Journal of Biotechnology 139 (2009) 68–77

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Enzymatic biotransformation of the azo dye Sudan Orange G with bacterial CotA-laccase

Luciana Pereira[a], Ana V. Coelho[a], Cristina A. Viegas[b], Margarida M. Correia dos Santos[c], Maria Paula Robalo[c,d], Lígia O. Martins[a,*]

[a] Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. da República, 2780-157 Oeiras, Portugal
[b] IBB-Instituto de Biotecnologia e Bioengenharia, Centro de Engenharia Biológica e Química, Instituto Superior Técnico, Av Rovisco Pais, 1049-001 Lisboa, Portugal
[c] Centro de Química Estrutural, Instituto Superior Técnico, 1049-001 Lisboa, Portugal
[d] Departamento de Engenharia Química, Instituto Superior de Engenharia de Lisboa, Rua Cons. Emídio Navarro, 1, 1959-007 Lisboa, Portugal

ARTICLE INFO

Article history:
Received 14 April 2008
Received in revised form 8 September 2008
Accepted 16 September 2008

Keywords:
Laccase
Synthetic dyes
Biotransformation
Toxicity
Decolourisation

ABSTRACT

In the present study we show that recombinant bacterial CotA-laccase from *Bacillus subtilis* is able to decolourise, at alkaline pH and in the absence of redox mediators, a variety of structurally different synthetic dyes. The enzymatic biotransformation of the azo dye Sudan Orange G (SOG) was addressed in more detail following a multidisciplinary approach. Biotransformation proceeds in a broad span of temperatures (30–80 °C) and more than 98% of Sudan Orange G is decolourised within 7 h by using 1 U mL^{-1} of CotA-laccase at 37 °C. The bell-shape pH profile of the enzyme with an optimum at 8, is in agreement with the pH dependence of the dye oxidation imposed by its acid-basic behavior as measured by potentiometric and electrochemical experiments. Seven biotransformation products were identified using high-performance liquid chromatography and mass spectrometry and a mechanistic pathway for the azo dye conversion by CotA-laccase is proposed. The enzymatic oxidation of the Sudan Orange G results in the production of oligomers and, possibly polymers, through radical coupling reactions. A bioassay based on inhibitory effects over the growth of *Saccharomyces cerevisiae* shows that the enzymatic bioremediation process reduces 3-fold the toxicity of Sudan Orange G.

1. Introduction

Laccases are oxidoreductases that have a great potential in various biotechnological processes mainly due to their high non-specific oxidation capacity, the lack of a requirement for cofactors, and the use of readily available oxygen as an electron acceptor (Xu, 1999; Riva, 2006). Laccases constitute a large subfamily of the multicopper oxidase family of enzymes and catalyze the four-electron reduction of oxygen to water (at the T2–T3 trinuclear Cu centres) by the sequential one-electron uptake from a suitable reducing substrate (at the T1 mononuclear copper centre) (Solomon et al., 1996; Stoj and Kosman, 2005). Most of the known laccases have fungal (e.g. white-rot fungi) or plant origins although a few laccases have recently been identified and isolated in bacteria (Gianfreda et al., 1999; Claus, 2003). Laccases have been implicated in various biological activities related with lignolysis, pigment formation, detoxification and pathogenesis (Gianfreda et al., 1999). Chemically, all these functions are related to the oxidation of a range of aromatic substrates such as polyphenols, diamines and even some inorganic compounds.

Colour is usually the first contaminant to be recognized in a wastewater, as very small amounts of synthetic dyes in water (10–15 mg L^{-1}) are highly visible, affecting the aesthetic merit, transparency and gas solubility of water bodies. Azo dyes account for about 50% of all dyes in the textile, food, pharmaceutical, leather, cosmetics and paper industries and are, along with anthraquinonic dyes, the most common synthetic colourants released into the environment (O'Neil et al., 1999). Dye removal from wastewaters with traditional physicochemical processes, such as coagulation, adsorption and oxidation with ozone is expensive, can generate large volumes of sludge and usually require the addition of environmental hazardous chemical additives (Robinson et al., 2001). On the other hand, most of the synthetic dyes are xenobiotic compounds which are poorly removed by the use of conventional biological aerobic treatments (Chen, 2006). Through microbial anaerobic reductions, compounds such as aromatic amines, known to be more toxic than the original dye, are generally generated (Chen, 2006). Fungal laccases have been confirmed for their ability to degrade several azo dyes (Abadulla et al., 2000; Adedayo et al., 2004;

* Corresponding author. Tel.: +351 214469534; fax: +351 214411277.
E-mail address: lmartins@itqb.unl.pt (L.O. Martins).

0168-1656/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2008.09.001

Husain, 2006; López et al., 2004; Tauber et al., 2005). The laccase enzymatic processes are considered environmental friendly since the degradation mechanisms proceeds through the release of molecular nitrogen which prohibits aromatic amines formation (Chivukula and Renganahathan, 1995).

This is the first study where a bacterial laccase was tested in the biotransformation of synthetic dyes. We have used recombinant CotA-laccase, a bacterial thermoactive and intrinsically thermostable enzyme from *Bacillus subtilis*, extensively studied at the biochemical and structural level, which has the predictable robustness for biotechnological applications (Martins et al., 2002; Enguita et al., 2003; Durão et al., 2006; Durão et al., 2008). We found that this bacterial enzyme does not require the addition of redox mediators for the decolourisation of a wide range of structurally different dyes and presents optimal activity in the alkaline pH range, distinctive features when compared with fungal laccases. The enzymatic oxidation of the azo dye Sudan Orange G (SOG) was studied in more detail using a multidisciplinary approach that combined enzymology, electrochemistry, mass spectrometry and microbiology. The aim is to get mechanistic insight that could help us to understand the enzymatic biotransformation process and could guide us to further develop, by using protein engineering tools, a useful enzymatic technology.

2. Materials and methods

2.1. Enzyme production and purification

Recombinant CotA-laccase from *B. subtilis* was produced and purified as previously described (Martins et al., 2002). Enzyme activity was determined by monitoring the oxidation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, Sigma) as previously reported (Martins et al., 2002). One unit of activity was defined as the amount of enzyme that transformed 1 μmol of substrate per min. The protein concentration was measured by using the absorption band of CotA-laccase at 280 nm (ε_{280} = 84,739 M^{-1} cm^{-1}).

2.2. Dyes

Twenty two dyes were screened for decolourisation by using CotA-laccase; three anthraquinone dyes, Acid Blue 62 (Yorkshire Europe, Belgium), Reactive Blue 19 and Alizarin (Sigma–Aldrich) and nineteen azo dyes, Acid Black 210, Acid Yellow 49, Acid Yellow 194, Acid Red 266, Direct Yellow 106, Direct Red 80, Reactive Blue 222, Reactive Yellow 145, Reactive Red 195 (all from Town End, Leeds, UK) and Acid Red 299, Direct Blue 1, Direct Black 38, Direct Red R, Disperse Blue 1, Disperse Yellow 3, Reactive Black 5, Reactive Red 4, Reactive Yellow 81 and Sudan Orange G from Sigma–Aldrich.

2.3. Dye decolourisation

Enzymatic reactions were followed at 37 °C at a Molecular Devices Spectra Max 340, 96-well microplate reader. Dye decolourisation was monitored after 24 h of reaction by measuring the difference at the λ_{max} absorbance for each dye as compared with control experiments without enzyme. Assays were performed by using 50 μM of dye in Britton–Robinson (B&R) buffer (100 mM phosphoric acid, 100 mM boric acid and 100 mM acetic acid mixture with 0.5 M NaOH to the desired pH). The addition of the redox mediator ABTS, 1-hydroxy-benzotriazole (HBT, Sigma) and violuric acid (VA, Sigma) were tested at a final concentration of 10 μM.

