Bacterial Enzymes and Multi-enzymatic Systems for Cleaning-up Dyes from the Environment

Sónia Mendes, Maria Paula Robalo and Lígia O. Martins

1 Introduction

Synthetic dyes are xenobiotic compounds that are being increasingly used in several industries, with special emphasis in the paper, textile and leather industries. Over 100,000 commercial dyes exist today and more than 7×10^5 tons of dyestuff is produced annually, of which $1–1.5 \times 10^5$ tons is released into the wastewaters (Rai et al. 2005). Among these, azo dyes, characterized by the presence of one or more azo groups (–N=N–), and anthraquinonic dyes represent the largest and most versatile groups. Synthetic dyes are highly visible pollutants and can hardly be removed from the effluents by conventional wastewater treatments. They are anthropogenic pollutants causing deterioration of water quality, affecting photosynthesis, decreasing dissolved oxygen levels and severely disturbing the aquatic ecosystems (Rai et al. 2005; van der Zee and Villaverde 2005). Moreover, dyes have become a health hazard as many of them and/or their breakdown products have been found to be toxic and potentially carcinogenic (Golka et al. 2004; Pinheiro et al. 2004; Schneider et al. 2004; van der Zee and Villaverde 2005; Chen 2006). Physico-chemical treatment processes, such as coagulation, precipitation, filtration, adsorption, photolysis and oxidation with hydrogen peroxide or ozone, can generate a large volume of sludge and usually require the addition of other

S. Mendes · L.O. Martins (✉)
Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa,
Av. Da República, 2780-157 Oeiras, Portugal
e-mail: lmartins@itqb.unl.pt

M.P. Robalo
Área Departamental de Engenharia Química, Instituto Superior de Engenharia de Lisboa,
Rua Conselheiro Emídio Navarro, 1959-007 Lisbon, Portugal

M.P. Robalo
Centro de Química Estrutural, Instituto Superior Técnico, Universidade de Lisboa,
Av. Rovisco Pais, 1049-001 Lisbon, Portugal

S.N. Singh (ed.), *Microbial Degradation of Synthetic Dyes in Wastewaters*,
Environmental Science and Engineering, DOI 10.1007/978-3-319-10942-8_2

environmental hazardous chemical additives (Forgacs et al. 2004; Chen 2006). Biological treatment technologies are attractive alternatives to the traditional physicochemical methods, as they are low-cost, environmentally friendly and can selectively provide a complete degradation of organic pollutants without collateral destruction of either the site's flora or fauna (Anjaneyulu et al. 2005; Chen 2006; Husain 2006; Rodriguez Couto 2009a). It has been demonstrated that microorganisms are able to degrade synthetic dyes to non-colored compounds or even mineralize them completely under certain environmental conditions (dos Santos et al. 2007; Saratale et al. 2011; Solís et al. 2012; Chengalroyen and Dabbs 2013; Khan et al. 2013). However, the fact that most of dye pollutants persist for long periods in the environment indicates the natural inadequacy of microbial activity to deal with these xenobiotic compounds. Biological systems need to exhibit not only a high catalytic versatility towards the degradation of a complex mixture of structurally different dyes, but also a superior robustness against the toxic effects of the dyes and their products, in addition to the salts, detergents, surfactants, and metals present in the dye-containing effluents, often at extreme pHs or high temperatures (Anjaneyulu et al. 2005; Chen 2006). Considering these requirements, there is currently no simple solution for the biological treatment of dye-containing effluents.

Enzymatic processes are particularly sought for the treatment of dye-containing effluents, mainly because of their specificity and relatively ease of engineering towards improved robustness; enzymes only "attack" the dye molecules, while valuable dyeing additives or fibers are kept intact and can potentially be re-used (Kandelbauer and Guebitz 2005). Likewise, new recycling technologies will allow a huge reduction in water consumption in the textile finishing industry. Although dye molecules display high structural diversity, they are only degraded by a few enzymes that share common mechanistic features as they all catalyze redox reactions and, exhibit relatively wide substrate specificities. The most important dye degrading enzymes are: azoreductases, laccases and peroxidases (Kandelbauer and Guebitz 2005). Azoreductases are oxidoreductases, which are particularly effective in the degradation of azo dyes through reduction of the azo linkage, the chromophoric group of azo dyes (Kandelbauer and Guebitz 2005; Rodriguez Couto 2009b). The majority of characterized azoreductases are FMN or FAD dependent enzymes that require the addition of NAD(P)H as electron donors for the reduction of azo dyes releasing aromatic amines as products (Stolz 2001; Deller et al. 2008). Laccases are multi-copper oxidases that couple the one-electron oxidation of four substrate molecules to the four electron reductive cleavage of the O–O bond of dioxygen to water. These enzymes have a great potential in various biotechnological processes mainly due to their high non-specific oxidation capacity, the lack of requirement for cofactors, and the use of the readily available molecular oxygen as an electron acceptor (Stoj and Kosman 2005; Morozova et al. 2007; Haritash and Kaushik 2009; Mikolasch and Schauer 2009). These include the detoxification of industrial effluents (Rodriguez Couto and Toca Herrera 2006), mostly from the paper and pulp, textile and petrochemical industries, and bioremediation to clean up herbicides, pesticides and certain explosives in soil (Morozova et al. 2007; Haritash

and Kaushik 2009). The capacity of laccases to produce polymeric products also makes them a useful tool for organic synthesis (Riva 2006; Madhavi and Lele 2009) and in addition, are also potential enzymes for biosensors or biofuel cells (Wheeldon et al. 2008; Willner et al. 2009). Peroxidases are heme-containing proteins that use hydrogen peroxide (H_2O_2) or organic hydroperoxides (R-OOH) as electron accepting co-substrates while oxidizing a variety of compounds. Due to their catalytic versatility and enzymatic stability, peroxidases are of particular interest for industrial redox conversion processes (Hofrichter et al. 2010). Among peroxidases, a new super family has arisen, the so-called dye-decolorizing peroxidases (DyPs) (Sugano 2009; Hofrichter et al. 2010; Colpa et al. 2013). These enzymes are known to successfully oxidize a wide range of substrates, but most importantly, they highly degrade high redox synthetic dyes, such as anthraquinone and azo dyes. In this paper, we have reviewed the enzymatic properties, mechanisms and toxicity of dye-degradation products of different bacterial enzymes and also the properties of in vitro and in vivo multi-enzymatic systems for the decolorization of synthetic dyes.

2 Biotransformation of Dyes Using Laccases

Laccases are a part of the large multi-copper oxidase family of enzymes that catalyze the four-electron reduction of oxygen to water (at the T2–T3 trinuclear Cu centre) 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. However, many laccases have been isolated from bacteria in the last decade (Claus 2003; Giardina et al. 2010). Fungal laccases are the enzymes used in the vast majority of the studies in the literature, but bacterial laccases show advantages for biotechnological processes due to the lack of post-translational modifications, their higher yields of production, easiness of manipulation and improvement by protein engineering approaches.

2.1 Decolorization Capacity of Bacterial CotA-Laccase

The first study, using bacterial laccases for synthetic dyes decolorization, was performed with recombinant CotA-laccase from *Bacillus subtilis*, which is a bacterial thermoactive and intrinsically thermostable enzyme (with half-life of 2 h at 80 °C), showing the predictable robustness for biotechnological applications (Pereira et al. 2009a, b). Twenty two synthetic dyes, both anthraquinonic and azo dyes, were found to be degraded to different extents, after 24 h of reaction by CotA-laccase (Fig. 1).

