ScienceDirect

Enzyme and Microbial Technology 40 (2007) 1748–1752

MICROBIAL TECHNOLOGY

www.elsevier.com/locate/emt

Enzymatic synthesis of Tinuvin

M. Schroeder [a], L. Pereira [a], Susana Rodríguez Couto [b], A. Erlacher [a], K.-U. Schoening [c], A. Cavaco-Paulo [d], G.M. Guebitz [a,*]

[a] *Department of Environmental Biotechnology, Graz University of Technology, Petersgasse 12, A-8010 Graz, Austria*
[b] *Department of Chemical Engineering, Rovira i Virgili University, Tarragona, Spain*
[c] *Ciba Specialty Chemicals, CH-4002 Basel, Switzerland*
[d] *Textile Engineering Department, University of Minho, Guimarães, Portugal*

Received 16 October 2006; received in revised form 27 November 2006; accepted 27 November 2006

Abstract

Coupling of 3-(3-*tert*-butyl-4-hydroxyphenyl) propionic acid methylester to 1*H*-benzotriazole using a laccase from *Trametes hirsuta* was studied. The potentially resulting coupling product Tinuvin 1130 is an important UV-absorber used in polymer based materials. Oxidation of the phenol by the laccase led to homomolecular coupling reactions while the laccase did not attack 1*H*-benzotriazole. Due to the homomolecular reaction of the phenol in the presence of laccase coupling of phenol and 1*H*-benzotriazole was only observed when 1*H*-benzotriazole was applied in four-fold molar excess. The reaction was monitored by UV/vis spectroscopy, TLC and MS (ion trap) analysis. Coupling of 1*H*-benzotriazole took place in *ortho* position according to the postulated mechanism.

Keywords: Laccase; Oxidative coupling; Tinuvin; *Trametes hirsuta*; Biotransformation

1. Introduction

Benzotriazol based UV-absorbers such as Tinuvin are a very important class of photo stabilisers. They play a key role in prolonging the lifetime of polymers and paintings. For several years such UV-absorbers are applied in the textile industry stabilising wool textiles against light and harmful gases [1–3].

Laccases are copper-containing polyphenol oxidases which oxidize polyphenols, methoxy-substituted phenols, diamines and a considerable range of other compounds using molecular oxygen as an electron acceptor. When used in combination with mediators, the substrate spectrum of laccases can be broadened to include compounds which are no laccase substrates on their own [4]. Laccases are widely studied enzymes due to their potential use in several areas such as textile [5], food [6], pulp and paper industries [7]. In bioremediation laccases as well as immobilized laccases have been used for the treatment of phenolic effluents [8], PAHs [9] and PCBs [10]. The enzymes render phenolic compounds less toxic via degradation or polymerisation reactions and/or cross-coupling of pollutant phenols with naturally occurring phenols [11]. Only few reactions for organic synthesis have been reported using laccase induced oxidation or polymerisation [12]. These include the synthesis of aromatic aldehydes [13], substituted imidazoles [14] and 3-(3,4-dihydroxyphenyl)-propionic acid derivates [15] as well as cross-coupling of 4-aminobenzoic acid with *para*-dihydroxylated compounds [16] and enzymatic oxidation of alkenes [17].

Laccase catalysed organic syntheses exploit the high specificity of enzymes and environmental friendly reaction conditions. In this study, a laccase from *Trametes hirsuta* was assessed for its ability to catalyse the coupling of 3-(3-*tert*-butyl-4-hydroxyphenyl) propionic acid methylester with 1*H*-benzotriazole. The reaction was monitored by TLC and MS–MS analysis and reaction conditions for the production of Tinuvin were investigated. Prior to this different phenols were screened for their potential for Tinuvin synthesis.

2. Materials and methods

2.1. Chemicals and enzymes

2,2-Azinobis-(3-etylbenzothiazoline-6-disulfonic acid) (ABTS) was obtained from Sigma and Coomassie Brilliant Blue was purchased by BioRad (Hercules, CA, USA). All other chemicals and solvents were

* Corresponding author. Tel.: +43 316 873 8312; fax: +43 316 873 8819.
E-mail address: guebitz@tugraz.at (G.M. Guebitz).