2.4. Enzymatic biotransformation studies of SOG

CotA-laccase activity towards Sudan Orange G dye (98%, Sigma–Aldrich) was measured by using a discontinuous method due to the high absorbance of the dye, even at low concentrations. The assay mixtures in a total volume of 50 mL contained 0.5 mM of dye and 1 U mL^{-1} of CotA-laccase in B&R buffer. Reactions were conducted at 37 °C, with shaking (180 rpm) and at appropriate time-points samples were withdrawn, diluted and analysed in a spectrophotometer (430 nm) or in a HPLC (see below). Control assays lacking enzyme were also carried out. The effect of increasing concentrations of ABTS, VA and HBT (1–100 μM) as redox mediators was evaluated. The effect of pH on the enzymatic activity was examined at 37 °C in B&R buffer (pH 5–10). The optimal temperature for activity was determined at values ranging from 30 to 80 °C at pH 8. Kinetic parameters were measured using reaction mixtures containing concentrations of SOG between 10 and 1000 μM. Kinetic constants K_m and k_{cat} were fitted directly to the Michaelis–Menten equation, taking into account substrate inhibition (OriginLab, Northampton, MA, USA). The molar extinction coefficients of SOG at 430 nm (ε_{430}) were estimated at different pH values from calibration curves by using its molecular weight and were as follows: 17,615 M^{-1} cm^{-1} at pH 5, 18,385 $M^{-1}cm^{-1}$ at pH 6, 30,154 M^{-1} cm^{-1} at pH 7, 26,205 M^{-1} cm^{-1} at pH 8, 39,077 $M^{-1}cm^{-1}$ at pH 9, 39,179 M^{-1} cm^{-1} at pH 10. The specific activity was expressed in nmol of dye oxidized min^{-1} mg^{-1} of protein. All enzymatic assays were performed at least in triplicate.

2.5. HPLC analysis

HPLC analyses were performed in a HPLC Merck Hitachi system, using a reverse phase C-18 column (250 × 4 mm length, 5 μM particle size and pore of 100 Å, from LiChrospher 100 RP-18, Merck). Samples were diluted 1:1 in acetonitrile (99.9%, Lab-SCAN, Dublin, Ireland) and centrifuged before injection. Compounds were separated using isocratic conditions over 30 min at a flow rate of 1 mL min^{-1}, at 40 °C. The mobile phase contained 60% acetonitrile in 0.1% (v/v) of trifluoracetic acid. Compounds elution was monitored at 430 and 254 nm.

2.6. Potentiometric measurements

The acid–base reaction of SOG (1 mM) was studied by potentiometry at 25 °C in a water–methanol (1:1 v/v) solution. The equipment and conditions used were as described before (Marques et al., 2006). The ionic strength of the solutions was kept at 0.10 ± 0.01 M KCl. The $[H^+]$ of the solutions was determined by the measurement of the electromotive force of the cell, $E = E'^{\circ} + Q \log[H^+] + E_j$, E'°, Q, E_j and $K_w = ([H^+][OH^-])$ were obtained as described previously (Costa et al., 2000). The value of K_w was found to be equal to $10^{-13.91}$ mol^2 dm^{-6} under our experimental conditions. Overall protonation ($\beta H_h L_l$) constants, were calculated by fitting the potentiometric data obtained for all the performed titrations with HYPERQUAD program (Gans et al., 1996), with $\beta H_h L_l = [H_h L_l]/([[H]^h[L]^l)$.

2.7. Electrochemical measurements

Cyclic voltammetry (CV) was used to investigate the redox behavior of SOG in 0.1 M B&R buffer at different pH values. Voltammetric experiments were performed using a potentiostat/galvanostat from ECO-CHEMIE, AUTOLAB/PSTAT 12 as the source of applied potential and as a measuring device. The whole system was controlled by the General Purpose Electrochemical System (GPES) software package from ECO-CHIMIE. The

three-electrode system consisted of a carbon rod counter electrode, a saturated Ag/AgCl reference electrode and a glassy carbon (BAS MF-2012) working electrode. Typical cyclic voltammetry experiments were performed in the potential range between −1.5 and 1.3 V and the scan rate, v, was varied between 0.01 and 1 V s^{-1}. Potential values are referred to a saturated Ag/AgCl electrode (205 mV vs. Standard Hydrogen Electrode, SHE), unless otherwise stated and are affected by an error of ±10 mV. Measurements were made in de-aerated solutions with high-purity type nitrogen and at 20 ± 2°C, whenever required.

2.8. LC-ESI-ion trap and MALDI-TOF analysis

The ESI-ion trap MS system was a LCQ ion trap mass spectrometer (Thermofinnigan) equipped with electrospray source and run by Xcalibur software. The HPLC separation was performed using the conditions described above. The injection volume was 100 μL. The following conditions were used in experiments with ESI source in positive mode: temperature of the heated capillary, 350 °C; source voltage, 4.5 kV. Nitrogen was used as sheath gas and auxiliary gas. The sheath and auxiliary gas flow rates were 80 and 20 arbitrary units, respectively. HPLC–MS was performed in the full scan mode from *m*/*z* 50 to 1000. All the fragmentation experiments utilized 35% collision energy. MALDI-time of flight (TOF) mass spectra were obtained using a PerSeptive Biosystems MALDI-time of flight MS Voyager-DE STR (Framingham, USA) equipped with delayed extraction, and a 337-nm N_2 laser. Mass spectra were acquired in reflectron delayed extraction mode using an acceleration of 20 kV and a low mass gate of 700 Da. The laser power was set to just above the threshold of ionization. Spectra were accumulated, averaging 500 shots taken across the width of the sample spot for *m*/*z* values between 700 and 5000. Biotransformation products co-crystallization was achieved by applying 0.5 μL of the sample on plate and adding on top an equal volume of re-crystallized matrix consisting of 2,5-dihydrobenzoic acid (10 mg mL^{-1}) prepared in acetonitrile (50%, v/v) with trifluoracetic acid (0.1%, v/v). The mixture was allowed to air dry (dried droplet method).

2.9. Toxicity analysis

A susceptibility toxicity assay based on the inhibitory effects on the growth of the yeast *Saccharomyces cerevisiae* BY4741 was used with minor adaptations from that described previously (Papaefthimiou et al., 2004). The yeast cell suspension used as inoculum was prepared from a mid-exponential phase culture, which was centrifuged and suspended to $OD_{640} = 0.10 \pm 0.02$ in a double strength minimal growth medium (MMB, Papaefthimiou et al., 2004). For the toxicity tests, 75 μL of the standardized yeast cell suspension were mixed with 75 μL of test or control solutions in 96-well polystyrene microplates (Greiner Bio-one) that were sealed and incubated at 30 °C for 24 h with constant agitation. Growth of the yeast cell population was assessed by optical density (OD_{640}) after 24 h of incubation. The toxicity was estimated based on the percentage of inhibition of yeast growth defined as $(1 - OD_{640}{}^{X}/OD_{640}{}^{0}) \times 100$, where $OD_{640}{}^{X}$ and $OD_{640}{}^{0}$ are the OD_{640} attained by the yeast cell population in the presence and in the absence of each test solution, respectively. The no-observed-effect-concentration (NOEC) value is defined as the maximum concentration of the dye that did not significantly affect yeast growth (i.e. that leads to ratio $OD_{640}{}^{X}/OD_{640}{}^{0} \times 100\%$ higher or equal than 98 ± 2%). The 50%-inhibitory-concentration (IC_{50}) value is defined as the concentration of dye at which toxic effects produce 50% inhibition of growth. Data reported are average values with standard deviations of results from at least two toxicity tests (in triplicate) from two independent biotransformation experiments.