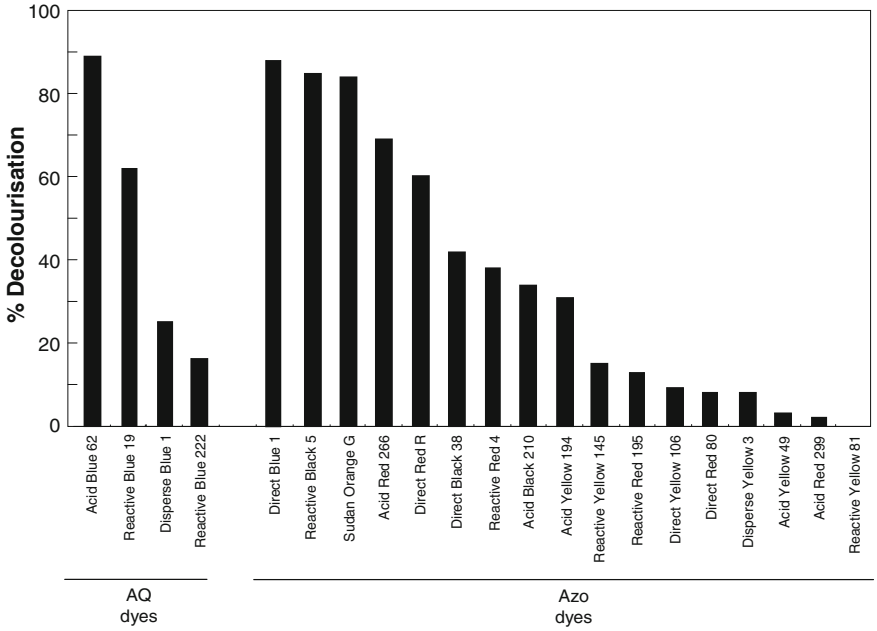

Fig. 1 Decolorization of several of anthraquinonic (AQ) and azo dyes after 24 h of reaction in the absence of redox mediators by using CotA-laccase (adapted from Pereira et al. 2009b)

Two major differences were observed when compared to dye degradation using fungal laccases: (1) the non requirement of redox mediators and (2) a maximal activity at the neutral to alkaline range of pH. The lack of a strict requirement for redox mediators exhibited by bacterial CotA-laccase constitutes a significant advantage over fungal enzymes from a technological perspective. Low-molecular weight compounds are expensive and a large quantity in relation to the substrate is often required. Moreover, some mediators give rise to highly unstable compounds that can lead to enzyme inactivation and are toxic upon release into natural environments. The requirement of redox mediators, acting as electron shuttles, is usually justified to overcome the steric hindrance of substrates that impairs its proper approach to the enzyme's catalytic center or the high redox potential of the substrates in comparison to the enzyme (Bourbonnais and Paice 1990). Interestingly, it was observed that CotA, a low redox laccase (E^o = 525 mV vs. NHE), is able to degrade high redox compounds, e.g. the azo dye reactive black 5 (E^o = 742 mV) to a higher extent in the absence of redox mediators, in contrast to what was observed with high-redox potential fungal laccases (E^o ~780 mV vs. NHE) which requires the presence of redox mediators (Abadulla et al. 2000; Zille et al. 2004; Camarero et al. 2005; Tauber et al. 2005). This indicates that redox potential is not the only or the most important parameter to be considered in what concerns substrate oxidation by laccases (Durão et al. 2006).

The optimal pH for dye-decolorization by CotA-laccase around 8–9 is a distinctive feature shared with other bacterial laccases from *Streptomyces ipomoea* (Molina-Guijarro et al. 2009), *Bacillus vallismortis* (Zhang et al. 2012) or *Bacillus subtilis* X1 (Guan et al. 2013) which is in contrast with the optimal pH values in the acidic range shown by laccases of fungal origin (Abadulla et al. 2000; Almansa et al. 2004; Maier et al. 2004; Camarero et al. 2005; Rodriguez Couto et al. 2005; Zille et al. 2005a, b; Pogni et al. 2007). In order to explore the enzymatic mechanism of azo dyes degradation, Sudan orange G (SOG) was selected for more detailed investigations. Two pKa values for SOG were measured using potentiometric measurements, 6.90 ± 0.02 and 11.74 ± 0.02, which were attributed to *ortho* and *para* hydroxyl groups of the azo dye (Pereira et al. 2009b). Based on this data, the oxidation of SOG is mostly dependent on the protonation-deprotonation equilibrium of the more acidic hydroxyl group of the substrate molecule, since maximal rates are found at pH 8, above the pKa value of the *ortho* hydroxyl group of SOG. This is in contrast with fungal laccases, which, in agreement with their optimal pH at acidic ranges, oxidise more easily the protonated form of the dye. The results obtained with CotA for the oxidation of SOG are consistent with data obtained using syringyl-type phenolic compounds, where maximal enzymatic rates were also observed at pH values above the pKa value of the compounds tested which confirmed the preference of CotA for deprotonated phenolic groups (Rosado et al. 2012). The differences in the optimal pH, as observed in bacterial and fungal laccases, are most probably related to the presence of a conserved negatively charged residue close to the substrate binding cavity of fungal laccases and absent in CotA or in any bacterial laccase identified so far and proposed to stabilize the formation of the phenoxy radical during the catalytic reaction of fungal laccases (Bertrand et al. 2002; Piontek et al. 2002; Madzak et al. 2006; Kallio et al. 2009; Rosado et al. 2012). Therefore, the oxidation of phenolic groups by bacterial laccases without any carboxylic acid residue in the substrate binding site is strictly dependent on the chemical nature of the substrates i.e. maximal rates are found at pH values above the pKa values, when phenolate anions, which are more prone to oxidation than the phenol form, are present at higher concentrations.

2.2 Azo Dyes Biotransformation

The transformation of SOG (Pereira et al. 2009a) resulted in a decrease in the intensity of the dye absorption band, at λ_{max} = 430 nm, with concomitant increase in absorption bands at 325 and 530 nm, indicating the generation of biotransformation products (Fig. 2a).

The time course of SOG biotransformation was additionally monitored by HPLC (Fig. 2b), where SOG was chromatographically separated from products of the enzymatic reaction. A major peak with R_t of 5 min, corresponding to the substrate which decreased over the time course of the reaction and disappeared after 7 h

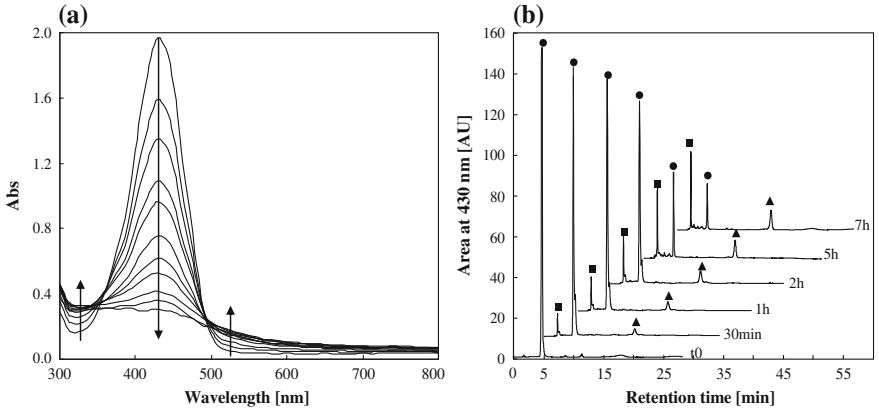

Fig. 2 Time course for Sudan orange G (SOG) biotransformation as monitored by absorbance (**a**) and by HPLC (**b**). [(**b**) *black circle* SOG and products: *black square* R_t 2.2 min and, *black triangle* R_t 15 min] (Pereira et al. 2009b)

(Fig. 2b). Two major products emerged at R_t of 2.2 and 15 min. The CotA efficiency of 8×10^3 M^{-1} s^{-1}, is in the same order of magnitude to those calculated for fungal laccases (Pereira et al. 2009b). The assay mixtures became browner in color over the course of reaction, presumably due to formation of products (Fig. 3). After centrifugation the final reaction mixture, the supernatant contained the compounds corresponding to the major peak with R_t of 2.2 min and the pellet contained the major product with R_t of 15 min. The full identification of this latter fraction was 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 was performed only in the soluble part of acetonitrile-dissolved fraction. The structural identification of twelve SOG biotransformation products (Fig. 4b) was based on ESI-MS and MALDI-TOF MS data in combination with a putative degradation pathway (Fig. 4a) based on the accepted model for azo dye degradation by laccases (Chivukula and Renganathan 1995; Zille et al. 2005a, b).