0141-0229/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2006.11.026

of analytical grade. 2,4-Di *tert* amylphenol (**A**), 4-*tert* octylphenol (**B**), 3-(3-*tert*-butyl-4-hydroxyphenyl) propionic acid methylester (**C**), its dimer (**C_2**) and 3-(3-benzotriazol-2-yl-5-*tert*-butyl-4-hydroxy-phenyl)-propionic acid methylester (Tinuvin 1130; **D**) were supplied by CIBA while 1*H*-benzotriazole (**E**) was purchased from Sigma.

T. hirsuta (IMA2002) is deposited at the culture collection of the Department of Environmental Biotechnology, TU-Graz, Austria. Growth conditions and the purification of the laccase are described elsewhere [18]. Briefly, the 62 kDa laccase was purified by ultra filtration (10 kDa), anion exchange and gel filtration chromatography.

2.2. Laccase activity and protein assay

Laccase activity was determined using 2,2-azinobis-(3-etylbenzothiazoline-6-disulfonic acid) as substrate. Fifty microlitres of the sample were mixed with 700 μL of a 5 mM ABTS solution in succinate buffer (25 mM, pH 4.5). The increase in absorbance at 436 nm due to the formation of a green radical was followed, using a Hitachi U-2001 spectrometer. For rapid identification, samples of 50 μL were mixed in microtiter plates with 50 μL ABTS.

2.3. HPLC analysis

The HPLC equipment used was a DIONEX P-580 PUMP (Dionex Cooperation, Sunnyvale, USA), with an ASI-100 automated sample injector and a PDA-100 photodiode array detector.

For analysis of the different phenols, the corresponding Tinuvins and the reaction products a reversed phase column RP-C8 (Zorbax 5RPC ST 4.6/150 Agilent Paulo Alto, USA) was used. Separation was achieved applying a linear gradient starting with 60% acetonitrile and 40% water to reach a final concentration of 90% acetonitrile as eluent. A flux of 1 mL min^{-1} and a temperature of 40 °C were adjusted.

2.4. Laccase induced coupling

Preliminary studies were performed in a 100 mL Erlenmeyer flask in 10 mL of acetate buffer (pH 4.5) and after dilution in 60% (v/v) acetonitrile in water, the reaction mixtures were analysed by HPLC as described above.

Further studies were elaborated in a 1 cm quartz cuvette (total volume of 3 mL) in acetate buffer (pH 4.5) and reactions were monitored in a UV/vis spectrophotometer (HitachiU-2001) by taking the spectra in the range of 205–800 nm for 1 h. Oxidation of 1.0 g L^{-1} **C** and **E** (concentration of 4 and 8 mM, respectively) by laccase (5 nkat mL^{-1}) separately and the mixture of both was carried out. The individual spectra of **C**, **D** and **E** were also recorded for comparison (Fig. 1). Calibration curves were generated from the stock solutions of **C** and **E** dissolved in the ranges of linearity in hexane and water, respectively.

Coupling reactions were performed in 250 mL Erlenmeyer flasks (total volume of 50 mL) in the conditions described above. The reactions were monitored in a UV/vis spectrophotometer at different times and the reaction mixture was extracted with hexane and spotted on TLC plates (Silica gel, 10 × 20 cm, fluorescent indicator at 254 nm, Fluka) and a mixture of hexane–acetone of 75% (v/v) as eluent.

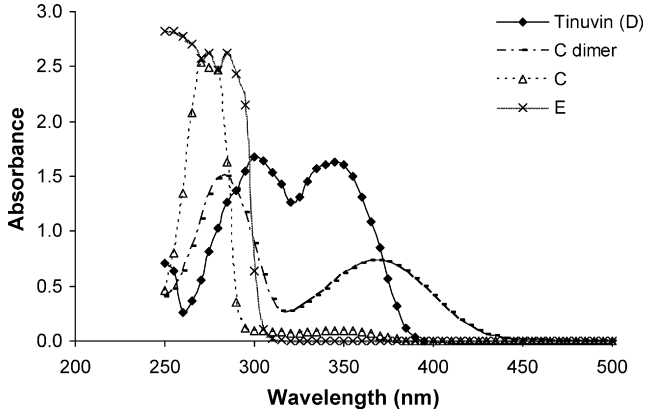

Fig. 1. Spectrum of the different reagents and standards.