3. Results and discussion

3.1. Dye decolourisation

All azo and anthraquinonic dyes tested, with the exception of Reactive yellow 81, were, at a different extent, oxidatively bleached by 1 U mL^{-1} of CotA-laccase in the absence of redox mediators (Fig. 1). Addition of redox mediators (ABTS, VA and HBT) to the assay mixtures resulted in similar final values of decolourisation for all the dyes tested. The lack of a strict requirement for redox mediators exhibited by CotA-laccase, presents, under a technological perspective, a benefit of this enzyme, as these molecules are expensive and need many times to be present in a large excess or, at least, a stoichiometric amount compared to the substrate is required (Bourbonnais et al., 1998). Moreover, some mediators give raise to highly unstable radical intermediates that can lead to enzyme inactivation and that are toxic upon release to the environment (Xu et al., 2000). Optimal pH for decolourisation occurred at pH 8–9 and no decolourisation was observed below pH 5 (data not shown). These results contrast strongly with data obtained with fungal laccases that shows optimal pH for dye degradation in the acidic range of pH (Abadulla et al., 2000; Almansa et al., 2004; Camarero et al., 2005; Couto et al., 2005; Kandelbauer et al., 2004a,b; Zille et al., 2005a,b; Pogni et al., 2007). Interestingly, CotA-laccase a low redox potential laccase (455 mV vs. NHE, Durão et al., 2006) is able to decolourise in a higher extension the Reactive Black 5 dye (E° 742 mV, unpublished data) in the absence of redox mediators, in contrast to what happen with the high-redox potential fungal laccases *Pycnoporus cinnabarinus*, *Trametes villosa* (E° 780 mV vs. SHE) (Camarero et al., 2005; Zille et al., 2004), *Tr. hirsuta* (Abadulla et al., 2000) or *Tr. modesta* (Tauber et al., 2005) that degrade this dye only in the presence of redox mediators. In laccases the substrate oxidation involves the Marcus "outer-sphere" mechanism in which the redox potential difference between the substrate and the T1 Cu site (together with the reorganization energy and transmission coefficient) determines the electron transfer and thus, the oxidation rate (Solomon et al., 1996). Nevertheless, other factors as for example, the composition, structure or p_{Ka} of substrates affecting, for example, the proper docking of compounds to the enzyme substrate pocket, are also known to play an important role in determining the oxidation rates (Xu et al., 2000; Tadesse et al., 2008; Chivukula and Renganahathan, 1995; Almansa et al., 2004; Kandelbauer et al., 2004b).

3.2. SOG oxidation as monitored by absorbance and HPLC

Sudan Orange G was chosen as a substrate due to its simple chemical structure with two hydroxyl groups, in the *ortho* and *para* positions relative to the azo group (see Fig. 8). Optical absorption and HPLC were used to detect and monitor the time course for the enzymatic biotransformation of 0.5 mM of SOG at 37 °C and pH 8 (the optimal pH for enzymatic activity, see below) (Fig. 2A, B). A decrease in the intensity of the 430 nm absorption band of SOG was observed during the enzymatic process, indicating the vanishing of the aromatic azo groups (Fig. 2A). Concomitant with this decrease at 430 nm, increased absorption intensities at ca. 325 nm and ca. 530 nm are observed, indicating the generation of biotransformation products. After 60 min of reaction, 50% of SOG was transformed and a rate of biotransformation of 23 ± 2 nmol min^{-1} mg^{-1} protein was calculated.

The time course of the enzymatic reaction was also monitored by HPLC where SOG was chromatographically separated from

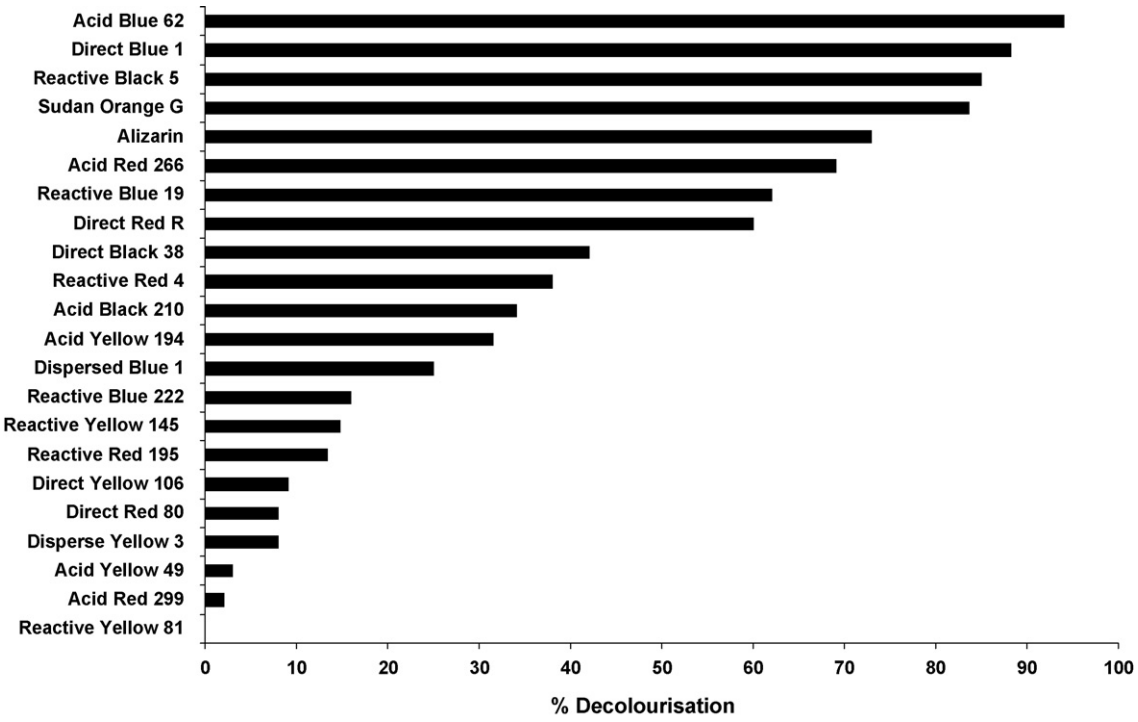

Fig. 1. Decolourisation of several anthraquinonic and azo dyes after 24 h of reaction in the absence of redox mediators by using CotA-laccase (1 U mL^{-1}).

products of the enzymatic reaction. A major peak with retention time of 5 min, corresponding to the substrate, decreased over the course of reaction and disappeared (≤2%) after 7 h of reaction (Fig. 2B). The emergence of two major peaks corresponding to reaction products is observed, with R_t of 2.2 min and 15 min (minor peaks with R_t of 2.6, 3.4 and 8 min are also observed upon amplification of the chromatograms). The assay mixtures became browner in colour over the course of reaction, presumably due to products formation. After centrifugation of the final reaction mixture, the supernatant contained the compounds corresponding to the major peak with R_t of 2.2 min and the three minor peaks. Therefore, this will be subsequently referred as the soluble (S) product fraction. The pellet contained the major product with R_t of 15 min, which is subsequently referred as the insoluble (I) product fraction.

The addition of redox mediators, ABTS, HBT and VA, to the reaction mixtures was tested and a two-fold increase in the rate of biotransformation (44 ± 4 nmol min^{-1} mg^{-1}) in the presence of 10 μM of ABTS was observed. The products obtained have similar retention times, as monitored by HPLC, and identical biotransformation yields were calculated, at the reaction equilibrium, to those obtained in the absence of ABTS. The presence of HBT and VA in the enzymatic assays was shown not to affect the reaction rates and yields (data not shown). This was correlated to the inability of CotA-laccase to oxidize these compounds (data not shown) probably due to the large difference between the redox potentials of CotA-laccase (455 mV vs. NHE) and of these mediator molecules (near 1000 mV vs. NHE, whereas ABTS has a E_0 of 670 mV vs. NHE (Bourbonnais et al., 1998).

3.3. Steady-state kinetics of SOG oxidation

The steady-state kinetic constants of the enzyme at 37 °C towards SOG oxidation were obtained following the equation $v = V_{max}/(1 + K_m[S] + [S]/K_i)$ (Fig. 3). The function that relates the enzyme rate vs. the concentration of substrate is a non-hyperbolic curve, indicating enzyme inhibition at high substrate concentrations. This behavior is clearly detected by the nonlinearity in the Lineweaver–Burk reciprocal plot, where a sudden and dramatic up-curving near the *y*-intercept could be observed (see insert of Fig. 3). A K_i of 474 ± 77 μM was calculated. The calculated K_m value is 44 ± 7 μM and V_{max} and k_{cat} values are 57 nmol min^{-1} mg $protein^{-1}$ and 0.05 s^{-1}, respectively. These shows that CotA-laccase presents a lower catalytic specificity (k_{cat}/K_m) towards SOG oxidation (0.008 s^{-1} μM^{-1}) when compared with its specificity to oxidize the classical substrates ABTS (0.25 s^{-1} μM^{-1}) and syringaldazine (1.84 s^{-1} μM^{-1}) (Durão et al., 2006). The lower reactivity of the azo dye is attributed to differences in the k_{cat} term (related to the substrate ability of surrendering electrons to the T1 copper catalytic site of CotA-laccase), as similar K_m values were measured for the different substrates (87 and 10 μM for ABTS and SGZ, respectively). The catalytic constants determined for SOG have broadly the same order of magnitude of those calculated for fungal laccases (Chivukula and Renganahathan, 1995; Kandelbauer et al., 2004a,b) but naturally, these values are highly dependent on the dye and on the enzyme under study. The optimal temperature for the SOG biotransformation reaction, 75 °C, is identical for ABTS oxidation, but an activation energy of 14.8 kcal mol^{-1} K was calculated for this dye oxidation, 3-fold higher then the calculated for ABTS (5.2 kcal mol^{-1} K) (Martins et al., 2002).

