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Effect of Natural Mediators on the Stability of *Trametes trogii* Laccase during the Decolourization of Textile Wastewaters

Rim Khlifi-Slama[1#*], Tahar Mechichi[2], Sami Sayadi[1], and Abdelhafidh Dhouib[1]

[1]*Laboratory of Environmental Bioprocesses, Centre of Biotechnology of Sfax, University of Sfax, Sidi Mansour Road Km 6, PB 1177, 3018 Sfax, Tunisia*
[2]*Ecole Nationale d'Ingénieurs de Sfax, Laboratory of Enzyme Engineering and Microbiology, University of Sfax, Soukra Road Km 4, BP 1173, 3038 Sfax, Tunisia*
[#]*Present address: Marine Ecotoxicology, UR 09-03, Sfax University, IPEIS, BP 805-3018, Sfax, Tunisia*

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The purpose of the present study was to determine the effect of natural mediators on the stability of the *Trametes trogii* crude laccase in the process of decolourization of textile effluents. Acetosyringone allowed the highest wastewaters decolourization rate of 25%. At higher concentrations of acetosyringone, the relative activity of laccase decreased approximately by between 38% and 88% after 5 days of incubation. *T. trogii* laccase was strongly inactivated at 3 mM syringaldehyde, after 3 days of incubation. However, laccase activity is more stable in the presence of the vanillin and *m*-coumarate. The *T. trogii* growth on solid effluent-based-medium was examined and evaluated by measuring the colony diameter in cm. *T. trogii* was completely inhibited on 100:0 and 80:20 effluent:water solid medium, however, colony diameter reached 5 cm on 60:40 effluent:water solid medium after 13–14 days incubation. When the textile effluent was pre-treated with laccase and laccase-acetosyringone system, the colony diameter of 2 cm of *T. trogii* on 80:20 effluent:water solid medium was reached after 14 and 10 days of incubation respectively.

***Keywords*:** laccase, stability, natural mediator, wastewater

Introduction

Laccases (EC 1.10.3.2: benzenediol, oxygen oxidoreductase, p-diphenol oxidase) from plants and fungi have been known for decades (Thurston, 1994). These multi-copper oxidases contain four copper ions of two different types: one type I Cu, whose redox potential determines the substrates to be oxidised and other three Cu ions transferring electrons to O_2. Laccases have found various biotechnological and environmental applications (Riva, 2006). Capability of laccases to act on chromophore compounds suggested their application in industrial decolourization processes (Abadulla *et al.*, 2000; Champagne and Ramsay, 2007; Svobodova *et al.*, 2008). The oxidation of a reducing substrate by laccase typically involves formation of a free (cation) radical after the transfer of a single electron to laccase. The oxidative efficiency of laccases depends on the redox potential differences between the reducing substrate and type 1 Cu in laccase. Due to its rather low redox potential (0.5–0.8 V), laccase is able to attack only the phenolic moieties in the lignin polymer. Some low molecular weight compounds that can be oxidized by laccase to stable radicals can act as redox mediators, oxidizing other compounds that in principle are not substrates of laccase due to its low redox potential. In addition to enabling the oxidation of compounds that are not normally oxidized by laccases, the mediators can diffuse far away from the mycelium to sites that are inaccessible to the enzyme itself.

Laccase has been studied recently, in both the pure form and in the presence of a mediator, for the degradation of lignin in wood, model compounds (Saparrat *et al.*, 2002; Saparrat, 2004) bleaching of paper pulp (Bourbonnais *et al.*, 1997, 1998) and dye detoxification and decolourization (Abadulla *et al.*, 2000; Wesenberg *et al.*, 2003; Mechichi *et al.*, 2006). The textile industry accounts for two-thirds of the total dyestuff market (Riu *et al.*, 1997) and consumes large volumes of water and chemicals for wet processing of textiles. Most currently existing processes to treat dye wastewater are ineffective and not economical (Cooper, 1995; Stephen, 1995). Therefore, the development of processes based on laccases seems an attractive solution due to their potential in degrading dyes of diverse chemical structure (Abadulla *et al.*, 2000; Blánquez *et al.*, 2004; Hou *et al.*, 2004), including synthetic dyes currently employed in the industry (Rodríguez *et al.*, 1999; Rodríguez Couto *et al.*, 2004; Rodríguez Couto, 2005). Several reports have been published on the decolourization of industrial dyes by laccase and laccase-producing fungi, in the course of which also different groups of dyes were examined (Ramsay and Nguyen, 2002; Radha *et al.*, 2005) as well as the environmental conditions of their bioconversion (Kapdan *et al.*, 2000) and the kinetic characteristics of laccase oxidations (Soares *et al.*, 2001). Many authors indicated the key role of mediators in decolourization (Soares *et al.*, 2002). In this way, (Claus *et al.*, 2002) found that the system laccase plus mediator enhanced dye decolourization and some dyes resistant to laccase degradation were decolourized.