Table 1
Screening of different phenols for coupling with 1*H*-benzotriazole by a laccase from *T. hirsuta*

	Phenol	Corresponding Tinuvin	Turnover of phenol (%)	Tinuvin
A	2,4-Di *tert* amylphenol	Tinuvin 328	99	None
B	4-*tert* Octylphenol	Tinuvin 327	<4.5	None
C	3-(3-*tert*-Butyl-4-hydroxyphenyl) propionic acid methylester	Tinuvin	>90	5.1%

2.5. Investigation of reaction conditions

In order to direct the reaction towards coupling of **C** and **E** different conditions were tested. Thereby different factors such as pH value (pH 2 to 6.5), incubation time (30 min to 12 h), laccase activity and molar ratio of the reactants (1, 2, 4 and 10) were elaborated to find best reaction conditions for coupling (Table 1).

2.6. Purification of the reaction products and characterisation using MS

The reaction mixture was extracted with hexane and concentrated under reduced pressure prior to purification by silica gel column chromatography (working volume of 1.5 L). Equilibration of the column with the mixture of hexane–acetone (75/25%, v/v, respectively) was performed three times. Twenty millilitres of the sample were applied and 2 L fractions were collected. Fraction containing the desired reaction product according to TLC was analysed by UV/vis spectroscopy and mass spectroscopy using an Agilent SL ion trap mass spectrometer (LC/MSD ion trap SL) in direct infusion mode. Atmospheric pressure electrospray ionization (API-ES) source was used in a negative or positive ionization mode and solvents were 25/75% (v/v) H_2O/MetOH with 0.1% (v/v) formic acid and 5 mM NH_4HCO_3, respectively.

Operating conditions were: dry temperature 325 °C, capillary voltage 3500 V, nebulizer 15 psi and dry gas helium 5.0 L min^{-1}. Ion trap full scan analyses were conducted from *m/z* 50 to 2200 with an upper fill time of 300 ms. For the MS–MS, the selected precursor ion was: *m/z* 354.4 (Tinuvin). Complete system control and data evaluations were done with Data Analysis (Agilent) for MS.

3. Results

3.1. Chromatographic analysis and calibration

For all different reactions a good separation of the phenols from their corresponding reaction products was achieved. Calibration curve showed accurate linearity within the range of applied substrate concentration (see Fig. 2). For the enzymatic reaction the interpretation was more complicated. Since one or more side products were detected but no standard was available not all peaks could be identified.

3.2. Laccase induced coupling

The aim of the first reactions performed directly in the spectrophotometer cuvette was to observe the effect of the enzyme on the reagents. Further, the reactions of **C** (3-(3-*tert*-butyl-

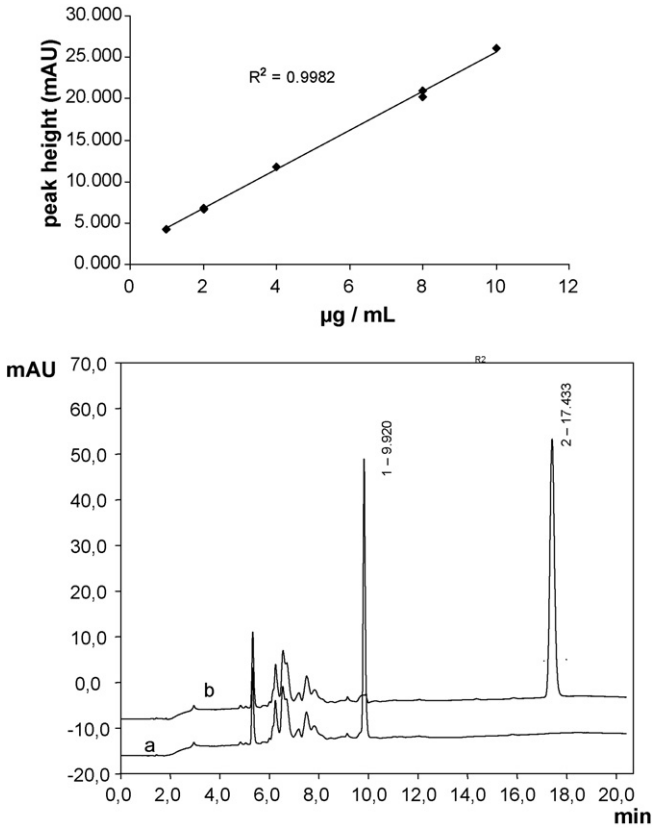

Fig. 2. Calibration (above) and HPLC-chromatograms (line a) of 4-*tert* octylphenol (**B**) and (line b) of Tinuvin 328.