3.4. Dependence on pH and E°

For the transformation of SOG by CotA-laccase, a bell-shape pH activity profile was found with an optimum at pH 8 (Fig. 4B). pH activity bell-shape profiles for phenolic substrates have been related with the opposite effects of the higher oxidation susceptibility of these substrates and the OH^- enzyme inhibition as pH increases (Xu, 1996). The UV–vis spectra of SOG was shown to be pH dependent (Fig. 5) and this dependence was correlated with changes in the dye structure, in particular, with its protonation/deprotonation equilibria. In fact, potentiometric measurements revealed two p_{Ka} values for SOG, at 6.90 ± 0.02 and

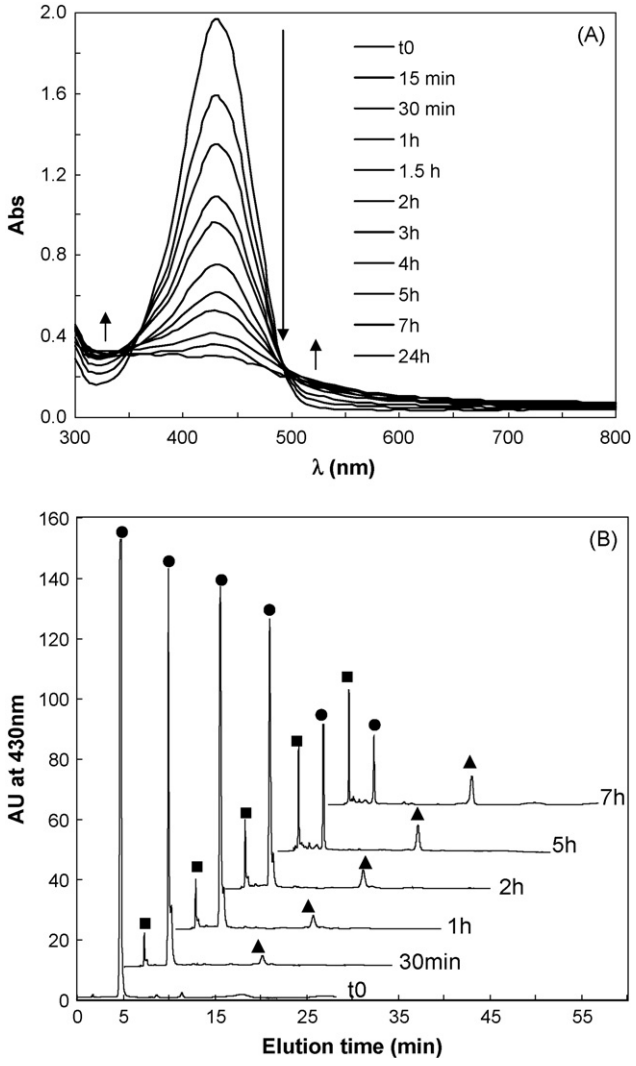

Fig. 2. Time course for SOG biotransformation as monitored (A) by absorbance and (B) by HPLC [(●), SOG; (■) product with R_t 2.2 min; (▲) product with R_t 15 min].

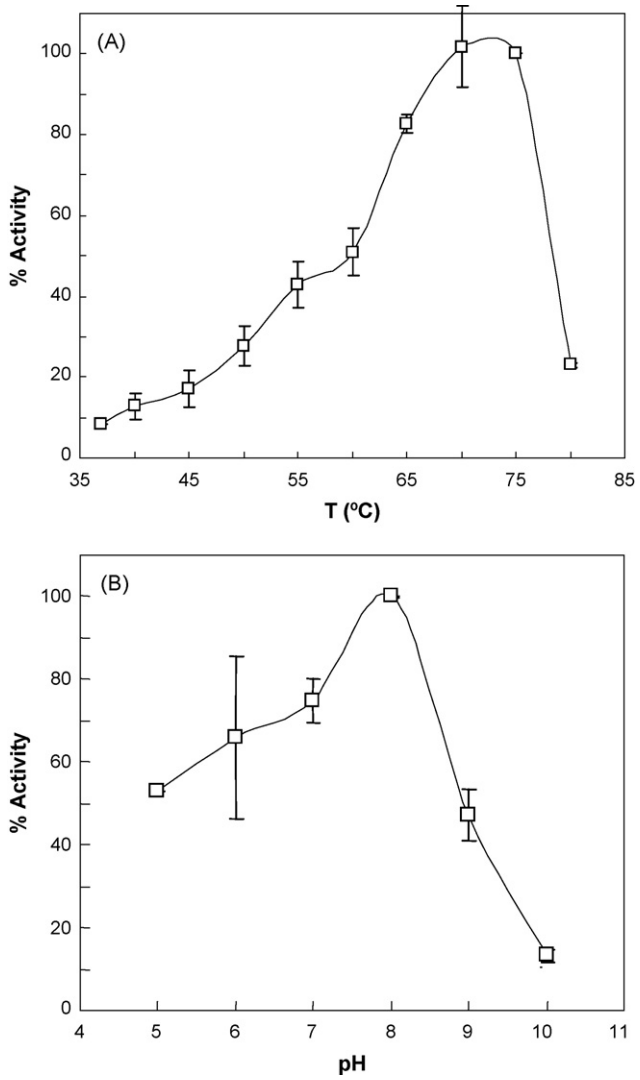

Fig. 4. (A) Temperature activity and (B) pH activity profile for SOG oxidation.

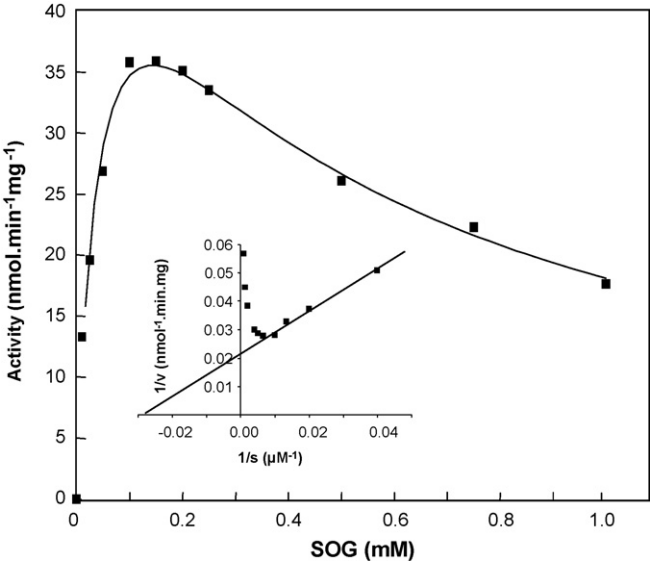

Fig. 3. Non-hyperbolic saturation kinetics of CotA-laccase with SOG as substrate. Insert: Lineweaver–Burk plot for SOG enzymatic oxidation.

