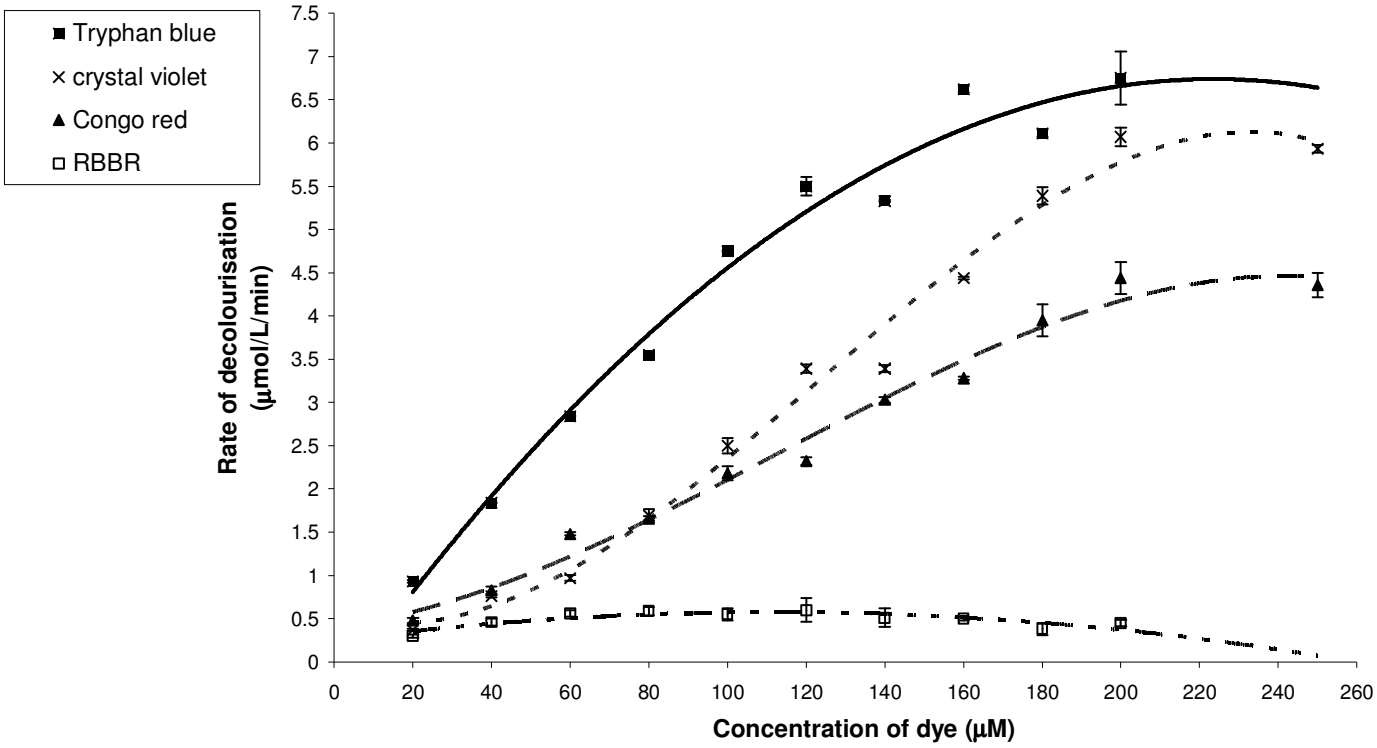


Figure 2. Effect of dye concentration on the rate of decolourisation of trypan blue, crystal violet, congo red and RBBR at pH 4.0 with 25 U of LiP from spent mushroom compost and 1 mM veratryl alcohol.