Our results indicate that the enzymatic electron transfer occurs upon oxidation of SOG deprotonated hydroxyl group. 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 dye chromophoric structure in accordance with previous reports (Chivukula and Renganathan 1995; Zille et al. 2005a, b). In addition, the presence of these products can undergo coupling reactions between themselves or with unreacted dye molecules, producing a large array of oligomeric products (Fig. 4b). The presence of these compounds is in accordance with the darkening of the enzymatic reactions, the high insolubility of products formed, and also with the reduced toxicity of the final reaction mixture as compared to solutions of intact SOG which was tested using a yeast-based bioassay (Pereira et al. 2009b).

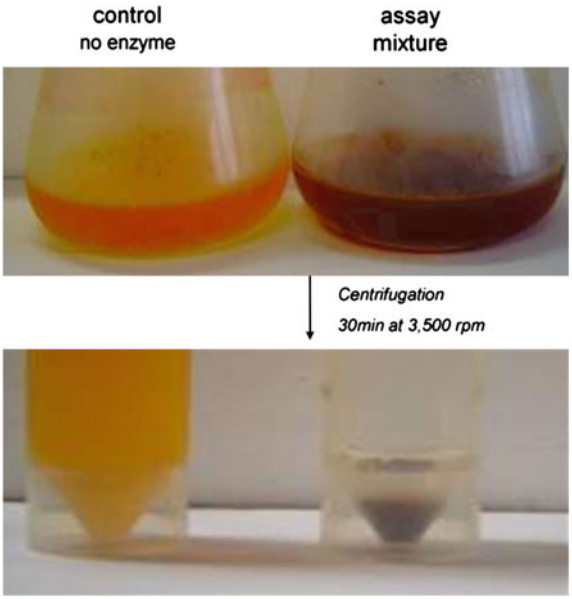

Fig. 3 Reaction mixtures: control and in the presence of enzyme after 24 h, showing the darkening of the enzymatic treated solution, most likely due to the high insolubility of the oligomeric products formed

2.3 Anthraquinonic Dyes Biotransformation

The transformation of the anthraquinonic model dye acid blue 62 (AB62) was carried out using CotA-laccase (Pereira et al. 2009b, Fig. 5a) or Lac3 from *Trametes* sp. C30 following the research initiated in Sophie Vanhulle group (Trovaslet et al. 2007; Vanhulle et al. 2008a, b). The degradation of AB62 resulted in a decrease in the intensity of the dye absorption bands, at λ_{max} = 600 and 630 nm, along with an increase in absorption around 500 nm due to the formation of reddish biotransformation products (Vanhulle et al. 2008a).

The time course of the biotransformation of AB62 was additionally monitored by HPLC (Fig. 5b). The AB62 biotransformation resulted in a product with a R_t of 13 min that appeared in the first minutes of reaction, although, as the reaction proceeded, it decreased concomitantly with the appearance of a new product with R_t of 50 min (Fig. 5b). The CotA steady-state the catalytic efficiency (k_{cat}/K_m) for AB62 oxidation is 5×10^3 M^{-1} s^{-1} around 2–3 fold lower when compared to other fungal laccases, including Lac3 from *Trametes* sp. C30 (Klonowska et al. 2002, 2005; Vanhulle et al. 2008a). The biotransformation products were identified after purification in the enzymatic reaction mixtures by NMR, MS/MS^n, LC-MS and GC-MS analysis. Using ^1H NMR and MS/MS^n was possible to identify the intermediate product DAAS (R_t = 13 min) and the final product of the reaction (4)

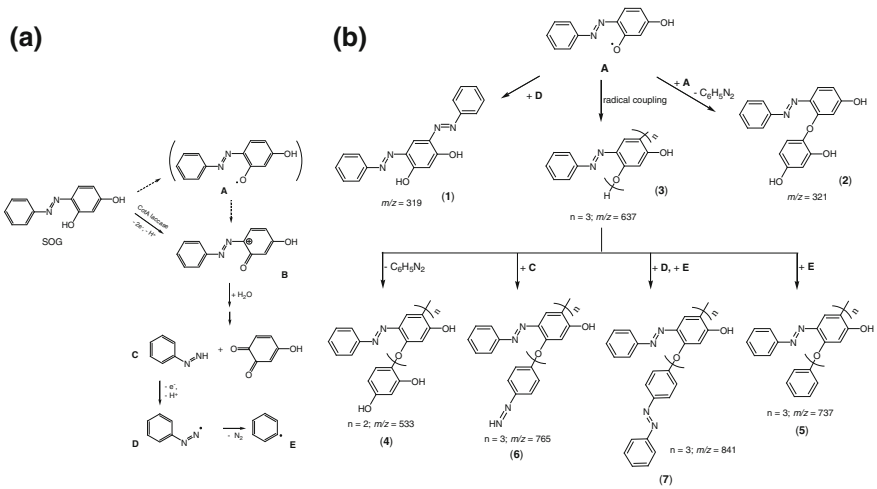

Fig. 4 Proposed mechanism for the biotransformation of SOG by CotA-laccase (**a**) and proposed structures (1)–(7) for the oxidation products (**b**). The oxidation of azo dyes occurs without the cleavage of the azo bond, through a highly non-specific free radical mechanism resulting in the formation of phenolic type compounds. Following this mechanism, CotA-laccase oxidizes one hydroxyl group of SOG generating the phenoxyl radical A, sequentially oxidized to a carbonium ion (B). The water nucleophilic attack on the phenolic carbonium, followed by N–C bond cleavage, produces diazenylbenzene (C) and the 4-hydroxy-1,2-benzoquinone. The diazenylbenzene (C) can lead to the radical (D) and then, to a benzene radical (E) upon loss of a nitrogen molecule. All these radicals were involved in further coupling reactions (adapted from Pereira et al. 2009b)

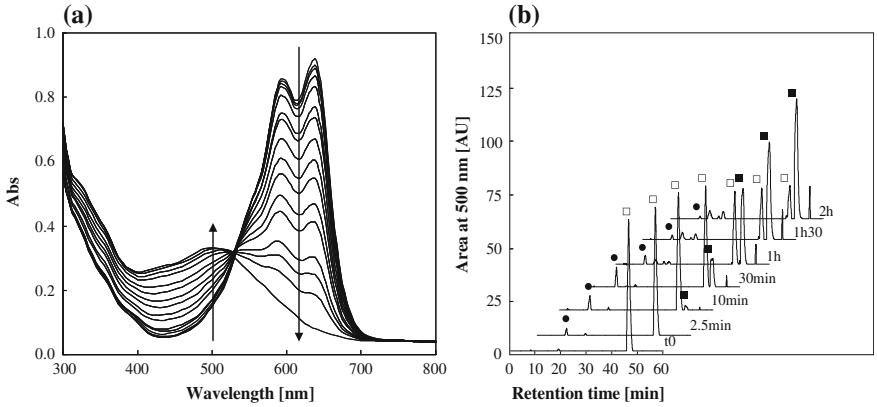

Fig. 5 Time course for acid blue 62 (AB62) biotransformation as monitored by absorbance (**a**) and by HPLC (**b**). [(**b**) *white square* AB62 and products: *black circle* R_t 13 min and, *black square* R_t 40 min] (adapted from Pereira et al. 2009a)

(R_t = 50 min) (Fig. 5b). The proposed mechanism of biotransformation of AB62 by laccases is illustrated in Fig. 6, showing the pathway for formation of an azo bound in (4) which is responsible for the color observed in the reaction mixtures.