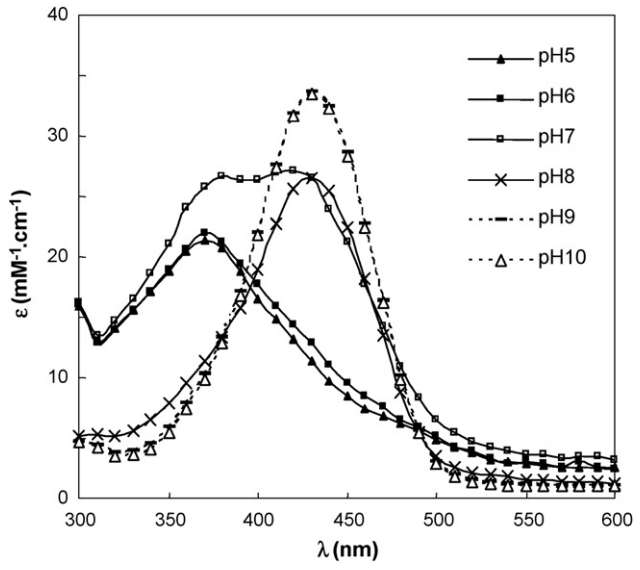

Fig. 5. UV–vis spectra of SOG in B&R buffer at different pH values.

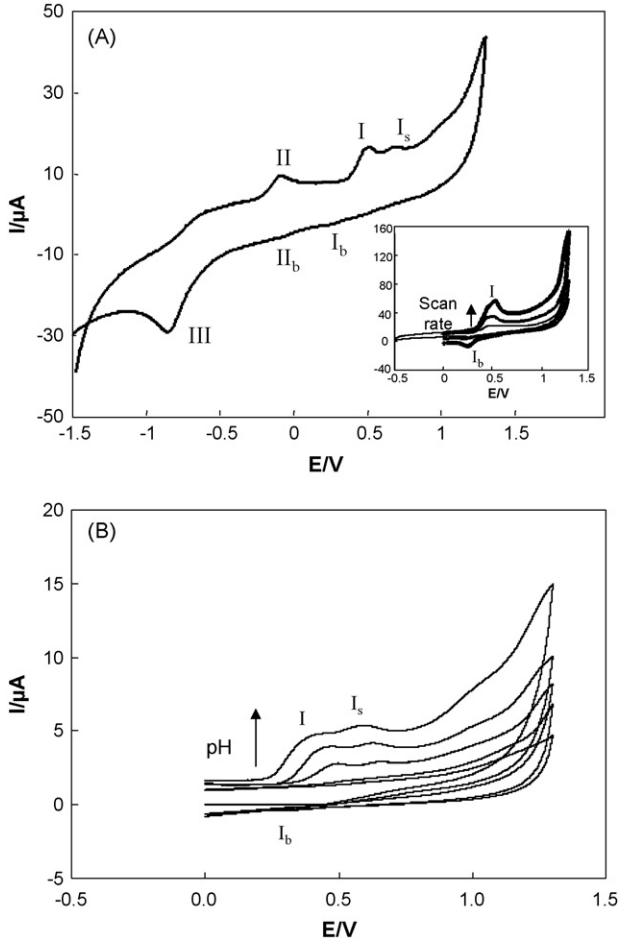

Fig. 6. (A) Cyclic voltammograms of SOG (1 mM) in 0.1 M B&R buffer, pH 8 in the potential range between −1.5 and 1.3 V at a scan rate of 0.1 V s^{-1} and (insert) from −0.5 to 1.3 mV at 0.1, 0.2 and 0.5 V s^{-1}. (B) Cyclic voltammograms at pH values from 6 to 10, from 0 to 1.3 V at a scan rate of 0.3 V s^{-1}.

11.74 ± 0.02, which were attributable to its *ortho* and *para* hydroxyl groups (data not shown) and thus, the higher rates of enzymatic oxidation measured at pH 8 implies that the dye is more prone to enzymatic oxidation after deprotonation of the more acidic hydroxyl group. Further electrochemistry studies were done to analyse the pH effect on the oxidation of SOG. Fig. 6A shows a cyclic voltammogram for SOG at pH 8. Two oxidation peaks are clearly seen, I and II, with peak potentials, E_p, close to 0.490 and −0.130 V, respectively, while a reduction peak exists with E_p close to −1 V (III). A closer inspection reveals in the positive scan a shoulder at potentials more positive than peak I (I_s) and in the reverse scan, two reduction peaks, I_b and II_b, that became more apparent for highest scan rates. No oxidation peak could be associated with peak III. As to peak I_s, current intensity increases considerably in comparison to that of peak I while increasing the scan rate (data not shown) and this is due to adsorption phenomena that becomes more relevant at higher scan rates (Bard and Faulkner, 2001). In CV in the potential range between −0.5 and 1.3 V, peak I remain while peak II does not appear, no matter the direction of the scan (insert of Fig. 6A). These shows that peaks II and II_b are associated with electroactive species formed only after the irreversible process occurring at peak III. Considering the mechanism of azo dye reduction, peak III is associated with the four-electron reduction leading to the cleavage of the N=N bond (Lund, 1991), and peaks II and II_b with the formation of unstable amine products (Chen, 2006). We have observed that for peak I_b current intensity increases with increasing v (insert of Fig. 6A) and this behavior points out to the reduction of a non-stable product formed after oxidation of peak I at E_p = 0.490 V and therefore I and I_b were associated. A good linear relationship with a null intercept were obtained for peak I current with the square root of scan rate corresponding to one electron transferred during oxidation. The difference $E_p - E_{p/2}$ = 0.062, where $E_{p/2}$ is the half peak potential, also compares with the theoretical value of 0.057 V for a reversible one-electron reaction. Taken together, these results show that the oxidation process at peak I is the one which can be associated with the enzymatic biotransformation of SOG (mechanism proposed below) and therefore the most important for this study. A formal redox potential for SOG at pH = 8, $E^{\circ'}$ = 0.70 V vs. SHE, was estimated from the oxidation peak potential using the relationship $E^{\circ'} = E_{1/2} = Ep - 1.109\,RT/nF$ (Bard and Faulkner, 2001). Electrochemical studies are consistent with the determined p_{Ka} values of 6.9 and 11.7 of the two oxidizable centres in SOG. The oxidation peak detected in the voltammetric experiments (peak I) should most probably correspond to the SOG more acidic group, i.e., the *ortho* hydroxyl group with p_{Ka} 6.9. Indeed, as can be seen in Fig. 6B, at pH 6 no oxidation signal could be detected up to potentials as positive as 1.3 V (close to the upper limit imposed by water oxidation), at pH 7, ill-defined peak appears for the highest scan rates at potentials more positive than 0.490 V and for pH 8, 9 and 10 peak 1 is clearly seen and its potential revealed to be pH independent.

3.5. Identification of products and proposed mechanism for SOG biotransformation

The identification of biotransformation products present in the aqueous soluble (S) and insoluble (I) fractions was performed by LC–MS and MALDI-TOF MS. The full identification of I fraction was

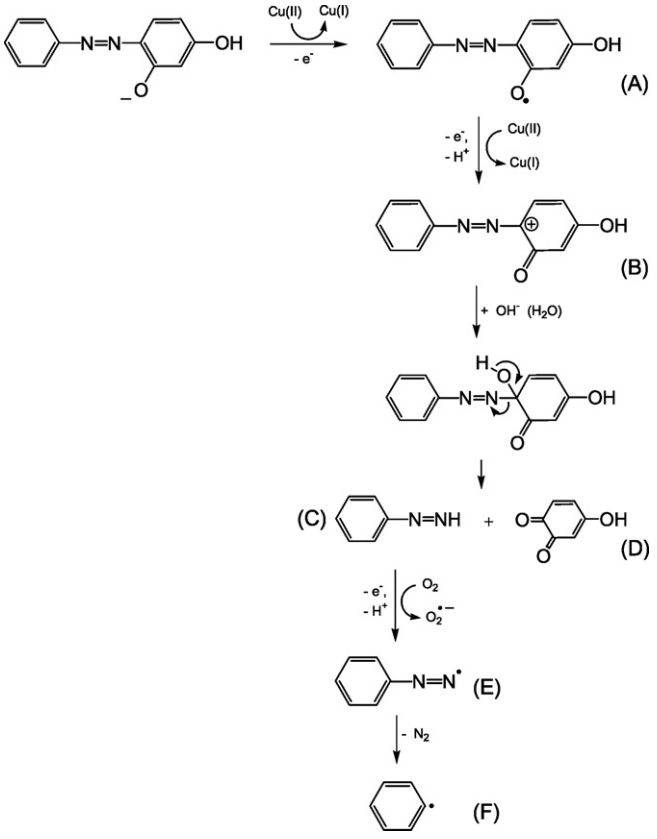

Fig. 7. Proposed mechanism for the biotransformation of SOG by the CotA-laccase.

