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Improving Phenol Red Decolourization Using Laccase-Mediator System

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ABSTRACT

Phenol red exhibits a slow rate of oxidation by laccase. A probable increase is suggested by the use of laccase-mediator systems (LMS). Nine compounds were tested and compared for their ability to act as mediators (1 mM) in the Decolourization of phenol red. Based on the dye decolorising ability, glycine and sulfanilic were selected as best mediators, achieving 64.63 and 63.8% decolourization, respectively with approximately 2.3 fold increase than the initial decolourization using laccase alone. The optimum conditions for attaining the highest decolourization were obtained at 30 h incubation time and 40^{o}C in dark under acidic conditions (pH 4). The fact that glycine being among the best chosen mediators offers a non toxic alternative to commonly used mediators. The nature of glycine suggests electron` transfer (ET) as a route of oxidation by LMS, while the decolourization by Fenton reagent (97%) suggests that hydroxyl (OH$^{\bullet}$) radical to be a successful route for phenol red oxidation. A combination of both routes promises an escalation for the oxidation process of this dye.

Key Words: Decolourization; Laccase; Mediators; Phenol red; Temperature; pH

INTRODUCTION

Laccase enzyme [benzenediol:oxygen oxidoreductases (EC 1.10.3.1)] belongs to a group of enzymes called blue copper oxidases, which are produced from white-rot basidiomycetes. This enzyme is capable of oxidising phenols and aromatic amines by reducing molecular oxygen to water (Thurston, 1994). Other functions of this enzyme include participation in lignin biosynthesis, degradation of plant cell walls and bacterial mineralization (Nyanhongo *et al*., 2002) The fact that it is considered a biocatalyst renders it suitable for bioprocesses such as biopulping, biobleaching and decolourization of industrial effluents most important of which is textile waste water (Bourbonnais & Demethylation, 1992). The discharge of textile waste water, which contains high concentration of reactive dyes is a well-known problem associated with dye stuff activities (Moldes *et al*., 2003)

There is a vast number of textile waste water treatment processes, however, they are not considered successful because of the expensive costs or the production of a huge amount of sludge which merely transfers the chemicals from waste water to solid waste (Bali, 2004). The development of processes based on laccase enzyme for dye removal offers a promising alternative for the treatments of such industrial effluents regarding cost and efficiency since this enzyme is able to degrade dyes of diverse chemical structures (Thurston, 1994)

Regardless of the success of using laccase in decolourization, some dyes such as phenol red (a sulfonephthalein indicator) exhibit an unusually slow rate of oxidation by laccase despite its being a phenol and, therefore, a natural substrate for this phenoloxidase enzyme (D'Acunzo & Galli, 2003). The decolourization of a model dye like phenol red is considered a way for assessing the aromatic degrading capability of ligninolytic enzymes (Lorenzo *et al*., 2002). Due to the slow rate, the oxidation of phenol red requires more than laccase alone. More research is being devoted to using mediators (oxidizable low molecular weight compounds) with laccase to achieve better decolourization results.

Mediators are found to extend or permit oxidation of non-substrate compounds (Johannes & Majcherczyk, 2000). They promote the catalytic activity of laccase towards different and more difficult oxidisable functional groups including benzyl and allyl alcohols, ethers and oxidation of alkylarenes (Cantarella *et al*., 2003). Polycyclic aromatic compounds and textile dyes have also been reported (Wong & Yu, 1999; Johannes & Majcherczyk, 2000; Soares *et al*., 2001; Nyanhongo *et al*., 2002; Moldes *et al*., 2003; Yesilada *et al*., 2003). The choice of the proper mediator substance plays a key role in the general applicability and effectiveness of the LMS (Johannes & Majcherczyk, 2000). Many organic compounds have been found to act as laccase mediators (Li *et al*., 1998, 1999). More than 100 possible mediator compounds have been described but the most commonly used ones are ABTS (2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)), HPI (N-hydroxyphthalimide), VLA (Violuric acid) and HBT (1-hydroxybenzotriazole). These have proved more effective in facilitating the oxidation of a variety of synthetic dyes (Johannes *et al*., 1996; Bourbonnais *et al*., 1998; Pickard *et al*., 1999; Johannes & Majcherczyk, 2000; Baiocco *et al*., 2003). The drawbacks

for using such mediators are cost and potential toxicity (Johannes & Majcherczyk, 2000). The routes by which the mediators act for increasing the oxidation of recalcitrant compounds are either though electron transfer (ET) and hydrogen abstraction (Biaocco *et al.,* 2003) or though hydroxyl radicals and superoxide anion radicals (Li *et al*., 1998).