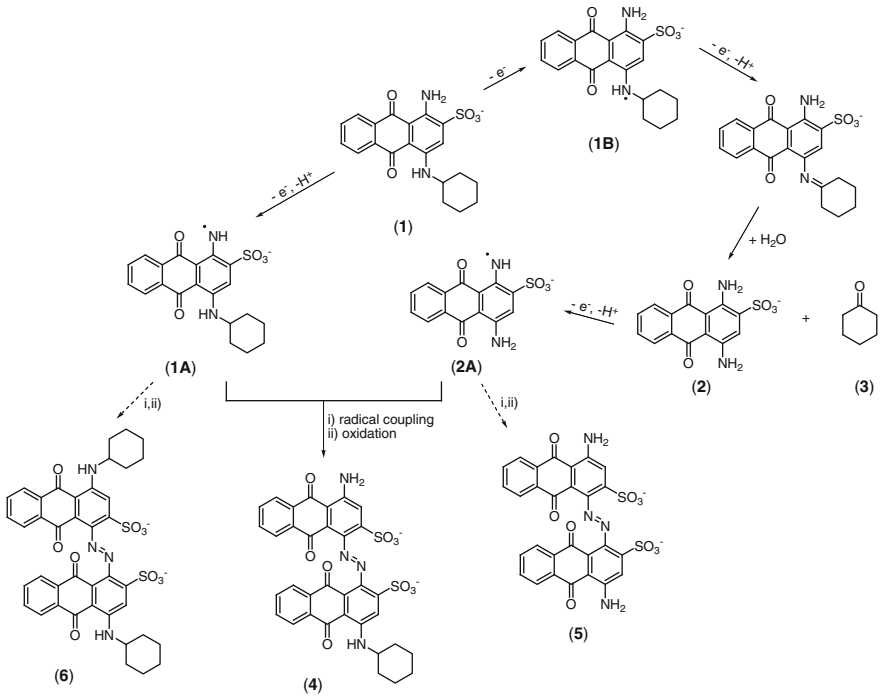

Fig. 6 Proposed mechanism of AB62 biotransformation by laccases. Two oxidative routes are possible, since laccases are able to catalyze a single-electron oxidation, either from the primary or the secondary amines of the compound 1 (AB62). The reactive radical species 1A and 1B are formed, and 1B was sequentially oxidized into an imine, which hydrolyzes leading to cyclohexanone (3) and the first intermediate 2 (DAAS). Compound 2 could be further oxidized and the resulted radical (2A) should lead to the formation of the main product of the reaction, the azo dimer (4) by cross-coupling reaction with 1A, followed by an oxidative step that could also be catalyzed by the enzyme. Similar dimerization processes of radicals 2A or 1A should end in the formation of compounds 5 and 6, but in a very low extent. The final product 4 was identified by both NMR and MS techniques and the formation of compounds 5 and 6 was supported by LC-MS^n analysis of the reaction mixtures (adapted from Pereira et al. 2009a)

The toxicity of synthetic dyes as well as of their bioconversion products presents a great environmental concern (O'Neil et al. 1999; Robinson et al. 2001). AB62 causes a significant inhibitory effect on yeast growth and values of LOEC and IC_{50} of around 7 and 420 μM (3 and 177 mg L^{-1}) respectively, were estimated. The IC_{50} is well above the expected dye concentrations in the environment, but is within the same order of magnitude of the typical dye concentration in spent dye baths (Robinson et al. 2001). The mixture, containing AB62 biotransformation products after 2 h of reaction with CotA-laccase, was significantly less toxic to the yeast cell population. Consistent with the reduced overall toxicity of AB62 solution, compound 4, the reddish azo product that accumulates during the biotransformation reaction, is significantly less toxic to the yeast (LOEC ~45 μM, $IC_{50>}$750 μM) than the parent molecule.

3 Biotransformation by Bacterial Azoreductases

Azoreductases is a generic name given to enzymes involved in the reduction of azo bonds (–N=N–) and azoreductase activity was identified in several organisms including algae, yeast and bacteria (Saratale et al. 2011; Solís et al. 2012; Chengalroyen and Dabbs 2013; Khan et al. 2013). These enzymes are flavin-independent or flavin-dependent oxidoreductases which utilize NADH and/or NADPH as an electron donor and catalyze the reductive cleavage of the azo bonds to produce colorless aromatic amine products under anaerobic or aerobic conditions. Flavin-dependent azoreductases share strong similarities with regard to sequence, structure, and reaction mechanism with the larger family of flavin-dependent quinone reductases that include Lot6p from *Saccharomyces cerevisiae* and the mammalian NQO1 (Deller et al. 2008). These enzymes are involved in the reduction of quinones, quinoneimines, azo dyes, and nitro groups to protect the cells against the toxic effects of free radicals and reactive oxygen species arising from electron reductions. They are assumed to take part in the organism's enzymatic detoxification systems; e.g., the azoreductases from *E. coli* and *B. subtilis* were recently implicated in the cellular response to thiol-specific stress (Towe et al. 2007; Leelakriangsak et al. 2008; Liu et al. 2009) and Lot6p, the azoreductase homologue in *S. cerevisiae* has been implicated in the response to oxidative stress (Sollner et al. 2007, 2009). Furthermore, as additional members of this family of enzymes are discovered, the list of transformed substrates continues to grow. Evolutionarily, these enzymes may provide a selective advantage to bacteria under various conditions of environmental stress (Khersonsky and Tawfik 2010).

3.1 Decolorization of Azo Dyes by PpAzoR from Pseudomona putida *MET94*

In an effort to find bacterial strains with a superior ability to degrade synthetic dyes, a collection of 48 bacterial strains was screened to select the strain *P. putida* MET94 for its superior ability to decolorize a diverse array of azo dyes to higher extent (Mendes et al. 2011b) (Fig. 7).