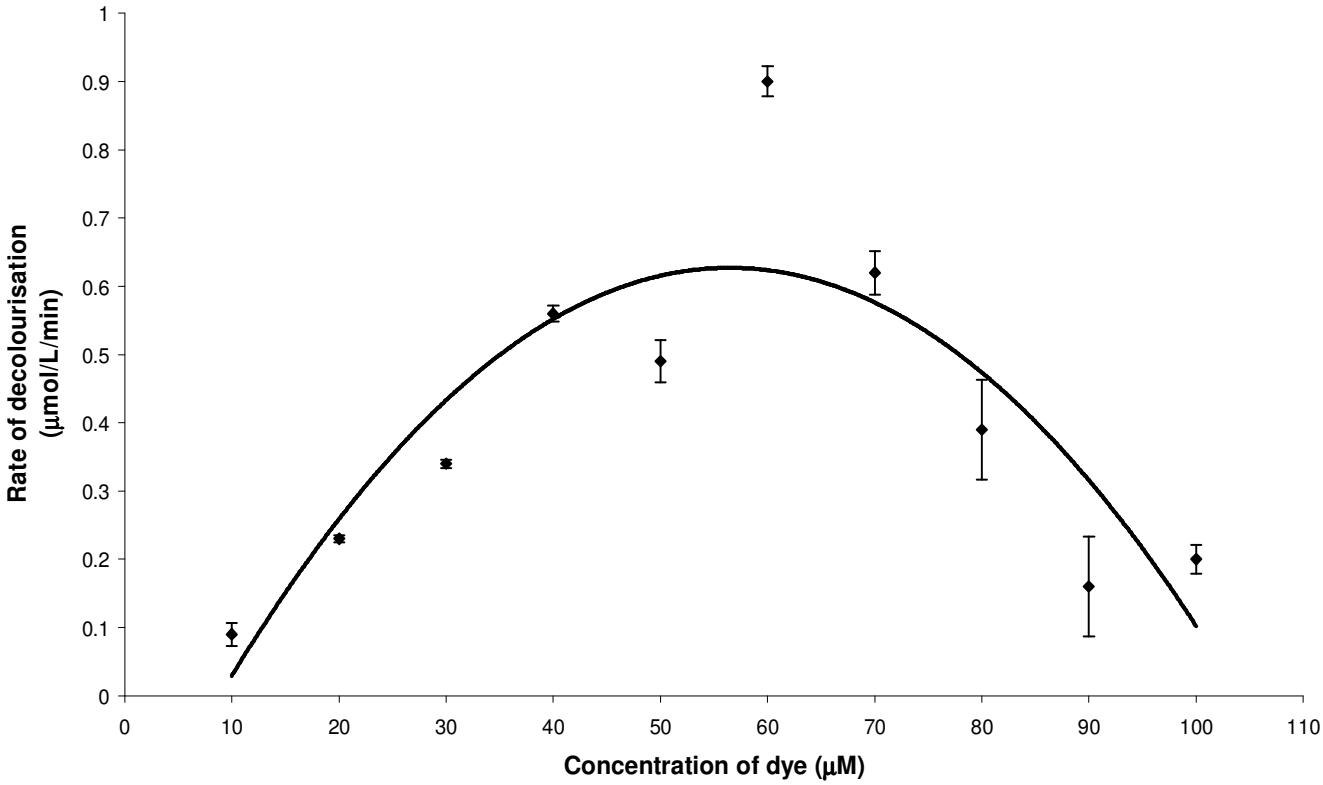


Figure 3. Effect of dye concentration on the rate of decolourisation of amido black at pH 4.0 with 25 U of LiP from spent mushroom compost and 1 mM veratryl alcohol.

Table 3. Optimum dye concentration and rate of decolourisation of dyes from the triphenylmethane, azo and polymeric/heterocyclic dye group by the enzymes from spent compost of *P. sajor-caju*.

Dyes	Optimum concentration (µM)	Rate of reaction (µmol/L/min)
Tryphan blue	160	6.62 ± 0.0
Amido black	60	0.90 ± 0.02
Congo red	180	3.95 ± 0.18
Crystal violet	200	6.07 ± 0.10
RBBR	100	0.55 ± 0.06