4-hydroxyphenyl) propionic acid methylester) were performed directly in the spectrophotometer cuvette. The phenolic compound (**C**) appeared to be oxidized by the laccase and an increase of absorbance in the full spectrum, more intensively in the UV region was clearly seen. With time course, a dislocation from 275 to 290 nm was achieved being the highest peak after 20 min at 290 nm due to possible dimerisation of **C**. No changes on the spectrum with time were seen for compound **E** (1*H*-benzotriazole) when incubated with laccase.

The reaction involving the laccase from *T. hirsuta* and both substrates **C** and **E** (in two-fold molar excess) resulted in a spectrum different from that of the initial compounds and different from that of **A** and laccase indicating a reaction between both reagents **C** and **E**. An increase in the UV region after 20 min was again obtained with peaks at 320, 350 which are in the same region like those obtained for Tinuvin.

On TLC, **C**, **D**, **E** and the dimer of **C** were used as standards. In the reaction mixtures remaining benzotriazol was only found in the aqueous phase while the other substances were in the organic phase (hexane). A TLC after enzymatic reaction is presented in Fig. 3. The solutions, where reaction had occurred, appeared as a yellow turbid liquid and, sometimes, a precipitate was formed, which can be due to the fact that the products formed were little soluble in water. Different phenols were tested for their potential as precursor in enzymatic Tinuvin synthesis. Only for **C** a Tinuvin formation could be detected. Interestingly, the phenol **B** was not affected by the laccase at all (Table 1).

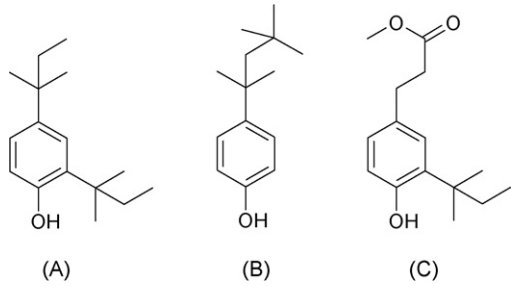

Fig. 3. Phenolic compounds used for laccase induced coupling reactions: (**A**) 2,4-di *tert* amylphenol, (**B**) 4-*tert* octylphenol and (**C**) 3-(3-*tert*-butyl-4-hydroxyphenyl) propionic acid methylester.

3.3. Investigation of reaction conditions

Various different factors such as pH-value, incubation time, molar ratio of the reactants were elaborated to find best reaction conditions for coupling of **C** and **E**. In order to reduce dimerisation of **C**, a molar excess (different levels) of **E** was used. A four-fold excess of **E** was necessary for coupling of **C** and **E** to take place while higher concentrations of **E** did not further improve the reaction (Table 2).

With the exception of pH 2.0, the result on TLC plates was the same in all cases and spots with similar rf as Tinuvin and to **C** were developed, more concentrated for the last. Reaction at pH 3.5 and 4.5 was repeated and samples were taken at different time intervals. The amount of **E** increased until an incubation time of 60 min was reached and remained constant thereafter (Fig. 4).

Furthermore, the reaction was performed in the presence of 0.10 μM ABTS, a well-known laccase mediator (Table 2). However, the mediated reaction went into a different direction (data not shown). After 1 min two peaks, at 410 and 345 nm, respectively, were formed, and decreased again after 10 min. The increase on the visible part may be from the oxidation of ABTS. No indication for the formation of **C** in the presence of ABTS was obtained in TLC analysis.

3.4. Purification and characterisation using MS

Chromatographic purification of the reaction mixture (sample 3, Table 1) was performed in order to conclusively identify products. A good resolution was achieved and five different fractions were separated. Thereof the main fraction (sample 3-A) was related to Tinuvin due to the same rf value on the TLC as the standard.

Table 2
Coupling of 3-(3-*tert*-butyl-4-hydroxyphenyl) propionic acid methylester (**A**) and 1*H*-benzotriazole (**B**) by a laccase from *T. hirsuta* (5 nkat mL^{-1}) at different reaction conditions (incubation time: 60 min)

Sample	Concentration (mM)		pH	Mediator	Yield of Tinuvin (%)
	A	**B**			
1	0.7	0.7	4.5	None	0.4
2	0.7	2.8	4.5	None	5.1
3	0.7	2.8	3.5	None	4.3
4	0.7	2.8	4.5	0.10 μM ABTS	n.d.