A BLAST search of the *P. putida* genome was performed and a 612-bp ORF encoding a 203 amino acid residue was identified containing all the conserved motif patterns of flavin-dependent azoreductases (Wang et al. 2007) and was, therefore, named PpAzoR (*Pseudomonas putida* azoreductase). The *ppAzoR* gene was cloned and expressed in *E. coli*. Subsequently, the recombinant FMN-dependent PpAzoR protein was purified and thoroughly characterized following kinetic, spectroscopic and biochemical and structural approaches (Correia et al. 2011; Mendes et al. 2011b; Gonçalves et al. 2013). It was observed that PpAzoR reduced several quinones (anthraquinone-2-sulfonic acid (AQS), 1,4-benzoquinone, catechol, 2-hydroxy-1, 4-naphtoquinone (Lawsone), 1,2-naphthoquinone) at rates 10–100 times higher than

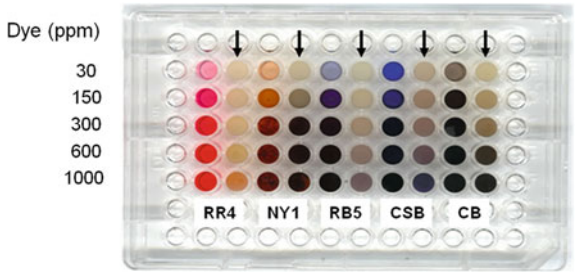

Fig. 7 Screening for decolorization of reactive red 4 (RR4), acid red 299 (NY1), reactive black 5 (RB5), direct blue 1 (CSB) and direct black 38 (CB) at increasing concentrations using growing cells of *P. putida* MET94

azo dyes (Mendes et al. 2011b; Brissos et al. 2014). The steady-state kinetic analysis, using 1,4-benzoquinone or reactive black 5 and NADPH, resulted in a family of parallel lines in a double reciprocal plot (Mendes et al. 2011b; Gonçalves et al. 2013) which is indicative of a ping-pong bi-bi kinetics as described for other flavin-dependent azoreductases. The efficiency for 1,4-benzoquinone is one and two orders of magnitude higher than azo dyes (V_{max} = 50 U mg^{-1}, $K_{m\ app}$ = 0.005 mM, $k_{cat\ app}$ = 49 min^{-1}, k_{cat}/K_m = 98 × 10^5), showing that quinones represent most probably the physiological substrates of this enzyme in *P. putida* cells.

PpAzoR (PDB code 4C0 W) is a homodimer and its tertiary structure adopts a flavodoxin-like fold characterized by a central twisted five parallel β-sheet connected by α-helices, which flank the sheet from the front and the back (Correia et al. 2011; Gonçalves et al. 2013). The arrangement of the α-helices and β-stands is identical to structures of azoreductases from *E. coli* (PDB code 2Z98), *Pseudomonas aeruginosa* (PDB code 2V9C), *Enterococcus feacalis* (PDB code 2HPV) and *Salmonella typhimurium* (PDB code 1T5B). Moreover, it contains the conserved motif patterns of flavin-dependent azoreductases, i.e. the sequence involved in the binding of FAD/FMN co-factors, the sequence involved in the dimerisation of the two monomers of the enzyme and the possible putative NAD(P)H binding motif. The crystal structures of native PpAzoR (1.6 Å) and PpAzoR complexed with anthraquinone-2-sulphonate (1.5 Å) or reactive black 5 (1.9 Å), were solved revealing the residues and subtle changes that accompany substrate binding and release. Such changes highlight the fine control of access to the catalytic site and tune the specificity offered by the enzyme towards different substrates. In particular, it helps to explain how PpAzoR allows for the accommodation of bulky substrates explaining its enlarged substrate utilization with similar catalytic efficiencies (Gonçalves et al. 2013).

The enzymatic activity of PpAzoR was tested using 18 structurally different synthetic dyes by measuring the decolorization levels after 24 h of incubation under anaerobic conditions. The results show that PpAzoR exhibits a broad substrate specificity with decolorization levels above 80 % for most of the dyes tested (Fig. 8).

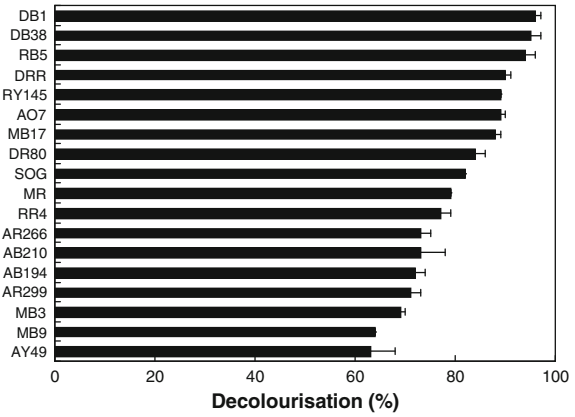

Fig. 8 Decolorization of several azo dyes after 24 h of reaction under anaerobic conditions using PpAzoR (Mendes et al. 2011a)

The specificity of PpAzoR was investigated by measuring the initial rates of reduction of a set of structurally different azo dyes under anaerobic conditions (Mendes et al. 2011b). PpAzoR uses either NADPH or NADH as electron donor, but the efficiency for NADPH is twice that of NADH (Gonçalves et al. 2013). The enzyme is particularly unspecific with regard to the azo dyes used, showing only smooth trends with methyl red and reactive black 5 representing the substrates reduced with higher specificity (around 1–2.5 × 10^3 M^{-1} s^{-1}) and mordant black 9 and acid orange 7 reduced at the lowest efficiency (around 0.3–0.4 × 10^3 M^{-1} s^{-1}). The affinity for dyes is reduced with K_m values between 0.1 and 4 mM indicating the need of adding 1–40 mM of dyes (10 × the K_m value) to the reaction mixtures in order to accurately measure the maximal rates of dye degradation (Mendes et al. 2011b). The high concentration of dyes leads to initial absorbance values out of the Lambert-Beer law's applicability range. Therefore, the reaction assays to determine the kinetic parameters for dye consumption need to be performed using a photometric discontinuous method, where samples are withdrawn from reactions at time intervals, diluted and the absorbance measured at the maximum wavelength for each substrate.

To further characterize the properties of PpAzoR, in particular the oxygen-sensitivity of PpAzoR to oxygen, the initial rate of reactive black 5 degradation was measured as a function of oxygen concentration (Fig. 9a). The results show that the rates of dye decolorization decreased with increased O_2 concentration. This is in conformity with the low levels of dye decolorization by growing or resting cells of *P. putida* MET94 cells under aerobic conditions. In order to test if oxygen is a non-competitive, competitive inhibitor or instead is substrate for the PpAzoR enzyme, oxygen consumption was measured in a reaction containing only enzyme, buffer and NADPH (Fig. 9b). The addition of catalase resulted in a 2-fold increase in the concentration of dioxygen in the mixture, showing that peroxide is in the solution most likely as a result of PpAzoR activity (Fig. 9b). When catalase was added at the beginning of the reaction, only half of the possible concentration of oxygen produced was attained (Fig. 9b). These results clearly show that oxygen is reduced to peroxide by PpAzoR.

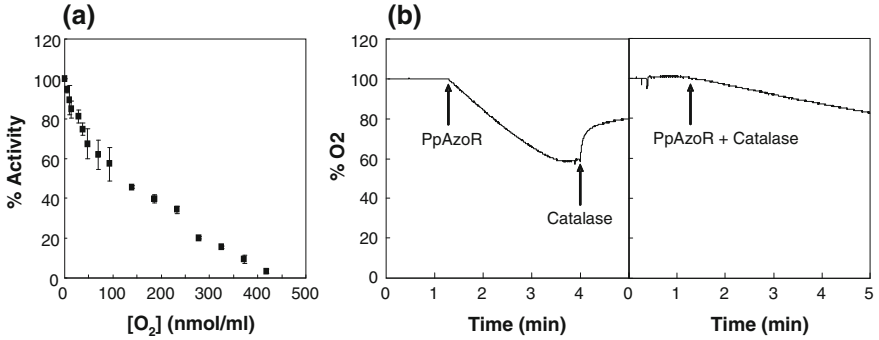

Fig. 9 **a** Inhibition of dye decolorization by increasing concentrations of dioxygen. **b** Consumption of oxygen with an "Oxygraph" equipped with a Clark oxygen electrode. The chamber volume (1 mL) contained 0.25 mM of NADPH in 20 mM Tris-HCl, pH 7.6 buffer. Reaction was initiated by the addition of 3.5 µM PpAzoR and 1,500 Units of catalase (Sigma) was added at different time periods, as indicated by the arrows

The steady state kinetics of PpAzoR for oxygen reduction (V_{max} = 5 U mg^{-1}, $K_{m\ app}$ = 0.1 mM, $k_{cat\ app}$ = 238 min^{-1}, k_{cat}/K_m = 7 × 10^4 M^{-1}.s^{-1}) showing higher affinity and one order of magnitude higher specifity as compared to dyes reduction which explains the competitive catalytic behaviour of oxygen and thus, the "oxygen sensitive" character of PpAzoR and the need to perform the decolorization of dyes under anaerobic conditions.