The fact that mediators might exert a toxic effect on the discharge water led to elaborating search for new classes of mediators, preferably with simpler structures and less toxicity. In this study a number of compounds have been tested in an attempt to improve the decolourization of phenol red. The decolourization is further investigated with respect to the effects of temperature and pH as factors possibly affecting phenol red oxidation by LMS.

MATERIALS AND METHODS

Chemicals. Laccase from *Trametes versicolor*, cysteine, glycine, imidazole, nicotinic acid were purchased from Sigma and all other chemicals were bought from Aldrich.

***In vitro* decolourization of phenol red.** This reaction was carried out in 30 mL vials at 30°C, the reaction mixture contains sodium acetate buffer, pH 4.5 (10 mM), phenol red (75 uM) and laccase (8 U L^{-1}) in a total volume of 3 mL. Phenol red and laccase are dissolved separately in the buffer to ensure solubility, then added together in the vials. The percentage of decolourization was determined by monitoring the decrease in the absorbance at 431 nm. The readings were taken after 1, 24, 48, 72 and 120 h. All samples were done in duplicates.

Laccase-Mediator system for phenol red decolourization. Nine mediators were chosen in an attempt to increase the decolourization of phenol red, namely: imidazole, sulfanilic, 4-dimethylamino benzaldehyde (DEAB), ampyrone, thiamine, glycine, glutamic, nicotinic acid and cysteine. All these mediators were dissolved in a buffer to achieve a final concentration of 1 mM. Total volume of reaction mixture was 3 mL, which was incubated at 30°C in the dark. The percentage of decolourization was monitored at 18, 24, 42, 48, 66 and 72 h.

Effect of temperature on LMS. For studying the effect of temperature on the laccase mediator system, the reaction mixture was prepared as described above. Five mediators were chosen according to the extent of decolourization achieved. They were sulfanilic, DEAB, ampyrone, glycine and glutamine. All these mediators, along with a control (laccase & dye), were incubated at different temperatures (25, 30, 40, 50 and 60°C) in the dark. The percentage of decolourization was estimated at narrower intervals, which were: 6, 24, 30, 48 h. The mediators with the highest decolourization rate were chosen for further experiments.

Effect of pH on LMS. Effect of pH on the decolourization of phenol red was studied using the best two mediators at the optimum temperature and incubation time. pH (3-10) was adjusted with HCl or NaOH before the addition of laccase the dye and the mediator. The decolourization of phenol red was monitored at 6, 24, 30 and 48 h.

Use of fenton reagent in decolourization of phenol red. This experiment was conducted to study the decolourization of phenol red using Fenton reagent (Fe SO_4 or $CuSO_4/H_2O_2$). Also, $FeSO_4$, $CuSO_4$ and H_2O_2 were all tested. Phenol red (75 uM) was dissolved in the buffer, reagents added, $FeSO_4$ (1 mM), $CuSO_4$ (1 mM) and H_2O_2 (100 mM). Incubation proceeded at 30°C in the dark; samples were taken after instant addition at 1, 18 and 24 h.