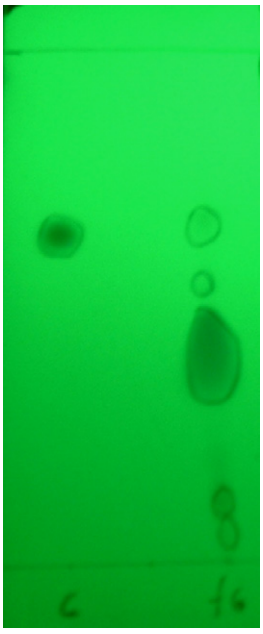

Fig. 4. TLC monitoring of enzymatic synthesis of Tinuvin: left lane standard Tinuvin (**D**); right lane: the reaction **C** and **E** in the presence of laccase.

Using mass spectroscopy, this fraction (3-A), the standards **C**, **D** and **E**, and the homomolecular reaction product of **C** with laccase were analysed. 1*H*-benzotriazole (**E**) was identified in positive ion mode (*m*/*z* 120.2 $[M+H]^+$) while 3-(3-*tert*-butyl-4-hydroxyphenyl) propionic acid methylester (**C**) was identified in negative ion mode (*m*/*z* 235.2 $[M–H]^-$). Tinuvin was analysed in positive ion mode at *m*/*z* 354.2 $[M+H]^+$ which was identical to the peak seen in the sample. MS^2 of both Tinuvin and the sample yielded fragment ions with *m*/*z* 266 $[M+H]^+$ (loss of propionic acid methyl ester), *m*/*z* 280 $[M+H]^+$ (loss of acetic acid methyl ester), *m*/*z* 298 $[M+H]^+$ (loss of the *tert*-butyl substituent), *m*/*z* 224 $[M+H]^+$ (loss of the *tert*-butyl substituent and acetic acid methyl ester) and *m*/*z* 323 $[M+H]^+$ (loss of methyl groups from the *tert*-butyl substituent).

4. Discussion

4.1. Laccase induced coupling

An initial screening using spectroscopic monitoring indicated the potential of a fungal laccase from *T. hirsuta* for coupling of 3-(3-*tert*-butyl-4-hydroxyphenyl) propionic acid methylester (**C**) to 1*H*-benzotriazole (**E**) yielding potentially the UV-absorber Tinuvin. The phenol compound **C** was oxidized by the laccase which resulted in dimerisation and other coupling products. Homolytic crosslinking is a common reaction used in different laccase activity assays measuring the rate of oxidative coupling reactions from substituted phenols such as dimethoxyphenol DMP to 3,3′,5,5′-tetramethoxydiphenylquinone [19]. Using laccase [15] coupled 3-(3,4-dihydroxyphenyl)-propionic acid to 4-aminobenzoic acid yielding 3-[6-(4-carboxyphenyl)amino-3,4-dihydroxyphenyl]-propionic acid. Like in our study for compound **C**, homomolecular coupling of 3-(3,4-dihydroxyphenyl)-propionic acid methylester was observed as unwanted reaction. Interestingly, homomolecular coupling decreased when hexylamine was used as a co-substrate. These authors hypothesize that the products are formed by a $R–NH_2$ attack of a cation radical of 3-(3,4-dihydroxyphenyl)-propionic acid.

1*H*-benzotriazole (**E**) was not a laccase substrate and no changes on the spectrum with time were seen. This result was expected since the compound **E** is already an oxidized product from 1-hydroxybenzotriazol (HOBT). To avoid laccase catalysed dimerisation of **A** the reaction was carried out with excess of **E**. In agreement with our results Niku-Paavola and Viikari reported that in laccase catalysed oxidation of organic molecules reaction conditions play a major role [17]. These authors studied