3.2 The Catalytic Mechanism of PpAzoR and the Toxicity of Dye Products

The ping-pong bi–bi mechanism is indicative that PpAzoR reduces the substrates in 2 distinct steps: first, a complete reduction of the FMN co-factor; and second, transfer of these electrons from the flavin to the substrate, resulting in the formation of the corresponding putative hydrazo derivatives. This reaction cycle proceeds a second time and delivers the necessary 4 electrons in order to obtain a complete reduction of the substrates into the final products. The mechanism of azo reduction by PpAzoR was supported by the detection of aromatic amines by HPLC (Fig. 10).

The reported high toxicity of the azoreductase products relates to the toxic nature of the aromatic amines formed (Pinheiro et al. 2004). Therefore, the toxicity of the azo dyes and PpAzoR enzymatic products was tested based on the inhibitory effects on the growth of *Saccharomyces cerevisiae* and on the reproduction inhibition of *Caenorhabditis elegans* (Mendes et al. 2011a). In general, the toxicity of intact dyes correlates well between the 2 model eukaryotic organisms tested (Fig. 11). Nevertheless, *C. elegans* seems more susceptible to the presence of intact dyes, since some dyes show more than 2-fold higher inhibition for the nematodes reproduction than for *S. cerevisiae* growth.

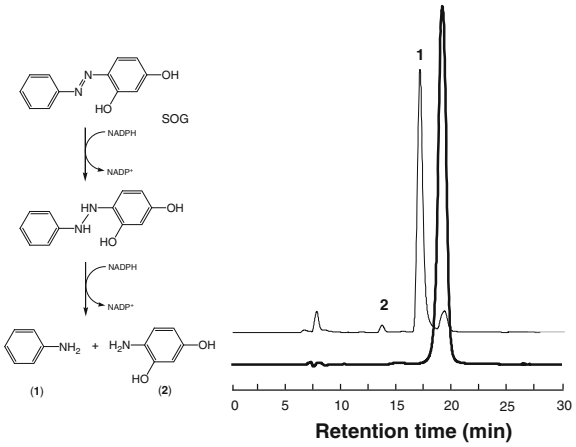

Fig. 10 Proposed mechanisms for the biotransformation of the azo dye Sudan orange G by PpAzoR and HPLC chromatograms of the reaction mixture after 24 h of reaction with (*thin line*) or without (*thick line*) PpAzoR. Products of the reaction were identified, in comparison to the standards: (1) aniline, (2) 4-aminoresorcinol

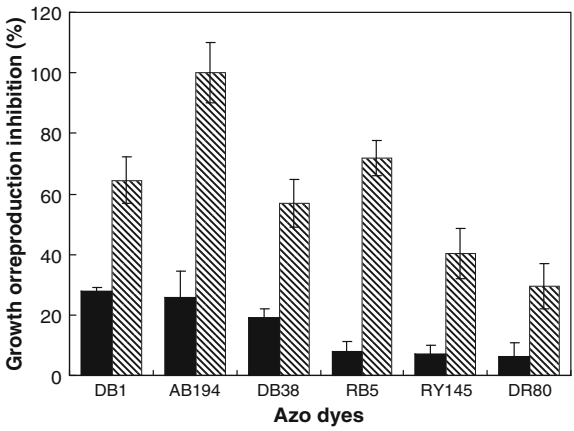

Fig. 11 Inhibitory effects of intact dyes over *Saccharomyces cerevisiae* (*dark bars*) and *Caenorhabditis elegans* (*dashed bars*) (adapted from Mendes et al. 2011a)

However, for the majority of the other dyes tested, the enzymatic products present a higher toxicity than intact dyes themselves, as assessed by the *S. cerevisiae* system, exhibiting 2 to 4-fold higher toxicity than intact dyes (Fig. 12) (Mendes et al. 2011a).

3.3 Engineering of PpAzoR for Improved Thermal Stability

PpAzoR broad substrate specificity makes it attractive for bioremediation processes, but its low thermal stability (half life of 13 min at 50 °C) impairs its full potential for environmental related applications. Thermal stability is a critical property, as it

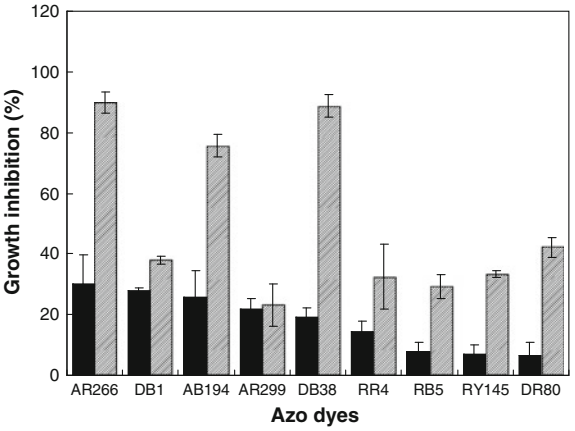

Fig. 12 Inhibitory effects of intact dyes on *Saccharomyces cerevisiae* (*dark bars*) and of 24 h-reaction mixtures treated with PpAzoR (*dashed bars*) (adapted from Mendes et al. 2011a)

correlates with longer life-times of enzymes and frequently relates also to higher tolerance to the presence of organic co-solvents, extreme pH values and high salt concentration or pressures, harsh conditions frequently found in industrial processes. Therefore, thermostable PpAzoR variants were generated by directed evolution (Brissos et al. 2014). Directed evolution is considered to be the most powerful approach for improving the thermostability of proteins. Different properties in various target enzymes have been successfully improved using directed evolution approaches (Kaur and Sharma 2006; Böttcher and Bornscheuer 2010; Wang et al. 2012). After five rounds of random mutagenesis, recombination and high-throughput screening, a thermostable 1B6 variant was identified. Noteworthy purified 1B6 variant enzyme maintains its full activity after incubation for 1 h at temperatures between 40 and 85 °C in clear contrast with the wild type enzyme that totally looses activity after 1 h at 50–55 °C (Fig. 13a). The kinetic or, the so called, long term stability was measured, showing that 1B6 is remarkably more stable than wild type with nearly a 300-fold higher half-life, i.e. retaining 50 % of activity after 58 h at 50 °C, while wild type enzyme takes 13 min to lose half of its initial activity (Fig. 13b). Therefore, a hit variant of PpAzoR was identified with increased resistance to inactivation, showing full reversibility of the unfolded state upon thermal inactivation i.e. it could be maintained at high temperatures for prolonged periods of time without losing its ability to be active at lower temperatures with an encouraging potential for biotechnological applications.

