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Laccase Production From *Trametes hirsuta* and Decolourisation of Phenolic Textile Dye In a Laccase Mediator System (LMS)

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ABSTRACT

In this study, fungal laccase was produced from *Trametes hirsuta* DSMZ 5072 at 28 °C in a submerged cultivation medium comprising: potato infusions from 200gm/L, glucose (1%) and malt extract (1%). The maximum laccase activity (129 U/L) was observed at day eight post-cultivation in the presence of 2mM of 2,5-xylidine, as an inducer. Fungal laccase from *Tramates versicolor* as a commercial enzyme, and home prepared laccase from *T. hirsuta*, were used to decolourise phenol red. The effects of initial medium pH, different systems, type of mediator and incubation temperature were investigated. It was observed that, for both commercial and homemade laccases, pH 4.5 allowed more efficient decolourisation of the dye at 30 °C. In an aqueous system, high decolourisation percentages, of 42.8% and 36.4%, were revealed within 72 hours in the presence of *T. versicolor* and *T. hirsuta* laccases respectively. The degree of decolourisation increased in the aqueous system when a different mediator was added. Results showed that the 1-hydroxybenzotriazole (HBT) has a much better decolourisation ability than the other mediators tested, such that decolourisation percentages as high as 73.6% and 79.4 were observed in the presence of 8mM HBT and laccase from *T. hirsuta* (40 °C/120 minutes) or *T. versicolor* (30 °C/120 minutes), respectively.

INTRODUCTION

Dyes are synthetic and aromatic molecular structural compounds. According to their dissociation in an aqueous solution, dyes can be classified as acid, direct reactive dyes (anionic), basic dyes (cationic) and disperse dyes (nonionic).

They are used on several substrates such as food, cosmetics, paper, plastic and in the textile industry. Solutions retain them by physical adsorption by making compounds with metals and salts using covalent bonds. Chemical dyes are increasingly being used in the textile and dyeing industries because of the ease and cost-effectiveness with which they can be synthesised, and their firmness and variety in colour compared to natural dyes. Coloured industrial effluents from the dyeing industries represent major environmental problems, however, since unbound reactive dyes undergo hydrolysis due to temperature and pH values during the dyeing processes and the strong colour of discharged dyes, even at very small concentrations, has a huge impact on the aquatic environment due to their turbidity and high pollution strength. In addition, toxic degradation products can be formed (Mishra and Bisaria, 2007; Moorthi *et al.,* 2007).

Wastewater discharged from textile and dye stuff industries therefore have to be treated due to growing public concern over their toxicity, carcinogenicity and impact on water bodies. The many different and complicated molecular structures of dyes, however, make dye wastewater difficult to treat by conventional biological and physico-chemical processes and, therefore, innovative treatment technologies need to be investigated. In this context, decolourisation of dye wastewater by fungal metabolic activity has been the subject of many studies (Abadulla *et al.,* 2000).

Fungi from the Basidiomycetes, known as white rot fungi, are a heterogeneous group of microorganisms that are able to degrade a wide variety of recalcitrant pollutants, including various types of dyes. Laccase based decolourisation treatments are potentially preferable to bioremediation technologies since the enzyme is produced in larger amounts. Laccases belong to the group of phenol oxidases; these copper containing enzymes are oxidative enzymes detected in many plants but mainly produced by numerous fungi (Li *et al.,* 2007).

Laccase Mediator Systems (LMS) have been widely studied in recent years, with three types of mediators having been proposed, and with the NOH-type and phenol-type having been found to be effective in the decolourisation of dyes. Moreover, a laccase-mediator system has been used in bleaching indigo carmine. In 1996, Novozyme (Novo Nordisk, Denmark) launched a new industrial application for laccase enzymes in denim bleaching: DeniLite®. Laccases can bleach indigo-dyed denim fabrics to lighter shades with the help of a mediator molecule (Morozova *et al.,* 2007; Hu *et al.,* 2008). The present study is to determine the ability of commercial and homemade laccases in the decolourisation of phenol red using aqueous and other nonconventional systems. Decolourisation under different temperatures, PH, mediators and mediator concentrations were investigated.

MATERIALS AND METHODS

Materials

Commercial laccase from *Tramates versicolor* was provided by Fluka Chemicals Company (Switzerland). Mediators like 2,2_azinobis (3-ethylthiazoline-6-sulfonate) (ABTS), (1-hydroxybenzotriazole) (HBT) were supplied by Fluka, and 2,2,6,6–tetramethylpiperidine –1-oxyl) (TEMPO) by Aldrich. All chemicals used as buffers, substrate and other mediators were commercial products of at least reagent grade, unless otherwise indicated.