RESULTS AND DISCUSSION

Phenol red decolourization by laccase. Laccase can be used in the decolourization of dyes either as extracellular culture fluid (Lorenzo *et al*., 2002) or as fungal pellets (Yesilada *et al*., 2003). *Trametes versicolor* laccase was incubated with phenol red for decolourization of the latter at different times. Data indicated an increase in the percentage of decolourization with the increase in time, though being slow initially (Fig. 1). A proportional relationship with time is suggested that 33.33% decolourization achieved after 120 h incubation might increase further if the incubation time was extended beyond the time limit of this experiment. It also reflected the impracticality if this process was to be applied to an on-site textile waste water treatment system. The results obtained confirms the ability of *Trametes versicolor* laccase to oxidise phenol red, this coincides with the results obtained by Moldes *et al*. (2003)

Laccase mediator systems. Phenol red is said to exhibit a slow oxidation rate by laccase (D'Acunzo & Gali, 2003), and the mediators are expected to increase this reaction. The oxidised mediator could rely on an oxidation mechanism, not available to the enzyme, thereby extending the range of substances accessible (Fabbrini *et al*., 2002). In this study nine compounds were tested to determine their ability to act as laccase mediators; some were of low molecular weight and others were amino acids. From the data it is evident that the mediators behaved differently when incubated with laccase/phenol mixture (Table I). Most mediators initially show a low percentage of decolourization, but a rapid increase was evident afterwards; all reaching a plateau when incubated for 42-48 h. Best mediators triggering the highest decolourization of phenol red were DEAB, sulfanilic, ampyrone, glutamine and glycine. It was expected that an aromatic compounds like thiamine or nicotinic acid would be more efficient in acting as mediators. However, high Decolourization by two amino acids was unexpected. Johannes and Majcherczyk (2000) stated that SH-group containing compounds could act as substrates for laccase. Therefore, thiamine, nicotinic acid and cysteine presumably gave good results as mediators. Cantarella *et al*. (2003) stated that the presence of >N-OH group ensures the efficiency of a compound to act as mediator. These results open up avenues of research on different compounds only containing –NH group.