The substrate range of laccases can be expanded to include non-phenolic compounds in the presence of synthetic or natural mediators such as ABTS (2.2′-asino-bis 3-ethylbenzothiazoline-6 sulfonate) and HBT (1-hydroxybenzo-

*For correspondence. E-mail: rimkhlifi@yahoo.fr; Tel.: +216-22-64-68-56; Fax: +216-74-275-615

triazole) (Sigoillot *et al*., 2005; Morozova *et al*., 2007). The joint use of the system laccase-HBT has proved a highly efficient choice for delignifying flax pulp (Camarero *et al*., 2004; Fillat and Roncero, 2009a) and for textile dye and wastewater decolourization (Wesenberg *et al*., 2003; Mechichi *et al*., 2006; Khlifi *et al*., 2010). Some synthetic mediators such as ABTS, HBT or violuric are suitable laccase mediators, however, major constraint for industrial application is their high price and toxic properties (Johannes and Majcherczyk, 2000b). Moreover, the enzyme can be denaturated by the active radicals generated. The advantage of using natural compounds resides in their low cost, environmental safety, and lack of toxicity since they are obtained from natural, renewable sources (Moldes *et al*., 2008). However, the cost of synthetic mediators tends to be prohibitive for implementation in, e.g., bio-bleaching. This has generated interest in mediators obtainable from plants or as industrial by-products. One of the first natural laccase mediators discovered was syringaldehyde (Kawai *et al*., 1988). Potentially cost-effective lignin-derived natural mediators (*p*-coumaric acid, syringaldehyde, and acetosyringone), obtainable from spent pulping liquors and plant materials, have recently been investigated by Camarero *et al*. (2007). The use of naturally occurring laccase mediators would present environmental and economic advantages. Compounds involved in the natural degradation of lignin by white-rot fungi may be derived from oxidized lignin units or directly from fungal metabolism (Martinez *et al*., 1994; Eggert *et al*., 1996; Johannes and Majcherczyk, 2000a). In addition to enabling the oxidation of compounds that are not oxidized by laccases (e.g., the nonphenolic lignin moiety), the mediators can diffuse far away from the mycelium to sites that are difficult to reach by the enzyme itself (e.g., the lignin macromolecule inside the plant cell wall). The natural mediators such as syringaldehyde and vanillin have similar structures, only differentiated by methoxylation of the aromatic ring: syringaldehyde has two methoxy groups in ortho position to the phenol group, while vanillin has only one methoxy group and thus a more sterically accessible phenolic group. Both compounds were already tested as mediators in decolourization of dyes showing promising results (Camarero *et al*., 2005).

To-date, there are many reports of the use of mediators to target non-laccase substrates for oxidation. In addition, there are several reports that indicate that mediators can enhance the conversion of natural substrates of laccase (Tsutsumi *et al*., 2001; Kang *et al*., 2002; Kim and Nicell, 2006a). However, little has been done to explore and optimize the use of mediators. In most reports, mediators were used at very high concentrations in the range of 6 to 57 mM (Li *et al*., 1999; Soares *et al*., 2001; Fabbrini *et al*., 2002a, 2002b). Such high concentrations may be suitable for some processes, particularly in closed-loop systems where the mediator is retained. However, high concentrations may not be appropriate for some applications, such as wastewater treatment processes, where important considerations include the potentially prohibitive costs of mediators and the possibilities of creating negative impacts on effluent toxicity (Kim and Nicell, 2006b) and on the effectiveness of downstream treatment processes, or in the environment upon their disposal into receiving waters. In previous study, it was shown that simple decolourization of textile industry effluents by crude laccase of *T. trogii* does not necessarily result in detoxification, as the products from use of the most effective synthetic mediator tested here, HBT, is still toxic (Khlifi *et al*., 2010). In addition, Khlifi *et al.* (2009) and Li *et al.* (1999) reported that the use of the mediators HBT at concentrations of 1–3 mM and 10 mM respectively, caused significant laccase inactivation. While the mediators concentrations used in that study were very high. These results indicated that enzyme inactivation due to mediators may limit the practicality of this approach and should be investigated further. In addition, the effectiveness of mediators for enhancing the conversion of target substrates has not been explored. The ideal mediator should be a good substrate for laccase, with stable reactive and with high oxidation power. Its oxidized and reduced intermediates forms must be stable, but must not inhibit the enzymatic reaction. In addition, its redox conversation must be cyclic and without side reactions. However, the mechanism of these processes is insufficiently understood in many cases (d'Acunzo and Galli, 2003; Morozova *et al*., 2007).