Table 1
Mass spectra of SOG biotransformation products.

Mass spectrometry method	Fraction	R_t (min)	m/z quasi molecular ions	MS/MS product ions
LC-ESI-ion trap	Soluble	2.2	148	130; 112
			189	(1)
			321	(1)
		8	637	544; 518; 502; 423
	Insoluble	15	319	301; 248; 226; 208; 198
			533	515; 440; 422; 395; 379; 335
MALDI-TOF	Insoluble		737	
			765	
			841	
			860	
			890	
			1036	

(1) No fragmentation ions were observed.

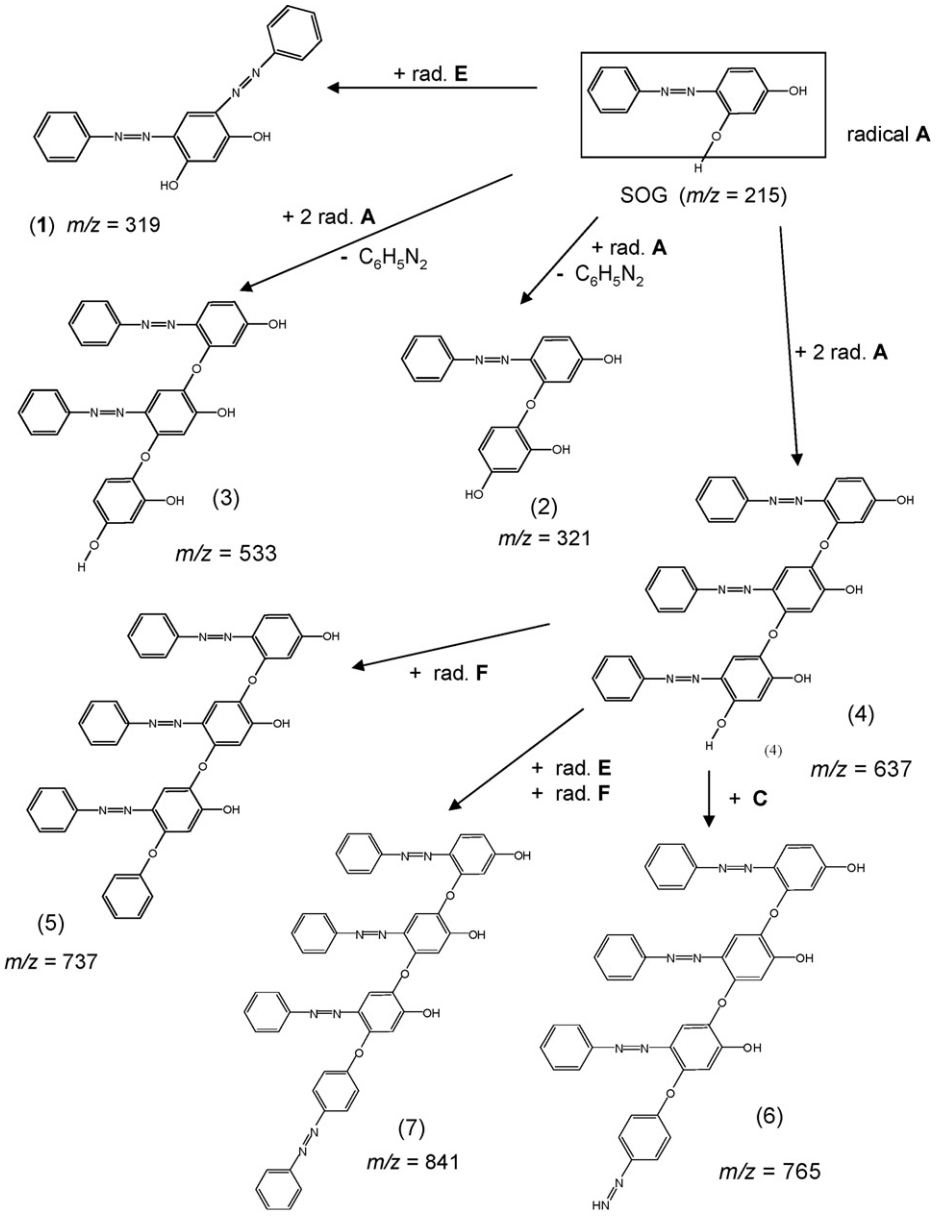

Fig. 8. Proposed structures (1)–(7) for the biotransformation products obtained from the oxidation of SOG by the CotA-laccase.

impaired by its low solubility in several solvents: acetone, ethanol, methanol, chloroform, dichloromethane, ethyl ether, toluene, hexane and tetrahydrofuran. A partial solubility (25%) was found on acetonitrile and thus, the identification of products in I fraction was performed in the soluble part of acetronitrile-dissolved I fraction. Positive ion mode MS analysis has shown to be more sensitive than the negative ion mode and was used for the MS analysis of SOG and its biotransformation products. Using positive mode ESI-MS and MALDI-TOF MS 12 SOG biotransformation products were detected.

The reaction pathways described in the literature for the enzymatic degradation of dyes could assist the process of identification of the biotransformation products. Therefore, we propose herein a mechanistic pathway for SOG biotransformation by CotA-laccase (Fig. 7) based on the accepted model for azo dye degradation by laccases (Chivukula and Renganahathan, 1995; Zille et al., 2005a). In these enzymatic pathways, azo dyes are oxidized without the cleavage of the azo bond, through a highly non-specific free radical mechanism, forming phenolic type compounds. This mechanism avoids the formation of toxic aromatic amines, obtained under reductive conditions (Chen, 2006). According to the described mechanisms, CotA-laccase oxidizes, in an one-electron transfer reaction, a hydroxyl group of SOG azo dye (presumably in the deprotonated form at pH 8) generating the phenoxyl radical A, that will be sequentially oxidized to a carbonium ion (B). The water nucleophilic attack on the phenolic carbonium, bearing the azo linkage, followed by N–C bond cleavage, produces diazenylbenzene (C) and 4-hydroxy-1,2-benzoquinone (D). The diazenylbenzene (C) can lead to radical (E) and then, to a benzene radical (F) upon loss of a nitrogen molecule. This benzene radical can undergo hydrogen radical addition or get involved in further coupling reactions. The free radicals resulting from the biotransformation process have a high reactivity and all species can participate in coupling reactions with intact dye and/or intermediate product molecules. The formation of oligomeric or polymeric condensation products as a result of coupling reactions between intermediates of a dye laccase-oxidative process have been described earlier (Moldes et al., 2004; Kandelbauer et al., 2004b; Zille et al., 2005a).

Considering the proposed mechanistic pathway for SOG degradation, we identified seven biotransformation products (see Table 1 and Fig. 8). The molecular ion 319 was attributed to 2,4-fenilazoresorcinol (1). This molecule can be formed through coupling between radical E and an intact SOG molecule. The fragmentation pattern found for this ion confirmed the presence of one azo, one hydroxyl and one benzene groups by the loss of 28, 18 and 77 amu, respectively. Moreover, the pattern is similar to that obtained for a 2,4-fenilazoresorcinol standard sample assayed in the same conditions. MALDI-TOF MS spectrum of the acetonitrile solubilised I fraction shows the presence of molecular ions 737, 765 and 841 which were identified as sodium adducts of compounds (5) (M = 714), (6) (M = 742) and (7) (M = 818), respectively (Fig. 8). The structures for compounds (5), (6) and (7) are proposed based on the possible successive coupling reactions between intermediates radicals A, E and F (for the (5) and (7) structures) and between radicals A and a diazenylbenzene molecule C in the case of structure (6). Considering the combination of radicals formed, as proposed in the SOG biotransformation mechanism, and the fragmentation patterns found, the molecular ions 533 from I fraction and 321 and 637 from the S fraction have been tentatively identified as compounds (3) (M = 534), (2) (M = 322) and (4) (M = 638). In fact, the analysis of fragmentation patterns obtained for products with m/z 533 and 637 confirmed the presence of azo and hydroxyl groups as well as one benzene group by the loss of 28, 18 and 77 amu, or through the combination of these values, which are in agreement with the proposed identification. However, their identification cannot be stated unambiguously mainly due to their unusual ionization process that seems to be achieved by the loss of an H^-. The proposed structures can be achieved by the coupling between radicals of A type (compound 4) or through nucleophilic attack to an intermediate B with the posterior loss of the azobenzene group by cleavage of the C–N bond.