Fig. 1. Effect of time on decolourization of phenol red by laccase

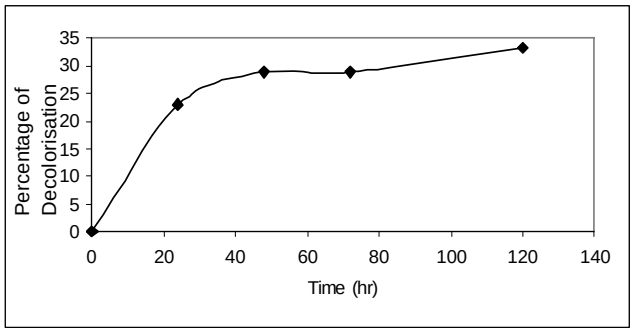


Fig. 2. Phenol red decolourization percentages by different LMS at 25°C

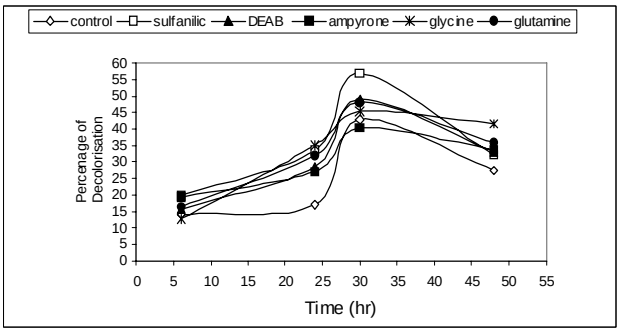


Fig. 3. Phenol red decolourization percentages by some LMS at 30°C

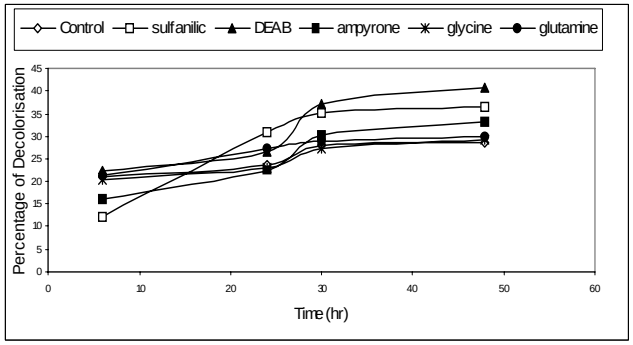


Fig. 4. Phenol red decolourization percentages by LMS at 40°C

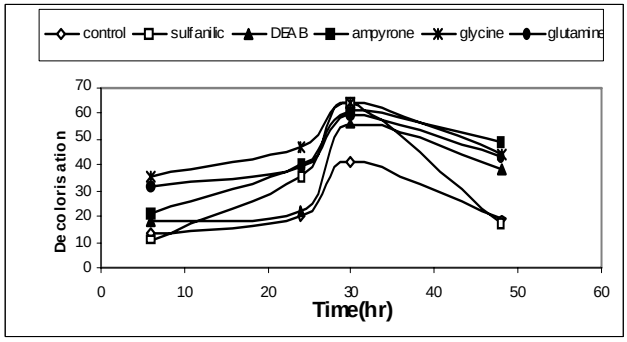


Fig. 5. Phenol red decolourization percentages by LMS at 50°C

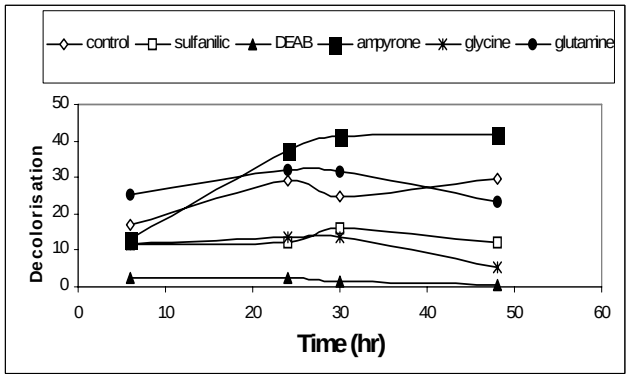


Fig. 6. Phenol red decolourization percentages by some LMS at 60°C

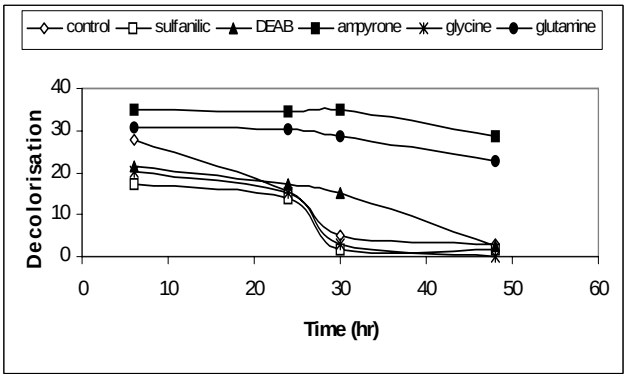


Fig. 7. Effect of pH variation on decolourization of phenol red using laccase

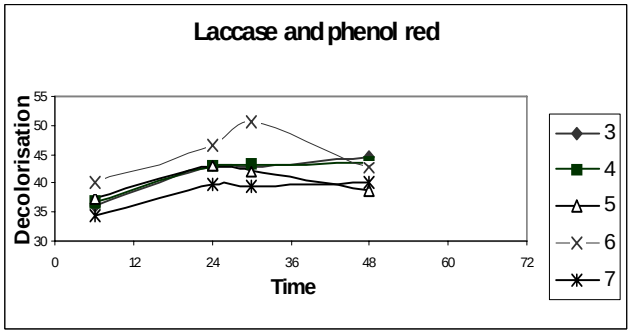


Fig. 8. Effect of pH variation on decolourization of phenol red using laccase/sulfanilic