Our previous studies had shown the ability of the laccase-mediator system of white-rot-fungi *Trametes* sp. and *T. trogii* to decolourize and detoxify malachite green dyes and textile industry wastewater (Maalej-Kammoun *et al*., 2009; Khlifi *et al*., 2010). Moreover, their role in the decolourization process has been studied and the stability of laccase from these white-rot fungi against the synthetic mediator HBT has also been studied (Khlifi *et al*., 2009; Maalej-Kammoun *et al*., 2009). However, the stability of *T. trogii* laccase against some natural mediators was not examined. The aim of the present study was to determine the effect of acetosyringone, syringaldehyde, vanillin and m-coumarate as laccase mediators, on the stability of crude laccase from *T. trogii* during the process of textile effluent decolourization. Therefore, the effect of the raw and pre-treated effluent with crude laccase and laccase-acetosyringone system on the growth of *T. trogii*.

Materials and Methods

Chemicals

2,6-Dimethoxyphenol (DMP), syringaldehyde, syringate, acetosyringone, vanillin, vanillate, *m*-, *o*-, and *p*-coumarate were obtained from Sigma-Aldrich.

Fungal isolate, media, and culture conditions

T. trogii CTM 50156=DSM 17 786, was provided by the culture collection of the Centre of Biotechnology of Sfax (Dhouib *et al*., 2005). For short term preservation, the fungus was grown on malt extract agar (Difco Laboratories, USA) at 30°C for 5 to 7 days, then Petri dishes were stored at 4°C. For laccase production, *T. trogii* was cultured on medium containing (g/L): glucose, 10; peptone, 5; yeast extract, 1; ammonium tartrate, 2; KH_2PO_4, 1; $MgSO_4{\cdot}7H_2O$, 0.5; KCl, 0.5; trace elements solution, 1 ml. Trace elements were added to give (g/L): $B_4O_7Na_2{\cdot}10H_2O$, 0.1; $CuSO_4{\cdot}5H_2O$, 0.01;

$FeSO_4 \cdot 7H_2O$, 0.05; $MnSO_4 \cdot 7H_2O$; 0.01; $ZnSO_4 \cdot 7H_2O$, 0.07; $(NH_4)6Mo_7O_{24} \cdot 4H_2O$, 0.01.

The pH of the solution was adjusted to 5.5 prior to autoclaving. Aliquots (3 ml) of homogenized mycelium in sterile medium prepared using an Ultra-turrax homogenizer were used to inoculate 300 ml of culture medium in 1,000-ml Erlenmeyer flasks. Cultures were incubated on a rotary shaker (160 rpm) at 30°C. Production of laccase was stimulated by adding 300 µM of $CuSO_4$ to the culture after 3 days incubation (Zouari-Mechichi *et al.*, 2006). Culture broth was collected at the time of maximum laccase production (10 d), filtered, and clarified by centrifugation at 7,000×g for 15 min. The resulting clear filtrate was decanted into dialysis tubing (10 kDa cut-off) and covered by an excess of polyethylene glycol 8000 (PEG 8,000). The procedure was repeated until the volume of enzyme preparation in the dialysis tubing decreased from 1,000 to 50 ml. The concentrated enzyme was then poured in a smaller size dialysis tube and dialysed against 10 mM acetate buffer pH 5. The experiments were performed with this concentrated clear filtrate.

Determination of laccase activity and properties

Laccase activity was assayed using 10 mmol/L DMP in 100 mmol/L sodium acetate buffer, pH 5 (ε_{469nm} = 27 500 M^{-1} cm^{-1}). Enzymatic reactions were carried out at room temperature (22–25°C). One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 µmol of substrate per minute at 22–25°C in 100 mmol/ L sodium acetate buffer, pH 5. Partial characterization of the laccase in the crude preparation, showed an optimal pH of around 4. Activity was stable in the crude extract at room temperature, pH 7 for 24 h; more than 50% activity was retained at pH 5. The laccase in the crude extract was also stable for 24 h at 50°C; more than 90% activity was lost at 60°C, however (Zouari-Mechichi *et al.*, 2006).

Effluent used

Industrial effluent was supplied by a textile factory (Ksar Helal, Tunisia) that utilises indigoid dyes and other chemicals including detergents, salts and surfactants. Indigo (C.I. Vat Blue 1, C.I. 73 000) is a vat dye used to dye cellulosic textiles, especially cotton. This blue dye was employed for dyeing cotton yarn in the manufacture of denims and blue jeans. The process of dyeing with indigo is complex. The indigo is reduced and dissolved in an alkaline solution such as sodium hydroxide, leading to production of effluent with an alkaline pH. The quantities of these chemicals in the effluents depend on the type of process that generates the waste. Effluent characteristics for the Ksar Helal factory are summarized in Table 1.