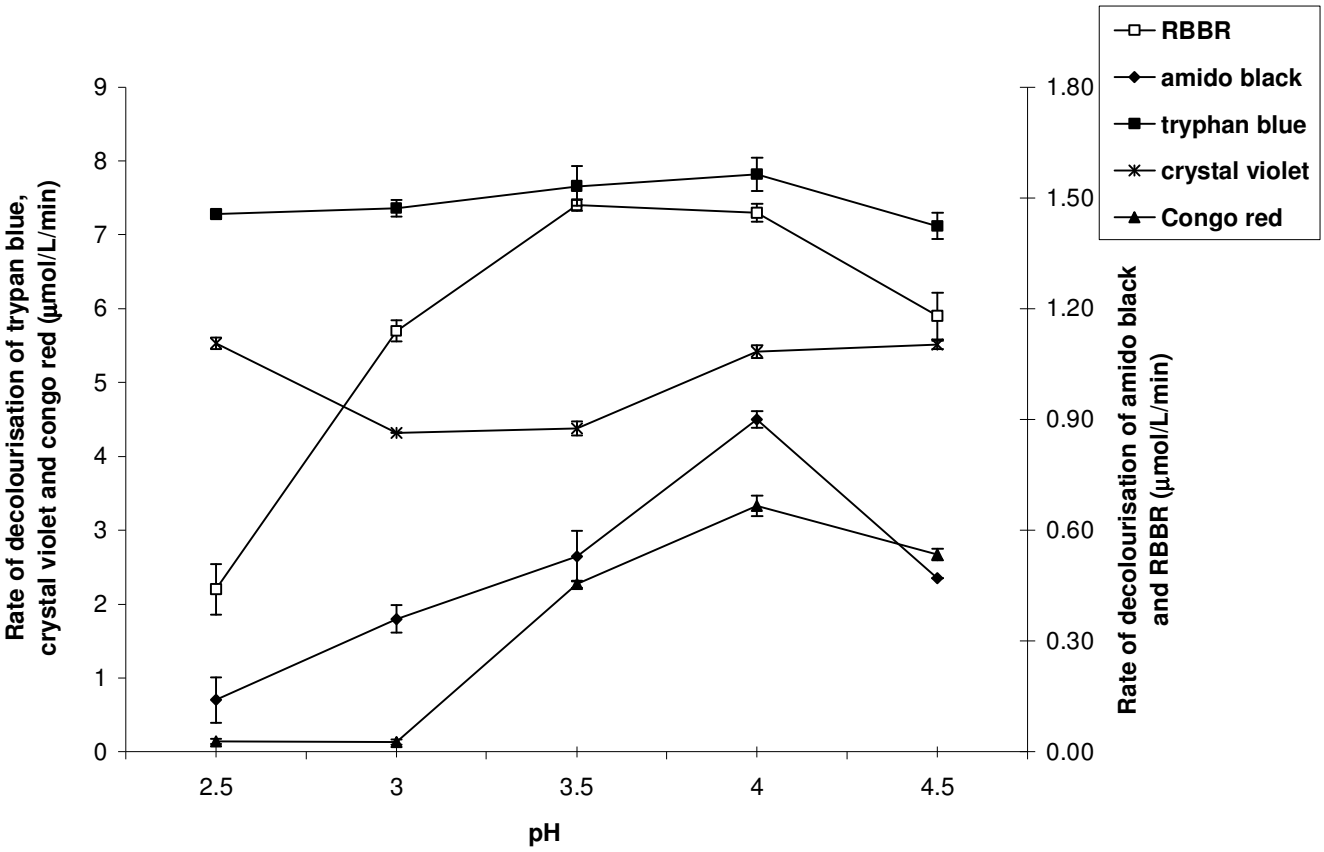


Figure 4. Effect of pH on the rate of decolourisation of trypan blue, crystal violet, congo red, amido black and RBBR. The reaction mixture contained 25 U of LiP from spent mushroom compost and 1 mM veratryl alcohol.

decolourisation for all the dyes when veratryl alcohol concentration was increased above optimum.

Based on the effect of H_2O_2 on the rate of decolourisation, the dyes could be divided into two groups. One group of dyes, which includes RBBR and amido black, had an optimum H_2O_2 concentration of 0.4 mM and a concentration above that caused a decrease in the rate of decolourisation (Figure 6). In the other group of dyes, which includes tryphan blue, congo red and crystal violet, the rate of decolourisation remained almost constant over the different H_2O_2 concentrations studied (Figure 6). A significant rate of decolourisation was also observed for tryphan blue and congo red in the absence of H_2O_2. Crystal violet exhibited a similar rate of decolourisation over all the H_2O_2 concentration but there was a decrease in the rate of decolourisation when H_2O_2 was omitted from the reaction mixture (Figure 6).

The optimum temperature for all the dyes except for crystal violet was between 30 - 35°C. There was a decrease in the rate of decolourisation at temperatures above 35°C (Figure 7). A significant rate of decolourisation was observed for crystal violet at all the temperatures studied. Temperatures above 35°C had no negative effect on the rate of decolourisation of crystal violet (Figure 7).