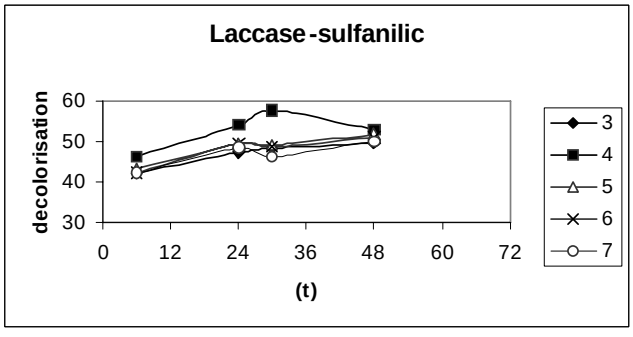
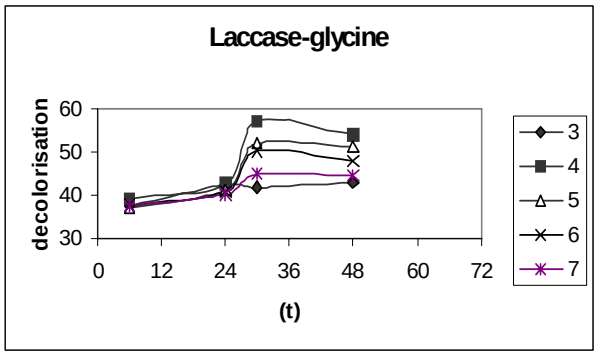


Fig. 9. Effect of pH variation on decolourization of phenol red using laccase/glycine



Effect of temperature on decolourization. Figs. (2-6) represent the effect of incubation temperatures on the decolourization percentage of phenol red under the laccase mediator system. Five chosen mediators, mentioned above, were incubated at five different temperatures and the percentage of decolourization was monitored at different time intervals. Fig. 2 indicated slow initial decolourization by all mediators, followed by an increase in it and reaching the highest at 30 h incubation time. The highest decolourization was noted for sulfanilic (56.86%) with an increase of 1.32 over control. Fig. 3 shows a relatively higher increase with the highest decolourization percentages attained after 48 h incubation; the maximum being for DEAB (40.65%), which was approximately 1.43 fold greater than control. The mediators exhibited a variation in initial decolourization, all of which exhibited a gradual built up in it later on at 40°C (Fig. 4). The maximum decolourization percentage was attained after 30 h of incubation that was almost double the initial value. Glycine exhibited the highest decolourization (64.63%), which was 1.6 fold higher over control, followed by sulfanilic (63.8%), ampyrone (60.69%), glutamine (59.29%) and DEAB (55.78%). At 48 h incubation, there was a sharp drop, which reached lowest point for sulfanilic (16.84%). This suggested that the temperature employed in this experiment was effective to a certain length of time after which the high temperature exerted an adverse effect, probably on laccase activity or the electron transfer (ET) by the mediators. This is obvious because of the fact that the decolourization percentages exhibited increases and decreases for mediators when the temperatures were varied. Another possibility is that phenol red and/or LMS experienced structural changes due to the high temperature. The effect of extremely high temperature is shown in Figs. 5 and 6, where the decolourization percentages are lower and in some cases negligible. It was evident that the temperature variation is a strong factor controlling the decolourization process. This coincides with the results obtained by Nerud *et al*. (2001) who stated that the decolourization of dyes are temperature related, a significant increase was noticed in non-enzymatic reaction as the incubating temperature increased up to 50°C. On the other hand, Soares *et al*. (2001) reported 60°C to be the optimum for decolourization for anthaquinone dyes which suggested that the process is only dye related and that laccase is not dysfunctional at high temperatures.

Sulfanilic acted as a mediator, probably though a resonance effect as it is considered a low redox potential compound and the Laccase is able to oxidise anilines (Johannes & Majcherczyk, 2000; Vandertol-Vanier *et al*., 2002). The fact that glycine gave good results as a mediator was unexpected in view of the fact that some amino acids containing aromatic rings or sulfhydryl groups are successful as mediators (Johannes & Majcherczyk, 2000). The Zwitterion effect may be a reason as to why glycine was able to induce laccase activity. Glycine, being low molecular weight, probably transferred electrons to laccase easily, thus acting as a good mediator. Sulfanilic and glycine were both employed in the upcoming experiment at 40°C.