Table 1. Effluent characteristics

Characteristics	Values
pH	12
$\lambda_{max\ (nm)}$	600
Main dye used	Indigo (CI)
COD	1
BOD_5	0.030
Total solids (g/L)	5,810
Volatile solids (g/L)	2,050
Suspended solids (g/L)	0.115
Volatile suspended solids (g/L)	0.112
Total nitrogen (g/L)	0.056
Ammoniacal nitrogen (g/L)	0.014
Luminescence inhibition (%)	98

Decolourization tests

Decolourization of effluent was examined using the crude *T. trogii* laccase preparation. Unless otherwise indicated, all experiments were performed in 3-ml disposable cuvettes in a 2 ml final reaction volume. The reaction mixture contained 100 mM acetate buffer pH 5, 20% effluent, and laccase with or without a laccase natural mediator. The reaction was initiated by the addition of laccase and incubated in the dark at 30°C. Decolourization of effluent was followed by measuring the absorbance at 600 nm after incubation.
Decolourization was defined as:

$$Decolourization\ (\%) = 100 \times \frac{(OD_{t0} - OD_{tf})}{OD_{t0}}$$

where $Absorbance_{t0}$ is the absorbance at 600 nm of the reaction mixture before incubation with the enzyme, and $Absorbance_{tf}$ is the absorbance at 600 nm of the reaction mixture after incubation.

Effect of effluent concentration on decolourization by laccase-natural mediator system

The effect of effluent concentration at 10, 20, or 40% in the reaction mixture, along with 5 U/ml laccase and 0.2 mM of syringaldehyde, vanillin, acetosyringone, syringate, vanillate, *o*-, *p*-, and *m*-coumarate as laccase mediators in 100 mM acetate buffer pH 5 on decolourization was studied in an initial experiment. The reaction incubated for 8 h in the dark at 30°C.

Effect of natural mediators on effluent decolourization

The effect of *m*-coumarate, syringaldehyde, vanillin, and acetosyringone was tested at 0.5 mM with 1 U/ml laccase and 20% of crude effluent in 100 mM acetate buffer pH 5. The reaction incubated for 6 h in the dark at 30°C. The decolourization of the effluent was followed by recording the spectra of the reaction mixture (between 230 and 800 nm) or by measuring the OD at 600 nm after 6 h of incubation.

Effect of natural mediators on the stability of laccase

Stability of laccase was examined in different concentrations of *m*-coumarate, siryngaldehyde, vanillin, and acetosyringone mediator: 0.05; 0.1; 0.5; 1; 2 and 3 mmol/L.

The initial laccase activity used was 1 U/ml. Unless otherwise indicated, assays were performed in 100 mmol/L acetate buffer pH 5 in 1.5 ml final reaction volumes in Eppendorf tubes. The reaction was initiated by the addition of laccase and incubation in the dark at 30°C. The reaction mixture comprised 100 mmol/L acetate buffer pH 5 and with an effluent concentration of 20%, effluent pH was adjusted to pH 5 prior to use.

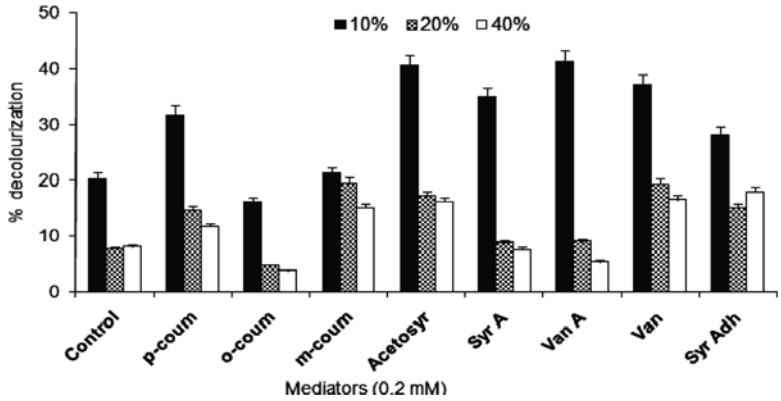

Fig. 1. Effect of laccase mediators on the decolourization of textile factory effluent at 10, 20, and 40% concentration by crude *T. trogii* laccase. p-coum, p-coumarate; m-coum, m-coumarate; o-coum, o-coumarate; acetosyr, acetosyringone; syr A, syringic acid; van A, vanillic acid; van, vanillin; syr Adh, syringaldehyde.

The stability of laccase was determined by measuring activity at a regular time intervals of 24 h. Relative activity was defined as:

$$\textit{Relative activity}\ (\%) = 100 \times \frac{\text{Activity}_t}{\text{Activity}_{t0}}$$

where Activity_{t0} is the activity of laccase at initiation of the reaction (t_0) and Activity_t is the laccase activity after incubation.

Effect of raw and pre-treatment effluent by laccase and system laccase-acetosyringone on the growth of fungi

The *T. trogii* growth was examined on solid medium (18 g/L agar-agar) containing different ratio effluent:water (0:100, 20:80, 40:60, 60:40, 80:20, and 100:0). Raw effluent, effluents treated between 0 and 8 h before incorporating into the culture medium with 5 U/ml crude laccase and with 5 U/ml laccase-0.5 mM^{-1} acetosyringone system, were tested for their effect on the growth of *T. trogii*. Growth was determined by measuring radial growth of the fungi in Petri dishes over 14 days incubation at 30°C.