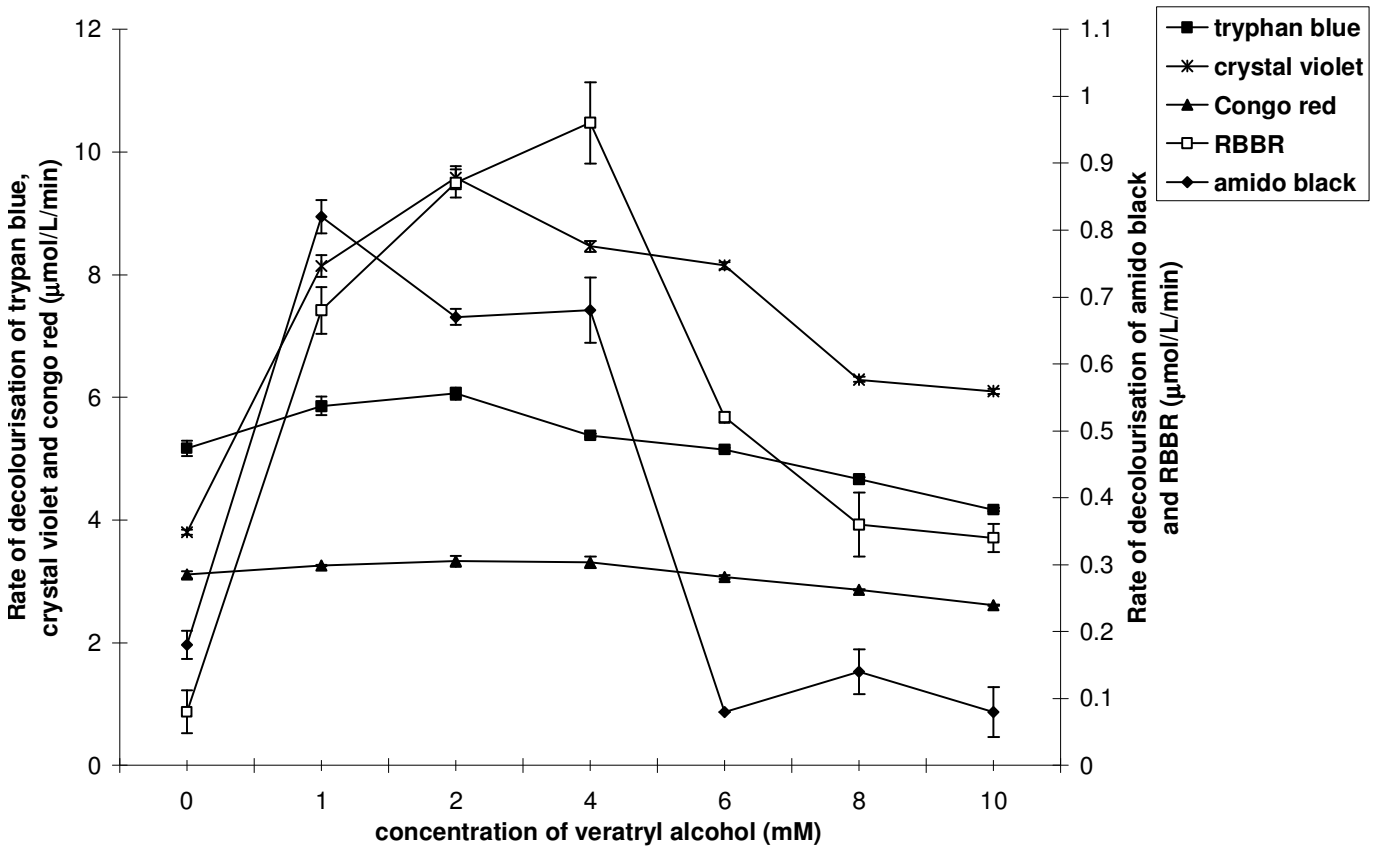


Figure 5. Effect of varying concentration of veratryl alcohol on the rate of decolourisation of trypan blue, crystal violet, congo red, amido black and RBBR.

DISCUSSION

A significant titers of cellulolytic and ligninolytic enzymes were extracted from five months old bags of *P. sajor caju* (Avneesh et al., 2003). During the decolourisation of triphenylmethane dyes, bromophenol blue was completely decolourized after 8 h as compared to crystal violet (64.3%) or methyl green (49.2%). A 62 and 100% decolourisation of 12.3 µM of crystal violet after 72 h by *Phanerochaete chrysosporium* cultures and also with purified LiP, respectively, has been reported (Bumpus and Brock, 1988; Yesilada, 1995). It has also been reported that crystal violet undergoes N-demethylation when treated with LiP as the metabolites detected by HPLC were penta, tetra or tri methylpararosaniline along with other uncharacterized pink coloured metabolites (Bumpus and Brock, 1988). A similar N-demethylation of crystal violet might be taking place in our study as the colour of the dye changed to pink upon decolourisation by LiP from SMC. No further change in the colour was observed even when the incubation time was increased. Bromophenol blue was completely decolourized although it contains no N-alkyl group, indicating a different mechanism of decolourisation from that of crystal violet (Bumpus and Brock, 1988).