Microorganism

Tametes hirsuta (DSMZ 5072), obtained from Prof. Dr Erko Stackebrandt (Deutsche Sammlung von Mikroorganism und Zellklturen GmbH, Germany), were grown on potato dextrose agar (PDA) plates at 30 °C for 10 days. Thereafter, the plates were maintained at 4 °C and inoculated once every three months.

Enzyme Production

For routine enzyme production in shake flasks, 250 ml of medium (potato infusion from 200gm/L, glucose (1%), malt

extract (1%), with different concentrations of 2,5 Xylidine and at pH5.5) in 1000-ml wide mouth Erlenmeyer flasks were inoculated with (5%) (vol/vol) of four days' old fungal mycelium growth and then incubated for up to eight days at 28 °C on a shaker at 180 rpm. The samples were collected every 24 hr under sterile conditions and then enzymatic activity was determined.

Determination of Enzymatic Activity

The laccase activity of the homemade enzyme from *T. hirsuta* was routinely assayed by measuring the rate of ABTS oxidation at room temperature. The activity was investigated in a reaction volume of 0.2mL, consisting of the appropriate amounts of phosphate buffer (100mM, pH 4.6) containing the laccase sample and 1mM of 2,2́-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS). The oxidation of ABTS was followed by an increase of absorbance at 405nm (ε_{405} = 36 000 $M^{-1}cm^{-1}$). One unit of activity was defined as the amount of laccase that oxidized 1 µmol of substrate per minute (Niku-Paavola *et al.*, 1990).

Dye decolourisation experiments

Decolourisation of phenol red was conducted according to Xin and Geng, (2010) with some modifications. The reaction was carried out in test tubes at 30 °C and 150 rpm shaking under darkness, and the reaction mixture contained 0.1 M sodium acetate (pH 4.5– 5.5 – 6.5 or 7.5), phenol red (100 µM), and laccase (0.12 u/ml) in a total volume of 2.5 ml. Commercial laccase from *T. versicolor* was used for comparison. A reaction mixture using distilled water in place of the enzyme solution was used as a control. Absorbance at 450nm (the maximum visible absorbance of phenol red) was monitored periodically (0, 2, 4, 8, 10, 12, 24, 48, 72 and 96 hr) as an indication of the dye concentration. All assays were carried out in duplicate. The degree of decolourisation was calculated according to following formula: $\mathbf{D = 100\ (A_{ini} - A_{obs})/A_{ini}}$ (**D**: is the degree of decolourisation (in percent), $\mathbf{A_{ini}}$: the initial absorbance, and $\mathbf{A_{obs}}$: the observed absorbance.

Decolourisation of phenol red in different systems

The study also investigated the ability of two different laccases, one commercially available fungal laccase from *T. versicolor*, and a homemade laccase from *T. hirsute*, to decolourise phenol substrate in a number of aqueous and non-conventional systems, including systems based on surfactant-less micro emulsions (with PT as a ternary system, 16% α-Pinene, 65.1% *t*-Butanol and 18.9% water) and organic co-solvents (t-Butanol 10 and 20%). In a typical reaction, the substrate (100 µM phenol red) was added to a vial containing the appropriate amounts of organic solvent, or the aqueous phase (100 mM acetate buffer pH4.5), containing 0.12 U ml^{-1} of enzyme and incubated at 25 °C. Controls were performed in the absence of the enzyme. Samples were withdrawn periodically.

The influence of laccase-mediator-systems on decolourisation

By adjusting the optimal pH and the reaction system of dye decolourisation, the reaction was initiated with the addition of different synthetic and natural mediators at different concentrations. The decolourisation percentage was determined and then monitored as previously described, and samples were withdrawn periodically.

Application of laccase-HBT mediator systems for dye decolourisation

HBT as a laccase mediator was introduced to the reaction mixture (100µM Phenol red and 0.12 U/ml of laccase in acetate buffer 0.1M pH 4.5) to reach the final concentrations of 1, 2, 4, 8, 16 and 32 mM. Decolourisation was determined as previously described.

The effect of incubation temperature on Laccase-HBT system decolourisation

The effect of temperature on laccase-HBT system decolourisation was studied by incubating 100µM of phenol red in the presence of 0.12 U/ml of laccase in a temperature range of 20 – 60 °C and pH 4.5.