Results and Discussion

Effect of effluent concentration on the decolourization by laccase-natural mediator system

In previous study (Khlifi *et al.*, 2010), we tested the effect of effluent concentration on their decolourization by laccase-HBT system and we showed that high effluent concentration decreased the decolourization rates (Khlifi *et al.*, 2010). However, the effect of effluent concentration on their decolourization by laccase-natural mediators system was not examined. Eight compounds (m-, o-, and p-coumarate, syringate, vanillate, vanillin, syringaldehyde, and acetosyringone), at 0.2 mM concentration, were screened as natural mediators for the decolourization of effluent by 5 U/L of *T. trogii* crude laccase. Effluent concentration affected the efficiency of colour removal. The laccase-mediator system tested here decolourized the effluent between 17–42%, 4.5–20% and 4–18% at the effluent concentrations of 10%, 20%, and 40% respectively, over 8 h incubation period (Fig. 1). This weak decolourisation rate may result from inhibition of the enzyme by excess of effluent. Colour removal efficiency also decreased when increasing concentrations of cotton bleaching effluent (Zhang *et al.*, 1999). Young and Yu (1997) and Maalej-Kammoun *et al.* (2009) also reported that high dye concentration decreased decolourization rate.

Effect of natural mediators on effluent decolourization

In this study, some aromatic compounds (*m*-coumarate, syringaldehyde, vanillin, and acetosyringone) were used as mediators in order to determine their effect on decolourization of the wastewater. The effect of the mediators on the decolourisation of the effluent was studied in presence of 1 U/ml laccase and 0.5 mM of the phenolic compound. Effluent was incubated in the presence of crude *T. trogii* laccase and laccase-mediator and the spectra of the reaction mixture were recorded after 6 h. Figure 2A shows the visible absorbance spectrum for effluent before and after treatment. A maximal absorbance was seen at 600 nm and this peak decreased with time, which is associated with oxidation of

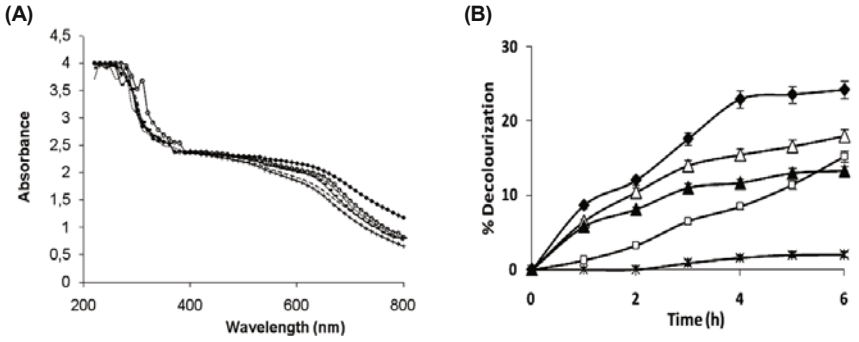

Fig. 2. Visible absorbance spectra of raw and treated effluent with crude laccase and system laccase-mediator (A): untreated effluent (◆), effluent treated with laccase (●), effluent treated with laccase-m-coumarate (...), effluent treated with laccase-vanillin (-), effluent treated with laccase-syringaldehyde (---), effluent treated with laccase-acetosyringone (+). Effect of mediators on effluent decolourization by crude laccase (B): *m*-coumarate (□), syringaldehyde (△), acetosyringone (◆), vanillin (▲) and no mediator (∗).

the effluent. Figure 2B shows that acetosyringone allows the best decolourization (25%) of the wastewater. The decolourization with m-coumarate, syringaldehyde, and vanilline was low and did not exceed 18%. Generally, the efficiency of these natural mediators differs according to the composition of the effluent, the nature and structure of the dye. Several studies on dye decolourization with laccase in the presence of naturally-occurring phenolic mediators of natural origin have been published recently.

Camarero *et al.* (2007) showed that, natural phenols as syringaldehyde and acetosyringone are promising laccase mediators, resulting in 25% lignin removal. Neifar *et al.* (2011) tested acetosyringone and a synthetic redox mediator ABTS and showed that highest decolourization yield after 1h of incubation of 29% was obtained by acetosyringone versus 21% obtained by ABTS. Acetosyringone and syringaldehyde, both dimethoxy substituted phenols derived from syringyl lignin units, where described as the fastest and most efficient laccase mediators, providing dye decolorization rates higher than those obtained with the powerful HBT mediator or other synthetic and natural mediators (Campos *et al.*, 2001; Camarero *et al.*, 2005; Dube *et al.*, 2008; Murugesan *et al.*, 2009). However, Khlifi *et al.* (2010) showed that HBT produce the strongest decolourization rate of effluent, >50% in 6 h by the *T. trogii* laccase and the decolourization increase with HBT concentration. These results corroborate those of Soares *et al.* (2001), in which higher HBT concentrations (up to 0.15%) led to higher decolourization rates of the lignin analogue dye Remazol Brilliant Blue R by a commercial laccase; concentrations above this value were inhibitory to the enzyme. Earlier Wong and Yu (1999) proposed a mechanism for increasing the decolourization capacity of *T. versicolor* laccase that involves the decolourization of non-substrate dyes in effluents via the substrate dyes acting as mediators in the laccase catalytic cycle. Therefore, several reports described the necessity of redox mediators (Soares *et al.*, 2001; Camarero *et al.*, 2005; Palmieri *et al.*, 2005; Khlifi *et al.*, 2010). Contrarily, Michniewicz *et al.* (2008) showed that efficient decolourization of textile dyes by laccase from *Cerrena unicolor* was achieved without any mediator.