Among the azo dyes, the percentage of decolourisation for congo red was not as high as the other two azo dyes, but a 60% decolourisation after 12 h was achieved for this dye. It has been reported that congo red can act as a substrate for LiP as a 54% decolourisation of congo red after 15 min by the crude LiP from the cultures of *P. chrysosporium* was observed both in the presence and absence of veratryl alcohol (Ollikka et al., 1993). In another study, a 97% decolourisation of 50 µM congo red and 25 µM amido black after 8 and 24 h respectively by *Thelephora* sp. has been reported by Selvam et al. (2003). However, the percentage of decolourisation decreased to only 8% and 12 - 15% for congo red and amido black, respectively, when the dyes were incubated with 15 U of laccase or LiP purified from the carbon–limited cultures of *Thelephora* sp. indicating both adsorption and degradation of dye molecules on the fungal hypae (Selvam et al., 2003). In this study, the decolourisation of azo dye was enzymatic and carried out by LiP as the main enzyme. Decolourisation of azo dyes by the cultures of *P. sajor caju* has already been reported (Chagas and Durrant, 2001). Since azo dyes make the largest group of synthetic colourants used in textile industries which were released into the environment, decolourisation of azo dyes by the enzyme cocktail from the

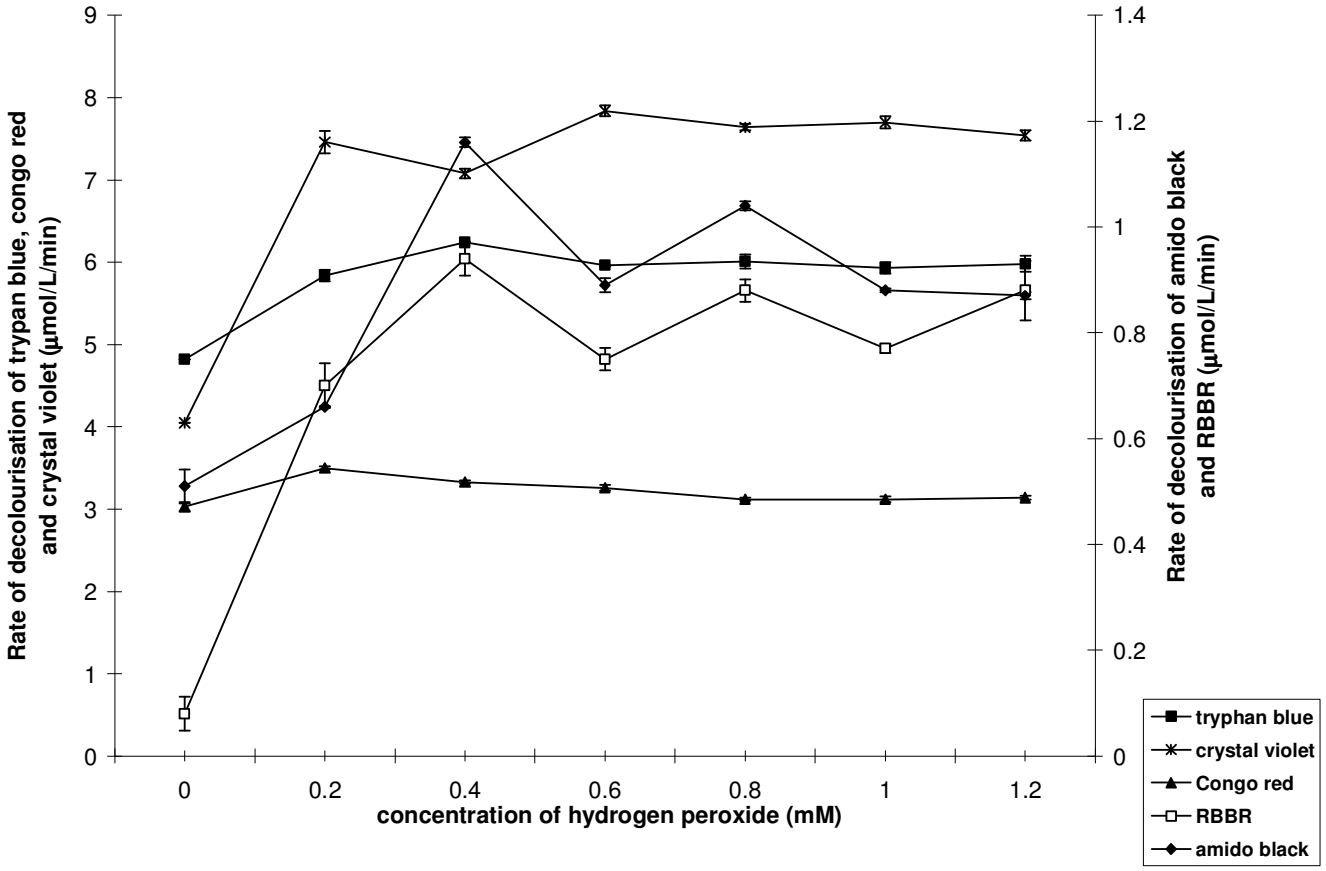


Figure 6. Effect of varying concentration of H_2O_2 on the rate of decolourisation of trypan blue, crystal violet, congo red, amido black and RBBR.

spent compost offers economical bioremediation alternative.