RESULTS AND DISCUSSION

Laccase production

T. hirsuta has been described as a very promising candidate for the production of laccase. The results in Figure 1 show that *T. hirsuta* (DSMZ 5072) produced higher laccase activity of 129 U/L at day nine in the presence of 2mM 2,5 Xylidine as an inducer. It was also observed that enzyme activity increased whenever the inducer concentration was increased in the potato infusion medium. Different production media were trialled for the ability to obtain high laccase activity in order to optimize the other production conditions (data not shown). Several foods and food wastes commonly used for laccase production by *T. hirsuta* , and other fungi in liquid culture or under solid-state conditions , and these materials were selected due to their availability and low cost (Abadulla *et al.*, 2000; Minussi *et al.*, 2000; Rosales *et al.*, 2002; Liers *et al.*, 2007).

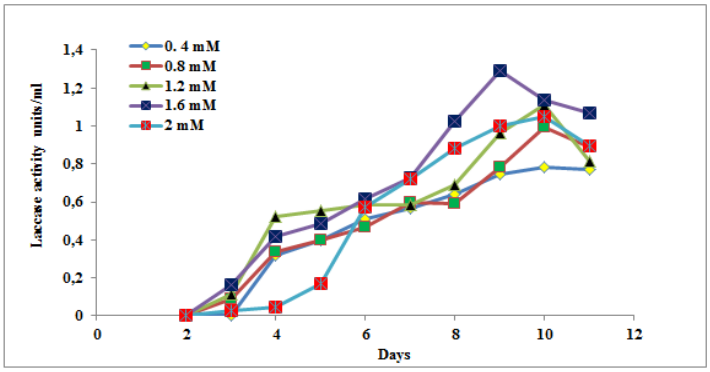

Fig. 1: The effect of xylidine concentration on laccase produced by *T. hirsuta* strain (DSMZ 5072) in potato infusion medium including 1% glucose and malt extract, at pH 5.5 and 28 °C.

Effects of pH on decolourisation

Both homemade and the commercial laccases revealed different degrees of decolourisation of phenol red , as a typical model dye for decolourisation , increased with incubation time at different degrees of pH . The initial decolourisation rate for commercial laccase was higher (49.18%), indicating that commercial laccase has better decolourisation performance than our own enzyme sample (47.39%) at pH 4.5 within 72 hr (Table 1). Blank decolourisation was also observed in the control sample (4.12%), indicating that the decolourisation was a biological reaction caused by laccase in the samples rather than by the activity of other ligninolytic enzymes.

Table 1: Decolourisation percentage of 100 µM phenol red under different degrees of pH by 0.12 U/ml of laccase from *T. versicolor* and *T. hirsute* within 72 hr at a reaction temperature of 30 °C. Blank: without enzyme (4.12 %).

pH	*T. versicolor*	*T. hirsuta*
4.5	**49.18**	**47.39**
5.5	**42.99**	**38.16**
6.5	**45.48**	**44.68**
7.5	**41.22**	**16.69**

This result suggests that commercial laccase has a high decolourisation ability and has great potential in the degradation of aromatic compounds.

The decolourisation percentage was affected by pH, temperature, enzyme dose, source of laccase and type of dye. As presented in this study, the optimum pH to achieve maximum decolourisation of 100 µM phenol red using either 0.12 u/ml home prepared laccase from *T. hirsuta* or commercially available laccase from *T.*

versicolor, was at a pH of 4.5 at 30 °C within 24 hr. Xin and Geng (2010) used 0.12 U/ml laccase from *T. versicolor* to obtain higher decolourisation of 50 µM phenol red at pH 7 and an incubation temperature of 30°C within 70 hr. Mirzadeh *et al.* (2014), meanwhile, found that free laccase (2 U/ml) from *Paraconio thyrium variabile* exhibited maximum decolourisation of Acid Blue 25 and Acid Orange at pH 5 and an incubation temperature of 40 °C within 65 minutes.

Effects of reaction systems on decolourisation

An experiment was conducted to study the effect of reaction system components on the extent of the decolourisation of the dye using aqueous, pinene *tert*-butanol (PT) and *tert*-butanol systems. The decolourisation ratio of laccase from *T. versicolor* had reached 42.82% within 24 hours in an aqueous system, as shown in Figure 2, while the percentage reached 36.43 for the enzyme produced from *T. hirsuta*.