In previous studies, some phenols, including syringaldehyde and acetosyringone, have been described as laccase mediators for indigo decolourization (Campos *et al.*, 2001) as well as for the transformation of the fungicide cyprodinil (Kang *et al.*, 2002). A comprehensive screening for natural mediators was performed (Camarero *et al.*, 2005). Among 44 tested natural lignin-derived compounds, 10 selected phenolic compounds derived from syringyl, guaiacyl, and p-hydroxyphenyl lignin units, characterized by the presence of two, one or no methoxy substituents, respectively (in *ortho* positions with respect to the phenolic hydroxyl). Syringaldehyde, acetosyringone, vanillin, acetovanillone, methyl vanillate, and *p*-coumaric acid have been found to be the most effective for mediated oxidation using laccases of *P.cinnabarinus* and *T. villosa*. Among them, syringaldehyde and acetosyringone belong to the main products of both biological and enzymatic degradation of syringyl-rich lignin (Kirk and Farrell, 1987).

Effect of natural mediators on the stability of laccase

In previous study (Khlifi *et al.*, 2009), it was shown that in the presence of 20% industrial effluent and 100 mmol/L acetate buffer pH5 laccase was stable within 5 days of incubation at 30°C (Khlifi *et al.*, 2009). Therefore, it would be of great importance to know the effect of the presence of such natural mediator on the stability of laccase in the textile wastewaters decolourization process. For this reason, the stability of *T. trogii* laccase in presence of natural mediators (acetosyringone, syringaldehyde, vanillin, and *m*-coumarate) was studied.

Figure 3A shows that in the presence of 0.05 and 0.1 mM acetosyringone laccase activity was stable within 5 days of incubation. However, with higher concentrations of acetosyringone, the relative activity of laccase decreased between 38% and 88% approximately, after 5 days of incubation.

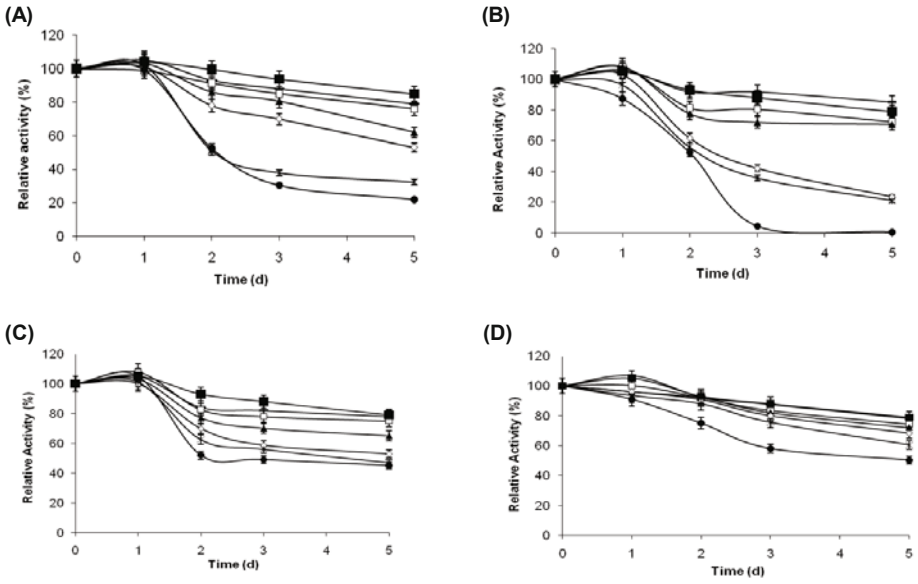

Fig. 3. Effect of natural mediator concentrations on the laccase stability (with effluent and with acetate buffer), 0 mM (■), 0.05 mM (-), 0.1 mM (□), 0.5 mM (▲), 1 mM (◇), 2 mM (∗), 3 mM (●). Acetosyringone (A), syringaldehyde (B), vanillin (C) and *m*-coumarate (D).