Almost complete decolourisation of RBBR (95%) was achieved after 8 h while methylene blue was partially decolourized with only 43% after 24 h. Ollikka et al. (1993) reported 85% decolourisation of 10 µM of methylene blue after 15 min of incubation with crude LiP of *P. chrysosporium*. However, only 20% decolourisation of methylene blue was observed when 10 µM of dye was treated with purified LiP isoenzymes of *P. chrysosporium*, indicating that different isoenzymes of LiP have different specificities for dyes as a substrate (Ollikka et al., 1993). RBBR has been reported to be decolourized by a number of enzyme systems including LiP (Ollikka et al., 1993), MnP (Boer et al., 2004) and laccase (Soares et al., 2001). Production of a novel peroxidase responsible for the decolourisation of RBBR by the cultures of *P. ostreatus* and *Geotrichum candidum* has also been reported (Vyas and Molitoris, 1995; Kim and Shoda, 1999). This RBBR-decolourizing peroxidase was catalytically different from LiP and MnP. RBBR is an anthracene derivative and structurally resembles lignin back-bone. This anthraquinone dye has been used to measure ligninolytic activity of white rot fungi (Thorn, 1993). Decolourisation of RBBR in our study indicates the involvement of LiP as the main enzyme in the decolourisation of dyes. Moreover, as RBBR resembles anthracene in structure, decolourisation of this dye also offers the potential of spent compost of *P. sajor-caju* in the bioremediation of polycyclic aromatic hydrocarbons (Eggen, 1999; Lau et al., 2003).

The rate of decolourisation for all the dyes except for congo red was optimum at 10 min and decreased at 20 or 30 min. The high rate of reaction at 10 min indicated that the reaction is at the initial stage when the enzyme sites were fully occupied. After 10 min, the rate of reaction decreased indicating that the enzyme sites were not fully occupied when reaction was caught at the later time intervals. For congo red, the rate of decolourisation was observed only at 20 min and no rate of decolourisation was observed at 10 min indicating that probably the binding of enzyme to the dye was a slow process. Although congo red also belongs to the azo dye group along with tryphan blue and amido black, the differences in the dye structure may affect the orientation of dye molecule with the active site(s) of enzyme during decolourisation.