Effect of pH on decolourization. In this experiment pH range of 3-10 was employed. There was almost no variation at 6 h incubation for laccase/phenol red, slightly enhancing at 24 h, but marker differences were noticed at 30 h incubation, with a maximum Decolourization of 50.81% at pH 6 (Fig. 7). A decrease is noticed at all pH values after 48 h incubation. After 30 h incubation, there was an increase in decolourization only at pH 4 when sulfanilic and glycine were used (Fig. 8 & 9). No variation was evident at other pH values even when incubation time increases. This suggested that pH variation is an enzyme-rather than a dye related factor. Nerud *et al*. (2001) experienced no alterations in decolourization at pH in the range of 3-9. From this, it can be assumed that the best decolourization results can be achieved at pH values suitable for laccase functioning.

Table I. decolourization percentages of phenol red at different times using different mediators

Sample	18 h	24 h	42 h	48 h	66 h	72 h
Control	21.82 %	23.68	28.47	28.82	28.82	28.82
Ampyrone	18.5	22.6	30.346	33.29	33.8	33.8
DEAB	24.39	26.64	35.6	40.65	40.65	43.6
Imidazole	24.76	24.76	28.25	29.06	29.3	29.3
Sulfanilic	13.13	30.9	35.6	36.46	36.66	38.38
Cysteine	4.83	11.46	15.73	19.10	19.10	20.78
Glutamine	23.44	27.41	29.89	30.1	32.25	33.97
Glycine	21.3	23.04	27.174	29.34	30.34	31.63
Nicotinic acid	15.172	20.68	27.01	27.50	27.58	31.03
Thiamine	6.88	13.879	20.327	20.87	21.8	26.22

Table II. The use of Fenton reagents on decolourization of phenol red

Substance added	0 h	1 h	18 h	24 h
$CuSO_4$	12.7 %	12.7	15.83	22.08
$FeSO_4$	24.58	28.33	17.91	21.25
$CuSO_4/H_2O_2$	37.41	55	97.33	97.75
$FeSO_4/H_2O_2$	47	59.96	97.54	97.54
H_2O_2	22.5	24.58	53	62.91

Use of fenton reagent. The Fenton reaction produces hydroxyl radicals by the Fe^{2+} dependent breakdown of H_2O_2. These radicals are known to be potent oxidizing agents in biological systems (Wood, 1994). Also, copper based systems can operate in the production of radicals. This experiment was conducted to assess whether complete decolourization of phenol red is achievable in short time. Our data negates report of Nerud *et al.* (2001) who stated that Fenton is only capable of decolorising 1% in 1 h and only 11% after 24 h incubation. As seen from Table II, the decolourization reached 59.96% after 1 h incubation with $FeSO_4/H_2O_2$ and 55% when incubated with $CuSO_4/H_2O_2$ for the same period of time. The decolourization increased dramatically to 97.54 and 97.75%, respectively after 24 h incubation. The incubated mixture was almost clear to the naked eye. Not all dyes underwent decolourization by the peroxide effect. Young and Yu (1997) stated that it is totally dependent on the dye structure. The results obtained in this experiment, highlights the assumption of $OH^{\cdot}$ being the reason or the mode of decolourization of phenol red.

CONCLUSION

It can be concluded that factors like temperature and pH are important in achieving better decolourization levels. Although little attended, the amino acids can act as potential mediators. They might be helpful in reducing cost and toxicity to discharged waste water, thus, eliminating further problems. Though very successful in decolourization, the addition of Fenton reagents to textile waste water has limitations considering the cost and the toxicity to water in streams. There is a possibility of using fungi capable of producing or generating H_2O_2 for the use of multi-enzyme systems or as mixed fungal cultures to obtain even higher levels of decolourization. The use of LMS offers a safer approach to decolourization, especially if one considers using an amino acid like glycine or compounds of diverse nature as mediators. Although the route by which laccase mediator systems (LMS) works has been described for a number of dyes, the assumption is strongly propelled towards the application of both electron transfer system (ET) and hydroxyl radicals in the decolourization of phenol red. Therefore, a combination of both routes in one process is likely to achieve higher percentages of decolourization.

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