In the presence of 3 mM syringaldehyde, *T. trogii* laccase was strongly inactivated after 3 days of incubation; higher concentration caused faster inactivation of the enzyme. However, at 1 mM and 2 mM of syringaldehyde concentration, the relative activity of laccase decreased by 77% and 79% respectively. Moreover, at low concentration (0.05, 0.1, and 0.5 mM) the relative activity of laccase decreased by 15%, 28%, and 30% after 5 days of incubation (Fig. 3B).

Laccase activity was more stable in the presence of the vanillin and m-coumarate. It lost approximately 50% of its activity within 3 mM mediator (Fig. 3C). This difference of stability could be explained by the nature of the formed radicals by the laccase from the various mediators.

In this work, inactivation was only observed with syringaldehyde (Fig. 3B); in fact, acetosyringone, vanillin and *m*-coumarate (Figs. 3A, 3C, and 3D, respectively) had the opposite effect (stabilizing the enzyme) in the textile wastewater decolourization process. Probably as a result of a lower reactivity in their radicals. A similar stabilizing effect was previously observed by Aracri *et al.* (2009), who found some natural mediators to prevent denaturation of *T. villosa* laccase. Moreover, Mai *et al.* (2000) found some phenolic compounds acting as enzyme substrates to stabilize laccase by binding to the active sites or suitable points in the protein chain of the enzyme. In addition, Fillat and Roncero (2010) found that both acetosyringone and syringaldehyde stabilise *P. cinnabarinus* laccase. Contrarily, we have observed that laccase was totally inhibited by syringaldehyde. In a recent study (Fillat *et al.*, 2010), the phenols syringaldehyde, acetosyringone and *p*-coumaric acid were used as natural laccase mediators in combination with laccase of *P. cinnabarinus* to bleach flax fibres. Their performance was compared with HBT in terms of enzyme stability, and pulp and effluent properties. HBT and *p*-coumaric acid were found to inactivate laccase in the absence of pulp. However, in the presence of unbleached flax pulp stability was increased for example with *p*-coumaric acid, laccase retained 77% of its initial activity, in contrast with complete inactivation in the absence of pulp. This suggests a protective effect of the pulp against the enzyme denaturation (Fillat *et al.*, 2010).

The natural mediators such as syringaldehyde and vanillin have similar structures, only differentiated by methoxylation of the aromatic ring: syringaldehyde has two methoxy groups in *ortho* position to the phenol group, while vanillin has only one methoxy group and thus a more sterically accessible phenolic group. Both compounds were already tested as mediators in decolourisation of dyes showing promising results (Camarero *et al.*, 2005). The advantage of the mediators, besides of acting as an electron shuttle between the enzyme and the substrates, is that they may follow oxidation pathway different from the enzymatic one. Laccase are copper-containing enzymes that catalyse the oxidation of electron-rich substrates such as phenols. The limiting step in the oxidation of phenols by laccase is the first electron transfer from the substrate to T1 copper, a reaction that is mainly governed by differences in redox potential between the phenol and the enzyme (Xu, 1996). The electron donor effect of methoxy substituents at the benzenic ring enhances laccase activity due to a decreased redox potential. In this way, acetosyringone and syringaldehyde (two S-type compounds) were rapidly oxidized by laccase than the G-type phenols (vanillin) and were also found to be the most rapid and efficient enzyme mediators (Camarero *et al.*, 2005). This was due to other factors influencing the oxidation rate by laccase, especially the pKa, since phenol oxidation to a phenoxy radical is favoured by the presence of the phenolate form (d'Acunzo and Galli, 2003). In this way, syringaldehyde and acetosyringone are more easily oxidized by laccase than vanillin due to their lower redox potentials (Camarero *et al.*, 2005). Indeed, the redox potentials of syringaldehyde, acetosyringone and vanillin were 0.66, 0.60, and 0.81V respectively (Fernandez-Sanchez *et al.*, 2002; Zheng *et al.*, 2010; Moodley *et al.*, 2011). Laccase from *T. trogii* was reported to have one of the highest redox potential among laccases (E 0.79 at pH 5.0) (Garzillo *et al.*, 2001), which makes this laccase particularly interesting since high redox potentials correlate with high laccase activity (Li *et al.*, 1999).

Some synthetic mediators such as HBT are known to degrade rapidly, and attack laccase diminishing its catalytic activity as a result in some cases (Sigoillot *et al.*, 2005; Aracri *et al.*, 2009; Fillat and Roncero, 2009a, 2009b). Moreover, the decolourization of the textile effluent was increased with the addition of this synthetic mediator (Khlifi *et al.*, 2010). These chemicals should be avoided because they could probably be a supplementary pollutant in the wastewaters. In a previous study (Khlifi *et al.*, 2010), we observed that decolourization textile effluent with laccase-HBT system gave more toxic effluent than that treated with crude laccase. Furthermore, they found that at HBT concentrations of 1–3 mmol/L, laccase activity was totally inhibited within 48 h of reaction initiation. Overall decolourization was not affected, however, as it occurred within the 48 h period, before enzyme inactivation (Khlifi *et al.*, 2009). Therefore, major constraint for industrial application is their high price and toxic properties. Moreover, the enzyme can be denaturized by the generated active radicals (Li *et al.*, 1998). In the presence of HBT, reactive compounds produce free radicals (d'Acunzo *et al.*, 2002; Fabbrini *et al.*, 2002a, 2002b). The reaction mixture, therefore, will contain significant quantities of reactive species, including free radicals. Given that the mediators had significant impacts on the stability of the enzyme, this evidence supports the hypothesis that laccase is subject to attack by free radicals. Thus, in a reacting system, where free-radical products are generated continuously either through the transformation of a substrate or the continuous cycling of mediators, free radicals may play a critical role in laccase inactivation.