The rate of decolourisation of each dye increased with

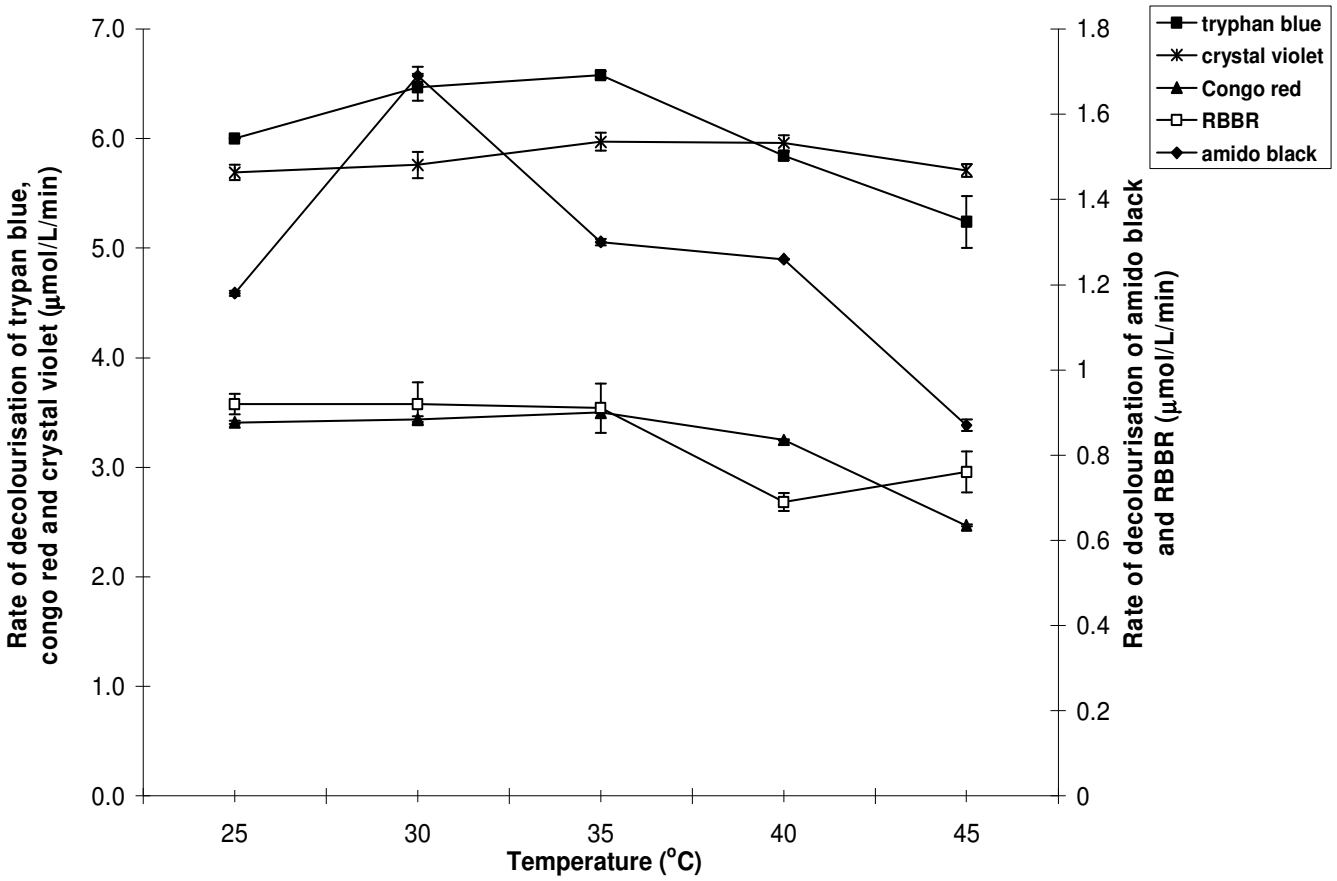


Figure 7. Effect of temperature on the rate of decolourisation of tryphan blue, crystal violet, congo red, amido black and RBBR.

the increasing dye concentration exhibiting apparent first order reaction. In general, high dye concentration will cause a slower decolourisation rate (Young and Yu, 1997). However, in the present studies, the rate of decolourisation increased to a certain optimum concentration of each dye after which the rate of decolourisation decreased. These results were in accordance with Radha et al. (2005). The decrease in the decolourisation of dyes with increasing concentration could be attributed to the fact that colour removal depends on the destruction of the chromophore. The peroxidases of the fungus need to attack one molecule of the dye several times, a lower concentration of the dye facilitates the destruction of the molecules and higher dye concentration implies less average attacks of enzyme to each dye molecule and hence slower colour removal rate (Young and Yu, 1997).

The rate of decolourisation for all the dyes was high in the pH range of 4.0-4.5, indicating that an acidic environment is required for a better rate of reaction as well as decolourisation. A similar requirement of an acidic pH range for the decolourisation of synthetic and commercial dyes by ligninases of *P. chrysosporium* has also been reported (Young and Yu, 1997; Radha et al., 2005). The pH effect on enzymatic decolourisation is associated with the pH dependence of the enzyme activity and the dye itself. The pH of 4.0 - 4.5 in this study was within the physiological pH range that is near optimum for the growth and lignin degradation by the ligninolytic enzymes (Kirk et al., 1978). A high rate of decolourisation for crystal violet was also observed at pH 2.5. A maximum rate of congo red oxidation at pH 2.5 with LiP isoenzyme H8 from *P. chrysosporium* has been reported by Tatarko and Bumpus (1998). Lignin peroxidase isoenzymes having pH optima of 2.5 have been reported from a number of white rot fungi including *P. chrysosporium* (Tien and Kirk, 1984) and *Bjerkandera* sp. strain BOS55 (Have et al., 1998). In the present study, the requirement of an acidic pH of 4.0 - 4.5 for optimum rate of decolourisation provided indirect evidence that the enzyme responsible for the decolourisation of dyes from SMC might be LiP and a few isoenzymes of LiP active at different pH might be responsible for broader pH optima for the rate of decolourisation.

The role of veratryl alcohol as a redox mediator, protecting ligninase from inactivation by H_2O_2 and helping in the completion of the oxidation-reduction cycle of ligninase is well explained (Harvey et al., 1986; Koduri and Tien, 1994). The optimum decolourisation of the synthetic dyes using partially purified ligninase from *P. chrysosporium* at the veratryl alcohol concentration of 0.5-1.0 mM has been