4 Biotransformation of Dyes Using Bacterial Dye-Decolorizing Peroxidases

Heme peroxidases catalyse the H_2O_2-dependent oxidation of a variety of substrates, most commonly small organic substrates, playing multiple physiological roles in a wide range of living organisms. Considering their broad specificity, these enzymes

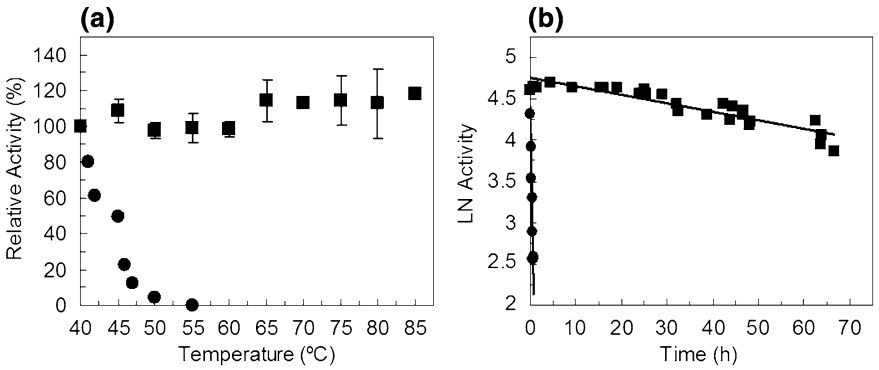

Fig. 13 **a** PpAzoR activity measured at 30 °C after incubation at different temperature (55–70 °C) for 1 h: wild type PpAzoR (*circles*) and 1B6 variant (*squares*). **b** Thermal inactivation of wild type PpAzoR (*circles*) and 1B6 variant (*squares*). Enzyme samples were incubated at 50 °C and catalytic activity was measured at known time intervals at 30 °C (adapted from Brissos et al. 2014)

have a considerable potential for application in many different areas. In particular, the interest in ligninolytic peroxidases, harbouring the highest redox potential among peroxidases, for biotechnological applications has increased rapidly in industrial areas related with the biorefineries, in particular for the selective delignification of lignocellulosic materials for production of biofuels (Martinez et al. 2009; Ruiz-Duenas and Martinez 2009). These enzymes are also suitable for environmental applications, including the treatment of toxic effluents, containing synthetic dyes, generated in various industrial processes (Wesenberg et al. 2003; Kandelbauer and Guebitz 2005; Husain 2006; Rodriguez Couto 2009b; Chacko and Subramaniam 2011; Khan et al. 2013). However, these enzymes are still not commercially available, in part due to constraints related to the genetic manipulation and relatively low levels of protein expression in both native and fungal host strains.

A new family of microbial peroxidases, known as dye decolorizing peroxidases (DyPs), was demonstrated to successfully degrade not only high redox anthraquinone-based, but also azo dyes, β-carotene (Scheibner et al. 2008), aromatic sulfides (van Bloois et al. 2010), phenolic or non-phenolic lignin compound units (Liers et al. 2010; van Bloois et al. 2010; Brown et al. 2012) and manganese (Roberts et al. 2011; Brown et al. 2012). The physiological function of these enzymes is at present unclear, but there are increasing evidences of their involvement in the degradation of lignin (Ahmad et al. 2011; Salvachua et al. 2013; Singh et al. 2013), and therefore, DyPs seem to have the potential to replace the high-redox fungal ligninolytic peroxidases in biotechnological applications. DyPs have primary sequence, structural and apparently, mechanistic features, unrelated to those of other known "classic" plant and animal peroxidases (Sugano et al. 2007; Liu et al. 2011; Yoshida et al. 2011; Singh et al. 2012; Strittmatter et al. 2012). The uniqueness of these enzymes is, therefore, interesting both at the fundamental and

applied perspectives. Importantly, DyPs, first discovered in fungi, were later identified in a wide range of bacterial strains. In fact, the increasing number of putative DyP-type peroxidases, identified in genomes and proteomes of bacteria, leads to the suggestion that this super family should be renamed into the super family of bacterial peroxidases (Colpa et al. 2013). DyPs have been classified into four phylogenetically distinct sub-families, with bacterial enzymes constituting A-C sub-families and fungal enzymes belonging to D sub-family (Ogola et al. 2009).

DyPs show characteristic conserved residues in the heme-binding site, in particular the characteristic GXXDG motif and an aspartate residue replacing the distal histidine, which acts as the acid-base catalyst in classical peroxidases (Sugano 2009; Hofrichter et al. 2010; Colpa et al. 2013). Structurally, these DyPs comprise two domains that contain α-helices and anti-parallel β-sheets, unlike plant and mammalian peroxidases, that are primarily α-helical proteins (Colpa et al. 2013). Both domains adopt a unique ferredoxin-like fold and form an active site crevice with the heme co-factor sandwiched in between (Colpa et al. 2013).

The cloning and characterization of two new DyPs, one from *Pseudomonas putida* MET94 designated as PpDyP (*P. putida* DyP) and another from *Bacillus subtilis*, called BsDyp (*B. subtilis* DyP) were recently described (Santos et al. 2014). The biochemical characterization of these bacterial enzymes allowed assessing their versatility and catalytic efficiency towards structurally different type of substrates as well as their stability properties. The constructed phylogenetic tree shows that BsDyP belongs to subfamily A and PpDyP belongs to subfamily B (Santos et al. 2014).

4.1 PpDyP and BsDyP Performance Towards Dye Decolorization

In order to characterize the catalytic specificity of PpDyP and BsDyP for synthetic dyes, an array of both anthraquinonic and azo dyes were tested as substrates (Table 1). All the dyes were degraded by both enzymes after 24 h of reaction to different extents (Fig. 14). However, no major differences were observed in the levels of decolorization of anthraquinonic as compared to azo dyes, in contrast to other DyPs that show typically lower activities for the azo dyes (Kim and Shoda 1999; Sugano et al. 2000; Ogola et al. 2009; Li et al. 2011).

The potential of these enzymes was well demonstrated by comparing the decolorization rates (V_{max}) of the studied DyPs with the azoreductase PpAzoR and the CotA laccase. Two to 40-fold higher activities were measured in DyPs (Table 1).

The *Bacillus* enzyme BsDyP is in general a less versatile and a weaker biocatalyst than PpDyP (Santos et al. 2014). Except for the oxidation of ABTS, *Pseudomonas* enzyme shows one to two orders of magnitude higher efficiency (k_{cat}/K_m) for the different synthetic dyes (Table 2 and data not shown), manganese

Table 1 Activities of PpDyP and BsDyP as compared to PpAzoR and CotA, using 2 mM of anthraquinonic (disperse blue 1, reactive blue 5, acid blue 62 and reactive blue 19) or azo (mordant black 9, acid black 194 and acid yellow 49) dyes as substrate (Santos et al. 2014)