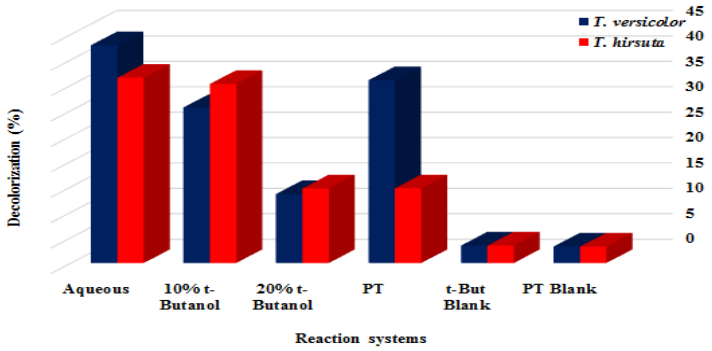

Fig. 2: Decolourisation percentage of 100 µM phenol red in different reaction systems by 0.12 U/ml of laccase from *T. versicolor* and *T. hirsuta* at pH 4.5 within 24 hr at a reaction temperature of 30 °C. (PT=Penine tert-butanol system). *Tert*-Butanol blank (3.43) and PT blank (3.22).

Effects of the type of mediator

In this study, six types of natural (Vanillin and *p*-coumaric acid and Syringaldazine) and synthetic mediators (ABTS, HBT and TEMPO were examined (Table 2). The results showed that HBT has much better decolourisation than the other mediators. The most effective synthetic mediator (0.5mM HBT) required 24 h to attain 83.16 and 71.49 % decolourisation with *T. versicolor* and *T. hirsuta*, respectively. Among the natural mediators, 0.1mM syringaldazine also required 24h to attain 45.06 and 33.18 % decolourisation by the two laccases. Reactions with the natural mediator (syringaldazine) may, therefore, require much higher mediator concentrations than for HBT.

Table 2: Decolourisation percentage of 100 µM phenol red in the presence of different mediators by 0.12 U/ml laccases from *T. versicolor* and *T. hirsuta*, at pH 4.5 and an incubation temperature of 30 ˚C within 24 hr.

Mediators	*T. versicolor*	*T. hirsuta*
0.1mM ABTS	**32.32**	**25.11**
0.1mM *p*-Coumaric acid	**31.72**	**28.43**
0.5mM HBT	**83.16**	**71.49**
0.1mM Syringaldazine	**45.06**	**33.18**
0.1mM TEMPO	**26.81**	**17.80**
0.5mM Vanillin	**35.26**	**29.09**

Phenol red exhibits a slow rate of oxidation by laccase. This is probably because the phenol red molecule has three benzene rings and this makes it difficult for the enzyme to access the molecular core. The application of laccase-mediator systems (LMS) can increase the oxidisation rate by acting rather like a vehicle shuttle to enable

the enzyme to reach sites that could not otherwise be accessed, and thereby reducing the time for and enhancing the extent of the decolourisation. Based on the decolourising of phenol red by LMS, Gomaa (2005) found that glycine and sulphanilic were the best mediators, achieving 64.63 and 63.8% decolourisation, respectively, with an approximately 2.3 fold increase compared to the initial decolourisation using laccase alone. Sadighi and Faramarzi (2013) determined higher relative decolourisation in the presence of HBT (0.5 mM) using laccase immobilized on chitosan nanoparticles.

Effects of HBT concentration

As illustrated in Figure 3, the HBT concentrations from 1 to 32 mM were used to determine the decolourisation percentages of both investigated laccases. The decolourisation percentage in the presence of 32mM HBT was 80.85 and 42.64 with laccases from *T. versicolor and T. hirsuta*, respectively, while it was 79.42 and 54.82 when the HBT concentration was reduced to 8mM HBT. Increasing the concentration of the mediator, however, comprised its solubility in the aqueous reaction system while the use of large amounts of chemically synthesised mediator may increase the toxic effects of LMS and have an environmental impact. Overall though, the laccase-HBT system is one of the most successful laccase-mediated systems for the removal of pollutants, elimination of some synthetic dyes and degradation of some pharmaceutical agents (Khilifi *et al.* 2010).

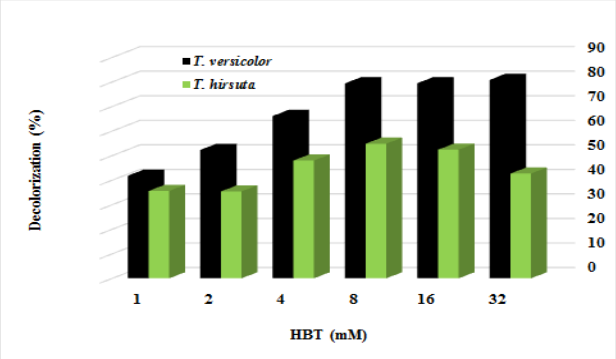

Fig. 3: Decolourisation percentage of 100 µM phenol red in the presence of different HBT concentrations by 0.12 U/ml laccases from *T. versicolor* and *T.hirsuta*, at pH 4.5 and an incubation temperature of 30 °C within 120 minutes. Blank: without enzyme is 5.64%.