reported (Young and Yu, 1997). However, there is little information on the effect of veratryl alcohol concentration above 2 mM on dye decolourisation. In this study, it was observed that the rate of decolourisation for all the dyes except RBBR decreased but was not inhibited at the veratryl alcohol concentration above 2 mM. A decrease in the rate of decolourisation of RBBR was observed at a veratryl alcohol concentration above 4 mM. It has been reported that the extent of phenol oxidation by LiP of *P. chrysosporium* also increased with increasing veratryl alcohol concentration due to the generation of veratryl alcohol cation radical (VA^{+}) by LiP in the presence of H_2O_2 (Chung and Steven, 1995). A similar phenomenon might be possible in this study whereby VA^{+} was formed by the oxidation of veratryl alcohol by LiP in the presence of H_2O_2 and oxidizes the dyes. The oxidized dyes undergo further chemical decomposition while VA^{+} was reduced back to veratryl alcohol. At low concentrations of veratryl alcohol, all the VA^{+} formed was used for the decolourisation of dyes, giving the appearance of inhibition of veratryl alcohol oxidation (that is, veratryl aldehyde formation). At this stage, the rate of decolourisation precedes the rate of veratryl alcohol oxidation. Both the veratryl alcohol and dyes compete for the catalytic sites of LiP and with veratryl alcohol being a preferred substrate for LiP as compared to dyes, the oxidation of veratryl alcohol to veratryl aldehyde at a higher concentration of the former precedes the oxidation of dyes resulting in a lower rate of decolourisation. However, as the oxidation of veratryl alcohol to veratryl aldehyde was not followed in this study, a further study is needed to prove this hypothesis.

An inhibitory effect of H_2O_2 concentration above 0.4 mM on the rate of reaction for RBBR and amido black was observed. The rate of reaction was relatively similar for crystal violet, congo red and tryphan blue at all the H_2O_2 concentrations. A similar effect of H_2O_2 on the decolourisation of acid dyes has been reported by Young and Yu (1997). Results from the present study showed that such a discrepancy might be due to two possible reasons which require further study. One reason is the formation of compound III (an inactivated enzyme form) from compound II (oxidized form) of LiP at high H_2O_2 concentrations in the case of RBBR and amido black. During the decolourisation of dyes, the degradation products of RBBR and amido black might not act as a substrate of compound II to convert it back to the native enzyme. This in turn might have lead to the accumulation of compound III in the presence of high doses of H_2O_2. The VA^{+} might also not be available for the oxidative conversion of compound III as it might be utilized for the oxidation of dyes thus resulting in the decrease in LiP activity as well as rate of decolourisation. A second possibility is that an enzyme system with a narrow H_2O_2 range may also be involved along with LiP in the decolourisation of RBBR and amido black. A dye decolourizing peroxidase (DyP) which is catalytically different from LiP and showed maximum decolourisation of azo and anthraquinone dyes at a H_2O_2 concentration of 0.1 - 0.2 mM has been reported (Kim and Shoda, 1999).

In this study, it was also observed that certain dyes like amido black, tryphan blue and crystal violet showed a significant rate of decolourisation even in the absence of H_2O_2. This can be explained on the basis of the complex enzyme mixture or “cocktail” employed in our study. The production of certain enzymes like glucose oxidase and aryl alcohol oxidase responsible for the generation of H_2O_2 by white rot fungi is well documented. The production and purification of aryl alcohol oxidase from the cultures of *P. sajor-caju* has already been reported (Bourbonnais and Paice, 1988). In our previous experiments, we observed significant titers of veratryl alcohol oxidase along with other ligninolytic enzymes during the solid substrate fermentation of rubber wood sawdust by selected *Pleurotus* spp. (unpublished data). Such enzymes might be responsible for the production of a certain amount of H_2O_2. However, the amount or concentration of H_2O_2 produced might not be enough and may require some exogenous addition of H_2O_2 as evident from the results of this study, as the rate of decolourisation increased after the addition of H_2O_2.

The optimum temperature for the rate of decolourisation of all the dyes except crystal violet was between 30-35°C, indicating that this temperature range is probably optimum for the main enzyme responsible for decolourisation. A temperature above 35°C decreased the rate of decolourisation, indicating a thermal denaturation of enzymes. In view of the economics of up scaling, 30°C can be a feasible temperature for the decolourisation of dyes. Thus all the experiments were performed at this temperature. No decrease in the rate of decolourisation was observed for crystal violet at temperature above 35°C. Whether a temperature range higher than the one employed in this study has an effect on the rate of decolourisation of crystal violet remains unanswered at this point and requires further study with a broader temperature range.

In conclusion, it has being shown that enzymes extracted from the spent compost of *P. sajor-caju* have potential in the decolourisation of chemically different dyes. In addition, the effect of certain physical parameters on the rate of decolourisation of each dye was also shown. The study also provided insight on how the knowledge of these parameters can help in the optimization of processes employing enzymes for bioremediation on an industrial scale. Moreover, the *in vitro* treatment of environmental pollutants with crude ligninolytic enzymes of white rot fungi represents a simpler and effective method as compared to the direct application of the fungi by eliminating the need to grow biomass and absorption effects of the pollutants on the mycelia. The utilization of SMC for the extraction of enzymes offers an economical advantage of obtaining industrially important enzymes without long incubation periods and additional

cost of specialized fermentations.

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