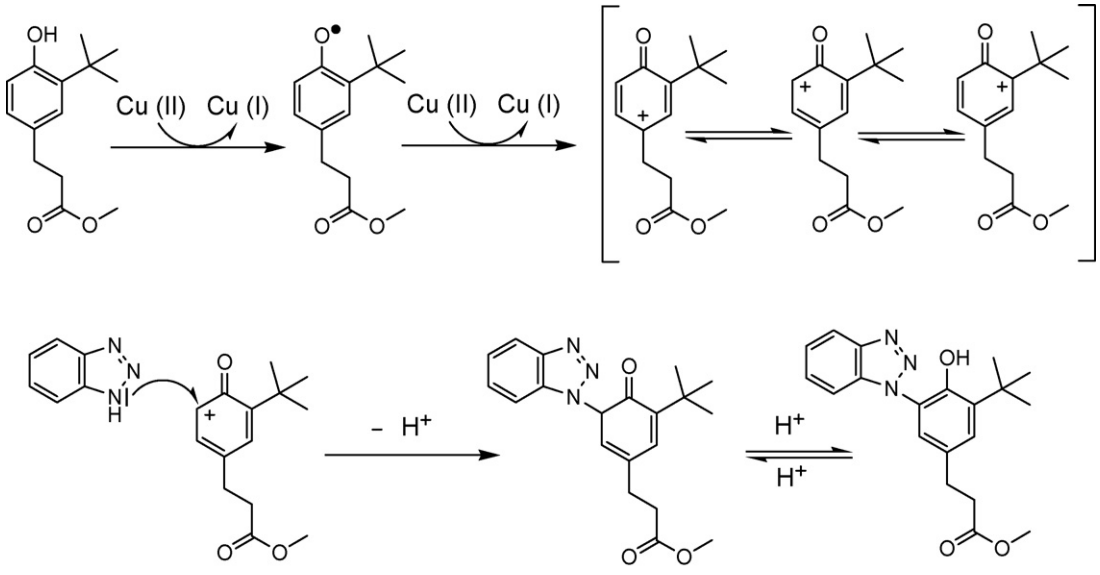

Fig. 5. Proposed mechanism of the synthesis of Tinuvin by a catalysed laccase reaction.

the oxidation of various alkenes and found that the nature of products formed depended strongly on temperature, incubation time, mediator, laccase and oxygen concentration.

Natural electron mediators secreted by fungi [20] and synthetic mediators [4] have been reported to expand the substrate range of laccases. In contrast to their definition we have recently shown with lignin model substrates that some mediators such as ABTS can even take part in the reaction being co-polymerised [21]. Thus, laccase catalysed oxidation reactions can follow different pathways in the presence of electron mediators as we have also experienced in this study. Other authors [22] have shown that the laccase/TEMPO system was able to oxidize primary and secondary benzyl alcohols and allyl alcohols into the corresponding aldehydes while tertiary benzyl alcohol was not accessible.

Due to the importance of pH on laccase catalyses, this parameter was studied performing the reactions at different values on the range of the laccase stability. The substrate specificity and affinity of laccase can vary with changes in pH and that makes necessary the study of a singular reaction with the pH [23]. Andreozzi et al. [24] concluded that benzotriazol in aqueous solution appears in two forms depending on the pH, and that the absorbance at 254 nm between pH 3.0 and pH 7.0 differs only 1%. The tautomer benzotriazolyl(sodium), a derivative less stable than **E** could be an intermediate to further reactions. Therefore, additional experiments were performed varying the ion strength. However, pH value and salt concentration did not show any significant impact.

Mass spectroscopy was used to identify the reaction product formed in laccase catalysed coupling of **C** and **E**. An identical fragmentation pattern proved the coupling of **C** and **E** while based on mechanistic considerations it seems very likely that coupling of 1*H*-benzotriazole took place at the free *ortho* position yielding Tinuvin. We hypothesize that in a first step two electrons are subsequently abstracted from the phenolic hydroxyl group from compound **C** leading to the structures shown in Fig. 5. In a second step, a nucleophilic attack of the nitrogen of **E** takes place most likely at the *ortho* position [16]. Coupling of both 1H-bentotriazol and 2H-benzotriazol would follow this reaction mechanism while due to higher stability the coupling of 1H-benzotriazol seems more likely. Concluding these results the potential of laccases to catalyse coupling reactions of phenolic substrates to H-benzotriazol has been demonstrated. However, further investigations with a wider range of phenolic substrates with different substiutents and enzymes with different redox potentials will be necessary to study mechanistic aspects and optimize coupling reactions towards higher yields.

Acknowledgement

We would like to thank the European Commission for financial support within the project Oxitex G1TR-CT-2000-00009.