3.6. Toxicity of SOG and biotransformation products

A yeast-based bioassay was used to compare the potential cytotoxicity of the Sudan Orange G with its enzymatic biotransformation products (Fig. 9). This is the first report of the toxicological properties of SOG as far as we are aware. We found that SOG has a significant cytotoxic effect on yeast cells growth and 24h-NOEC and 24h-IC_{50} values of approximately 90 and 300 μM, respectively, were estimated (Fig. 9A). The initial reaction mixture containing 300 μM of SOG exerted an inhibition of 65 ± 9 (in %) on yeast growth (Fig. 9B). Importantly, the final reaction mixture containing the biotransformation products is significantly less toxic to the yeast cell population (near 20% of inhibition of growth) than the untreated dye solution (Fig. 9B). The toxicity of the soluble (S) and insoluble (I) product fractions, 27 ± 5 and 20 ± 9%, respectively, were similar to that estimated for the total reaction mixture. The results obtained allowed us to estimate that the enzymatic treatment of the SOG solution leads to a final detoxification of 67 ± 6% (Fig. 9B). The information available in the literature regarding the detoxification of textile effluents with enzymes, namely fungal laccases, is

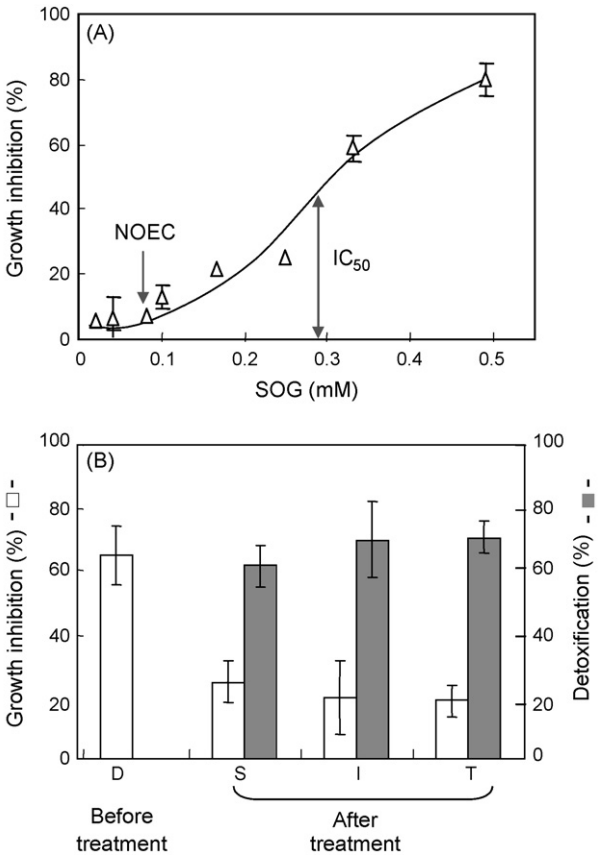

Fig. 9. (A) Toxicity of SOG to *S. cerevisiae* BY4741 assessed by growth inhibitory effect after 24 h at 30 °C in MMB medium (pH 6.5); NOEC, no-observed-effect-concentration and IC_{50}, 50%-inhibitory-concentration. (B) Comparison of the inhibition on yeast growth (□) exerted by 300 μM of SOG in B&R buffer, pH 6.5 (D) and the biotransformation products formed after 24 h of enzymatic reaction: total reaction mixture (T) and product soluble (S) and insoluble (I) fractions in B&R buffer, pH 6.5. The level of detoxification (%) is also indicated (■).

scarce and no strict correlation was found between decolourisation and detoxification, indicating that dye degradation products were still toxic in some cases (Abadulla et al., 2000; Adedayo et al., 2004; López et al., 2004; Tauber et al., 2005). The results obtained shows that the CotA-laccase enzymatic remediation process is effective in reducing the toxicity of dyeing effluents containing SOG, even if a more real picture of the environmental impact may be only obtained with additional toxicity tests using ecologically representative organisms of different taxonomic ranks.

4. Concluding remarks

Modern biocatalysis is achieving new advances in environmental fields, from enzymatic bioremediation to the synthesis of renewable and clean energies (Alcalde et al., 2006). From an environmental point of view, the use of enzymes instead of chemicals or microorganisms presents several advantages including the potential for their production at a higher scale, with enhanced stability and/or activity and at a lower cost by using recombinant-DNA technology. In the present work we show that bacterial CotA-laccase exhibits an optimum at pH 8 for the oxidation of a broad array of dyes in clear contrast with the vast majority of fungal laccases that show an optimal pH for dye decolourisation at the acidic range of pH. Moreover, CotA-laccase does not require the presence of redox mediators for dye decolourisation which represents another advantage of this enzyme under a technological perspective. The biotransformation of the azo dye Sudan Orange G by the recombinant bacterial CotA-laccase was studied in detail. The oxidation of SOG by CotA-laccase proceeds in a broad range of temperatures (30–80 °C) as this enzyme is robust and thermoactive with an optimum at 75 °C The acid–basic behavior of the electroactive groups of the SOG dye (p_{Ka} values of 6.9 and 11.7) was shown to affect its susceptibility towards enzymatic oxidation. Optimal for enzyme activity occurs at pH 8, when SOG *ortho* hydroxyl group is deprotonated. The one-electron oxidation of SOG molecule by the enzyme results therefore, in the formation of unstable radical molecules and in the concomitant destruction of the dye chromophoric structure. The radical products can undergo coupling reactions between themselves or with un-reacted dye molecules, to produce a large array of oligomeric products. This is in agreement with structures of final enzymatic products identified by LC–MS and MALDI-TOF analyses and foreseen from the mechanistic pathway proposed for SOG dye biotransformation. In addition, the presence of these compounds is in accordance with the darkening of the enzymatic treated solution and the high insolubility of products formed. This may explain the reduced toxicity of the final reaction mixture as compared with solutions of intact SOG and highlights the potential for the application of this bioremediation friendly system, as products can be removed from effluents in the form of a precipitate.

Acknowledgements

This work was supported by FP6-NMP2-CT-2004-505899 POCI/AMB/56039/2004, PTDC/AMB/64230/2006 and PTDC/BIO/72108/2006 project grants. L. Pereira holds a Pos-Doc fellowship (SFRH/BPD/20744/2004) from Fundação para a Ciência e Tecnologia. We wish to thank our colleagues at ITQB: A.L. Simplício for the HPLC facilities, S. Carvalho and R. Delgado for the potentiometric analysis, and P. Jackson for the English revision.

References

Abadulla, E., Tzanov, T., Costa, S., Robra, K.-H., Cavaco-Paulo, A., Gübitz, G.M., 2000. Decolourisation and detoxification of textile dyes with a laccase from *Trametes hirsuta*. Appl. Environ. Microbiol. 66, 3357–3362.

Adedayo, O., Javadpour, S., Taylor, C., Anderson, W.A., Moo-Young, M., 2004. Decolourisation and detoxification of methyl red by aerobic bacteria from a wastewater treatment plant. J. Microbiol. Biotechnol. 20, 545–550.

Alcalde, M., Ferrer, M., Plou, F.J., Ballesteros, A., 2006. Environmental biocatalysis: from remediation with enzymes to novel green processes. Trends Biotechnol. 24, 281–287.

Almansa, E., Kandelbauer, A., Pereira, L., Cavaco-Paulo, A., Gübitz, G.M., 2004. Influence of structure on dye degradation with laccase mediator system. Biocatal. Biotransform. 22, 315–324.

Bard, A.J., Faulkner, L.R., 2001. Electrochemical Methods, Fundamentals and Applications, 2nd ed. John Wiley & Sons, New York.

Bourbonnais, R., Leech, D., Paice, M.C., 1998. Electrochemical analysis of the interactions of laccase mediators with lignin model compounds. Biochim. Biophys. Acta 1370, 381–390.