The application of laccase in various biotransformation processes of industrial interest has hitherto been hindered by the low aqueous solubility of organic substrates. However, monophasic water–miscible organic solvent mixtures, i.e. ternary systems formed by a hydrocarbon with short chain alcohol and water, can be used as a reaction media for biocatalytic processes involving various enzymes (Zoumpanioti *et al.*, 2006; Wan *et al.*, 2008). The major advantages associated with the implementation of these systems are their thermodynamic properties, stability, optical transparency; high solubilisation capacity of both hydrophobic and hydrophilic substrates, which facilitates down-stream processing (Tzialla *et al.*, 2008). From our previous study, rapid inactivation of enzymes was detected in n-hexane based systems, while maximal enzyme stability was observed in a ternary system formulated with a-pinene (P), in which laccase from *T. hirsuta* retained about 90% of its initial activity, even after 24 hr incubation (Tzialla *et al.*, 2009). Despite the stability of laccase in low water contain systems; this study confirms that aqueous systems are the best for the decolourisation of phenol red by the different laccases that have been tested. This may be due to the effect on enzyme activity of the organic solvents that are included in other systems, in terms of oxidation of phenol red and the rapid dissolving of the dye in the water.

Effects of incubation temperature

The effects of the incubation temperature on the decolourisation of phenol red by *T. versicolor* and *T. hirsuta* laccases are shown in Figure 4. Dye decolourisation was observed at different rates at incubation temperatures of 20 °C, 30 °C, 40 °C, 50 °C and 60 °C. The highest decolourisation percentages, however, (79.4 and 73.6) were observed in the presence of 8mM HBT and laccases from *T. versicolor* at 30 °C or *T. hirsuta* at 40 °C, respectively, within 120 minutes and at pH 4.5.

Yang *et al.* (2009) and Yesilada *et al.* (2014) showed, however, that temperature variation had little effect on decolourisation of Remazol Brilliant Blue R (RBBR), an anthraquinonic dye, below 70 °C, with a maximum decolourisation percentage was observed at 50–60 ˚C after 10 min. Decolourization of Reactive Black 5 dye, meanwhile, was only 34% after 30s of incubation at 30 °C although the decolourisation increased to 62% at 50 °C with the same incubation period. On the other hand, the decolourisation values of Reactive blue 171 were above 60% at all temperatures in 30s). Nyanhongo et al. (2002), meanwhile, indicated that the rate of laccase-catalysed decolourisation of the dyes increased as the temperature increased up to certain point, above which, however, the dye decolourisation decreased or did not take place at all. This is probably due to the fact that the rate of enzyme inactivation became faster than the enzymatic catalysis rate at higher temperatures.

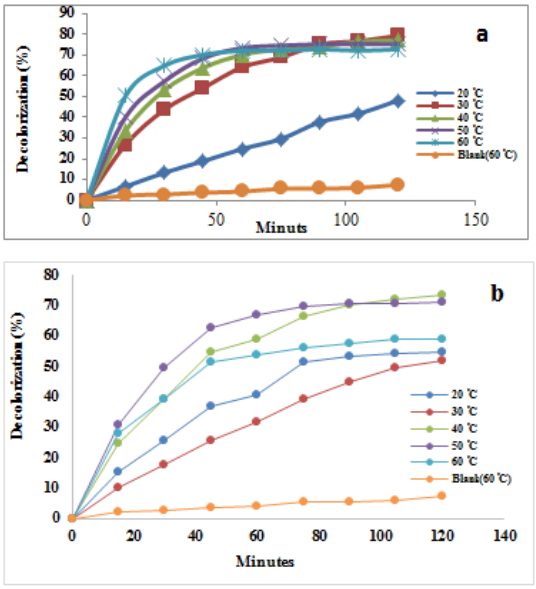

Fig. 4: Effects of incubation temperature on decolourisation percentages of 100µM phenol red by 8mMHBT and 0.12 U/ml of laccases from a) *T. versicolor* and b) *T. hirsuta* in aqueous systems. Blank: without enzyme 7.42%.

CONCLUSION

The use of appropriate conditions for rapid and high textile dye decolourisation could facilitate the development of more economical and environmentally friendly processes.The present study provides a comparison between natural mediators and synthetic mediators regarding their ability to decolourise phenol red dye. It was found that a synthetic mediator (8mM HBT) was able to attain a decolourisation percentage of 73.6 and 79.4 in the presence of 8mM HBT and homemade laccase from *T. hiruta* at an incubation temperature of 40 °C within 120 minutes, or in the presence of commercial laccase from *T. versicolor* at an incubation temperature of 30 °C within 120 minutes at pH 4.5, respectively.

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