References

[1] Schäfer A, Hocker H. Stabilizing wool textiles against light and harmful gases. Textilveredlung 2002;37:11–6.
[2] Crawford JC. 2(2-Hydroxyphenyl)2H-benzotriazole ultraviolet stabilizers. Prog Polym Sci 1999;24:7–43.
[3] Tsatsaroni EG, Eleftheriadis LC. UV-absorbers in the dyeing of polyester with disperse dyes. Dyes Pigm 2004;61:141–7.
[4] Thurston C. The structure and function of fungal laccase. Microbiology 1994;140:19–26.
[5] Galante YM, Formantici C. Enzyme applications in detergency and in manufacturing industries. Curr Org Chem 2003;7:1399–422.
[6] Minussi RC, Pastore GM, Durán N. Potential applications of laccase in the food industry. Trends Food Sci Technol 2002;13:205–16.
[7] Durán N, Esposito E. Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review. Appl Catal B: Environ 2001;28:83–99.
[8] D'Annibale A, Stazi SR, Vinciguerra V, Giovannozzi Sermanni G. Oxirane-immobilized *Lentinula edodes* laccase: stability and phenolics removal efficiency in olive mill wastewater. J Biotechnol 2000;77(2–3):265–73.
[9] Dodor DE, Hwang HM, Ekunwe SIN. Oxidation of anthracene and benzo[a]pyrene by immobilized laccase from *Trametes versicolor*. Enzyme Microb Technol 2004;35:210–7.
[10] Keum YS, Li QX. Fungal laccase-catalyzed degradation of hydroxy polychlorinated biphenyls. Chemosphere 2004;56:23–30.
[11] Nyanhongo GS, Gomes J, Gübitz GM, Zvauya R, Read JSA. Decolorization of textile dyes by laccases from a newly isolated strain of *Trametes modesta*. Water Res 2002;36:1449–56.
[12] Burton SG. Laccases and phenol oxidase in organic synthesis—a review. Curr Org Chem 2003;7:1317–31.
[13] Potthast A, Rosenau T, Chen CL, Gratzl JS. Selective enzymatic oxidation of aromatic methyl groups to aldehydes. J Org Chem 1995;60:4320–1.
[14] Schäfer A, Specht M, Hetzheim A, Francke W, Schauer F. Synthesis of substituted imidazoles and dimerization products using cells and laccase from *Trametes versicolor*. Tetrahedron 2001;57:7693–9.
[15] Mikolasch A, Hammer E, Jonas U, Popowski K, Stielow A, Schauer F. Synthesis of 3-(3,4-dihydroxyphenyl)-propionic acid derivates by N-coupling of amines using laccase. Tetrahedron 2002;58:7589–93.
[16] Manda K, Hammer E, Mikolasch A, Niedermayer T, Dec J, Jones AD, et al. Laccase-induced cross-coupling of 4-aminobenzoic acid with *para*-dihydroxylaed compounds 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide and 2.5-dihydroxybenzoic acid methyl ester. J Mol Catal B: Enzym 2005;41:86–92.
[17] Niku-Paavola ML, Viikari L. Enzymatic oxidation of alkenes. J Mol Catal B: Enzym 2000;10:435–44.
[18] Almansa E, Kandelbauer A, Pereira L, Cavaco P, Guebitz GM. Influence of structure on dye degradation with laccase mediator systems. Biocatal Biotransform 2004;22:315–24.
[19] Solano F, Lucas-Elio P, Lopez-Serrano D, Fernandez E, Sanchez-Amat A. Dimethoxyphenol oxidase activity of different microbial blue multicopper proteins. FEMS Microbiol Lett 2001;204:175–81.
[20] Temp U, Zierold U, Eggert C. Cloning and characterization of a second laccase gene from the lignin-degrading basidiomycete *Pycnoporus cinnabarinus*. GENE 1999;236:169–77.
[21] Rittstieg K, Suurnäkki A, Surnotti T, Kruus K, Guebitz GM, Buchert J. Polymerization of guaiacol and a phenolic β-*O*-4 substructure by *Trametes hirsuta* laccase in the presence of ABTS. Biotechnol Prog 2003;19:1505–9.
[22] Fabbrini M, Galli C, Gentili P. Comparing the catalytic efficiency of some mediators of laccase. J Mol Catal B: Enzym 2002;16:231–40.
[23] Xu F. Effects of redox potential and hydroxide inhibition on the pH activity profile of fungal laccase. J Biol Chem 1997;272:924–8.
[24] Andreozzi R, Caprio V, Insola A, Longo G. Photochemical degradation of benzotriazole in aqueous solution. J Chem 1998;73:93–8.