Collectively, these results suggest that mediators enhance the inactivation of laccase. The use of higher concentrations of mediators may promote more rapid reactions, but can also cause higher degree of enzyme inactivation. It is important, therefore, to limit mediator concentrations in reaction systems in order to maintain catalytic stability. This factor will be particularly important in applications requiring long-term stability of a laccase-mediator system. The search of natural compounds as new and environmentally safe mediators gained a lot of interest years (Camarero *et al.*, 2005, 2007). Perhaps, natural mediators such as acetosyringone are more

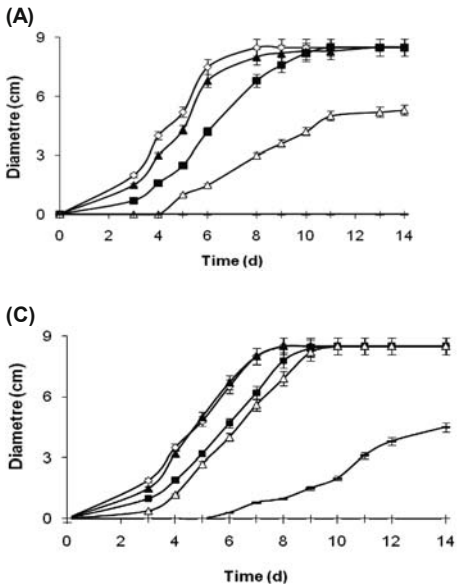

Fig. 4. Effect of raw effluent (A) and pre-treatment of effluent by laccase (B) and system laccase-acetosyringone (C) on the growth of fungi. 0% (◇), 20% (▲), 40% (■), 60% (△), 80% (-), and 100% (+).

efficient at the stability of laccase than HBT. At 1-3 mmol/L HBT concentrations, laccase activity was totally inhibited within 48 h of reaction initiation (Khlifi *et al.*, 2009). Moreover, natural mediators (such as acetosyringone) are more sustainable approach and even may lead to further to detoxification (Khlifi *et al.*, 2010). However, *T. trogii* laccase-acetosyringone represents a potential single step to decolourize and detoxify the textile effluent and to stabilise laccase.

Effect of the pre-treatment of the textile effluent by laccase and laccase-acetosyringone system on the growth of fungi

Textile effluent can be of high complexity, containing a mixture of auxiliaries, salts, surfactants, degradation products, metals and unknown components of refractory pollutants. Raw, crude laccase and laccase-acetosyringone-system pre-treated textile effluents have been used as basis of solid media to test the effect of the laccase pre-treatment on the fungal growth. Figs. 4A, 4B, and 4C shows that, medium composed of 100% of treated or not treated effluent inhibited *T. trogii* growth completely. However, in 80:20 pre-treated effluent with laccase or laccase-acetosyringone system: water, *T. trogii* grew after 7 and 5 days of incubation at 30°C, respectively (Figs. 4B and 4C). In 60:40 raw effluent:water, an acceptable growth was obtained after 11 days of incubation (Fig. 4A). While this same growth of *T. trogii* was obtained in 60:40 pre-treated effluent with laccase or laccase-acetosyringone system:water, after 7 days of incubation (Figs. 4B and 4C). These results showed that the treatment by laccase detoxifies the textile effluent and that this detoxification by laccase is more important in presence of a natural mediator. Moreover, in previous study (Khlifi *et al.*, 2010), we demonstrated that treatment of textile industry effluent with the laccase-acetosyringone system reduced the toxicity of raw effluent and, based on the parameters suggested by Joutti *et al.* (2000), even detoxified it. These observations suggest that the products from acetosyringone oxidation of textile effluents are not toxic to *V. fischeri* and appear to contribute to a considerable detoxification of the effluent. Also, Zouari-Mechichi *et al.* (2006) found that *T. trogii* growth well in the plates and decolourize the dyes, Neolane yellow, Neolane pink, and Bezaktiv S-BF turquoise but slow growth was obtained on the plates containing Basacryl yellow and Maxilon blue and no decolourization was obtained for these dyes. Moreover, Maalej-Kammoun *et al.* (2009) observed that *Trametes* sp. grew on agar containing laccase-treated malachite green.

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