Camarero, S., Ibarra, D., Martinez, M.J., Martinez, A.T., 2005. Lignin-derived compounds as efficient laccase mediators for decolourisation of different types of recalcitrant dyes. Appl. Environ. Microbiol. 71, 1775–1784.

Chen, H., 2006. Recent advances in azo dye degrading enzyme research. Curr. Protein Pept. Sci. 7, 101–111.

Chivukula, M., Renganahathan, V., 1995. Phenolic azo dye oxidation by laccase from *Pyricularia oryzae*. Appl. Environ. Microbiol. 61, 4374–4377.

Claus, H., 2003. Laccases and their occurrence in prokaryotes. Arch Microbiol. 179, 145–150.

Costa, J., Delgado, R., Drew, M.G.B., Félix, V., Saint-Maurice, A., 2000. A new redox-responsive 14-membered tetraazamacrocycle with ferrocenylmethyl arms as receptor for sensing transition-metal ions. J. Chem. Soc., Dalton Trans., 1907–1916.

Couto, S.R., Sanroman, M., Gübitz, G.M., 2005. Influence of redox mediators and metal ions on synthetic acid dye decolourisation by crude laccase from *Trametes hirsuta*. Chemosphere 58, 417–422.

Durão, P., Bento, I., Fernandes, A.T., Melo, E.P., Lindley, P.F., Martins, L.O., 2006. Perturbations of the T1 copper site in the CotA-laccase from *Bacillus subtilis*: structural, biochemical, enzymatic and stability studies. J. Biol. Inorg. Chem. 11, 514–526.

Durão, P., Chen, Z., Silva, C.S., Soares, C.M., Pereira, M.M., Todorovic, S., Hildebrandt, P., Bento, I., Lindley, P.F., Martins, L.O., 2008. Proximal mutations at the T1 copper site of CotA-laccase; spectroscopic, redox, kinetic and structural characterization of Ile494 and Leu386 mutations. Biochem. J. 412, 339–346.

Enguita, F.J., Martins, L.O., Henriques, A.O., Carrondo, M.A., 2003. Crystal Structure of a bacterial endospore coat component. J. Biol. Chem. 278, 19416–19425.

Gans, P., Sabatini, A., Vacca, A., 1996. Investigation of equilibria in solution. Determination of equilibrium constants with the HYPERQUAD suite of programs. Talanta 43, 1739–1753.

Gianfreda, L., Xu, F., Bollag, J.-M., 1999. Laccases: a useful group of oxidoreductive enzymes. Biorem. J. 3, 1–25.

Husain, Q., 2006. Potential applications of the oxidoreductive enzymes in the decolourisation and detoxification of textile and other synthetic dyes from polluted water: a review. Crit. Rev. Biotechnol. 26, 201–221.

Kandelbauer, A., Maute, O., Kessler, R., Erlacher, A., Gübitz, G.M., 2004a. Study of dye decolourisation in an immobilized laccase enzyme-reactor using online spectroscopy. Biotechnol. Bioeng. 87, 552–563.

Kandelbauer, A., Erlacher, A., Cavaco-Paulo, A., Gübitz, G.M., 2004b. Laccase-catalysed decolourisation of the synthetic azo-dye diamond black PV 200 and some structurally related derivatives. Biocatal. Biotransform. 22, 331–339.

López, C., Valade, A.-G., Combourieu, B., Mielgo, I., Bouchon, B., Lema, J.M., 2004. Mechanism of enzymatic degradation of the azo dye Orange II determined by ex situ ^{1}H nuclear magnetic resonance and electrospray ionization-ion trap mass spectrometry. Anal. Biochem. 335, 135–149.

Lund, H., 1991. In: Lund, H., Baizer, M.M. (Eds.), Organic Electrochemistry. Marcel Dekker Inc., New York, pp. 422–423.

Marques, F., Gano, L., Campello, M.P., Lacerda, S., Santos, I., Lima, L.M.P., Costa, J., Antunes, P., Delgado, R., 2006. 13- and 14-membered macrocyclic ligands containing methylcarboxylate or methylphosphonate pendant arms: chemical and biological evaluation of their ^{153}Sm and ^{166}Ho complexes as potential agents for therapy or bone pain palliation. J. Inorg. Chem. 100, 270–280.

Martins, L.O., Soares, C.M., Pereira, M.M., Teixeira, M., Jones, G.H., Henriques, A.O., 2002. Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the *Bacillus subtilis* endospore coat. J. Biol. Chem. 277, 18849–18859.

Moldes, D., Lorenzo, M., Sanromán, M.Á., 2004. Degradation or polymerisation of phenol red dye depending to the catalyst used. Process Biochem. 39, 1811–1815.

O'Neil, C., Hawkes, F.R., Hawkes, D.L., Lourenço, N.D., Pinheiro, H.M., Delée, W., 1999. Colour in textile effluents-sources, measurement, discharge consents and simulation: a review. J. Chem. Technol. Biotechnol. 74, 1009–1018.

Papaefthimiou, C., Cabral, M.G., Mixailidou, C., Viegas, C.A., Sá-Correia, I., Theophilidis, G., 2004. Comparison of two screening bioassays, based on the frog sciatic nerve and yeast cells, for the assessment of herbicide toxicity. Environ. Toxicol. Chem. 23, 1211–1218.

Pogni, R., Brogioni, B., Baratto, M.C., Sinicropi, A., Giardina, P., Pezzella, C., Sannia, G., Basosi, R., 2007. Evidence for a radical mechanism in biocatalytic degradation of synthetic dyes by fungal laccases mediated by violuric acid. Biocatal. Biotransform., 1–7.

Riva, S., 2006. Laccases: blue enzymes for green chemistry. Trends Biotechnol. 24, 219–226.

Robinson, T., McMullan, G., Marchant, R., Nigam, P., 2001. Remediation of dyes in textile effluent on current treatment technologies with a proposed alternative. Bioresour. Technol. 77, 247–255.
Solomon, E.I., Sundaram, U.M., Machonkin, T.E., 1996. Multicopper oxidases and oxygenases. Chem. Rev. 96, 2563–2605.
Stoj, C.S., Kosman, D.J., 2005. Copper proteins: oxidases. In: King, R.B. (Ed.), Encyclopedia of Inorganic Chemistry, vol. II, 2nd ed. John Wiley & Sons, pp. 1134–1159.
Tadesse, M.A., D'Annibale, A., Galli, C., Gentili, P., Sergi, F., 2008. An assessment of the relative contributions of redox and steric issues to laccase specificity towards putative substrates. Org. Biomol. Chem. 6, 868–878.
Tauber, M., Gübitz, G.M., Rehorek, A., 2005. Degradation of azo dyes by laccase and ultrasound treatment. Appl. Environ. Microbiol. 71, 2600–2607.
Xu, F., 1996. Oxidation of phenols, anilines, and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition. Biochemistry 35, 7608–7614.
Xu, F., 1999. Laccase. In: Flickinger, M.-C., Drew, S.W. (Eds.), The Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Bioseparation. John Wiley & Sons Inc., New York, pp. 1545–1554.
Xu, F., Kulys, J.J., Duke, K., Li, K., Krikstopaitis, K., Deussen, H-J.W., Abbate, E., Galinyte, V., Schneider, P., 2000. Redox chemistry in Laccase-catalysed oxidation of N-hydroxy compounds. Appl. Environ. Microbiol. 66, 2052–2056.
Zille, A., Ramalho, P., Tzanko, T., Millward, R., Aires, V., Cardoso, M.H., Ramalho, M.T., Gübitz, G.M., Cavaco-Paulo, A., 2004. Predicting dye biodegradation from redox potentials. Biotechnol. Prog. 20, 1588–1592.
Zille, A., Górnacka, B., Rehorek, A., Cavaco-Paulo, A., 2005a. Degradation of azo dyes by *Trametes villosa* laccase under long time oxidative conditions. Appl. Environ. Microbiol. 71, 6711–6718.
Zille, A., Munteanu, F.-D., Gübitz, G.M., Cavaco-Paulo, A., 2005b. Laccase kinetics of degradation and coupling reactions. J. Mol. Catal. B: Enzym. 33, 23–28.