Substrates	V_{max} (U mg^{-1})			
	PpDyP	BsDyP	PpAzoR	CotA
AQ dyes				
Disperse blue 1	10 ± 3	3 ± 0.1	nd	0.6 ± 0.04
Reactive blue 5	11 ± 1	9 ± 1	nd	0.3 ± 0.01
Acid blue 62	9 ± 1	10 ± 0.1	nd	1.3 ± 0.9
Reactive blue 19	9 ± 2	5 ± 0.2	nd	nd
Azo dyes				
Mordant black 9	26 ± 2	4 ± 0.1	2 ± 0.1	1 ± 0.3
Acid black 194	12 ± 2	2 ± 0.1	3 ± 0.4	0.9 ± 0.2
Acid yellow 49	10 ± 1	3 ± 0.2	2 ± 0.3	2 ± 0.2

nd not detected

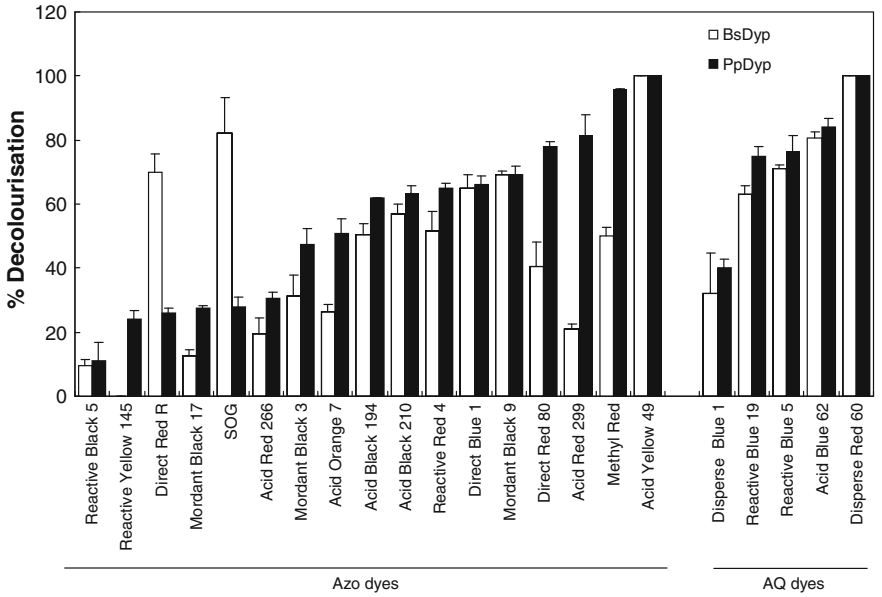

Fig. 14 Dye decolorization by the enzymes PpDyP (*black bars*) and BsDyP (*white bars*). Decolorization was measured by HPLC after 24 h of reaction (adapted from Santos et al. 2014)

or phenolic substrates tested, than the *Bacillus* enzyme. Moreover, PpDyP is able to oxidise the high redox non-phenolic veratryl alcohol compound (1.4 V) in the absence of redox mediators as DyPB from *R. jostii* and DyPs from C-D subfamilies

Table 2 Steady-state apparent catalytic constants of purified recombinant PpDyP and BsDyP (Santos et al. 2014)

Substrates	PpDyP			BsDyP		
	V_{max} (U mg^{-1})	$K_{m\ app}$ (μM)	k_{cat}/K_m (M^{-1} s^{-1})	V_{max} (U mg^{-1})	$K_{m\ app}$ (μM)	k_{cat}/K_m (M^{-1} s^{-1})
AQ dyes						
Reactive blue 5	15 ± 0.2	40 ± 3	2×10^5	11 ± 0.6	157 ± 46	5×10^4
Acid blue 62	14 ± 0.3	30 ± 4	2.4×10^5	12 ± 0.2	444 ± 45	2×10^4
Azo dyes						
Mordant black 9	32 ± 0.2	320 ± 47	5×10^4	5 ± 0.1	385 ± 46	1×10^4

of DyPs (Liers et al. 2010, 2011; Ahmad et al. 2011; Brown et al. 2012) and shows a reasonable metalloxidase activity towards ferrous ions, not detected in the *Bacillus* enzyme.

The different catalytic characteristics between members of the DyPs sub-families point to distinct heme micro-environments. The UV-visible absorption spectra of the Bs and Pp enzymes obtained upon addition of hydrogen peroxide reveal the accumulation of different catalytic intermediates. The accumulation of compound I in PpDyP is in accordance with results obtained for all other DyPs and the majority of classical peroxidases, while the accumulation of compound II intermediate in BsDyP was previously observed in A-type DypA from *R. jostii RHA1* (Roberts et al. 2011). The reasons behind the distinct spectral behaviour of BsDyP and PpDyP are possibly related to the higher redox potential of BsDyP which contributes to a relatively lower stability of Fe^{3+} and thus to a lower stability of compound I upon addition of hydrogen peroxide (Fig. 15). The poorer catalytic activity of BsDyP, as compared to the Pp enzyme, must rely to a highly abundant

$$P_{ox} + H_2O_2 \longrightarrow \text{Compound I} + H_2O \quad (1)$$

$$\text{Compound I} + AH \longrightarrow \text{Compound II} + A^{\bullet} \quad (2)$$

$$\text{Compound II} + AH \longrightarrow P_{ox} + A^{\bullet} + H_2O \quad (3)$$

Fig. 15 The three-step catalytic cycle in the classical peroxidation reaction catalyzed by peroxidases, where P_{ox} is the resting enzyme containing a ferric heme iron, Compound I is the first enzyme intermediate, which contains an oxyferryl iron center and a second oxidizing equivalent stored as a radical ($Fe(IV)=OR^{+\bullet}$) to give a formal oxidation state of +5, and Compound II is the second enzyme intermediate in which the radical is discharged leaving only the oxyferryl iron (formal oxidation state +4). AH represents the reducing substrate and $A^{\bullet}$ the radical product

catalytic incompetent 6-coordinated low spin state in the Bs enzyme, while the major population in PpDyP is the 5-co-ordinated quantum mechanically mixed spin state, as observed by resonance Raman (Sezer et al. 2013).

4.2 The Catalytic Pathway for Biotransformation of Anthraquinonic Dyes by DyPs

The transformation of the anthraquinonic dye reactive blue 5 was carried out using DyP from *Thanatephorus cucumeris* Dec 1 (Sugano et al. 2009). Changes in the visible spectrum of RB5 treated with DyP resulted in a decrease in the intensity of the dye absorption band, at λ_{max} = 600 nm, along with an increase in absorption at 400–500 nm as the color of the solution became red-brown.

Analysis of the final enzymatic reaction mixtures by NMR and MS techniques showed that the anthraquinone dye reactive blue 5 was transformed by DyP from *T. cucumeris* Dec 1 to three reaction products detected by their distinct molecular ion signals. The first product (**1**) was identified as phthalic acid by comparison with an authentic sample. The second one (**2**), with a molecular mass of 472 g mol^{-1}, can be attributed to a reactive blue 5 molecule without the anthraquinone frame (see Fig. 16). Finally, the third product (**3**) can be obtained from compound (**2**) which loss a 2,5-diaminobenzene sulfonic acid (ABS) molecule.

Based on these results, a reasonable degradation pathway of reactive blue 5 by DyP was proposed as shown in Fig. 16. The final red-brown color of the reaction mixture of reactive blue 5 biotransformation and the absence of *o*-ABS and *m*- or *p*-ABS as final products, suggest the presence of other products resulting from the dimerisation and polymerization reactions of ABS type substrates by DyP action. In fact, this was confirmed with the *o*-ABS reaction with DyP, leading to the formation of high weight colored products, from which compound **4,** containing an azo group, was identified.

4.3 Combined Sequential Enzymatic Treatment for Dye Degradation and Detoxification

In order to set-up enzymatic processes for maximal decolorization as well as detoxification, a sequential enzymatic procedure was performed combining the PpAzoR reduction of azo dyes to the oxidation of aromatic amines by CotA-laccase. It is know that laccases catalyze the oxidation of ortho- or para-substituted phenolic or aromatic amine substrates by one electron abstraction to form free radicals that undergo further coupling, polymerization, demethylation, or quinone formation (Abadulla et al. 2000; Kandelbauer and Guebitz 2005). In particular, we have shown recently that CotA-laccase catalyzed the homocoupling of primary

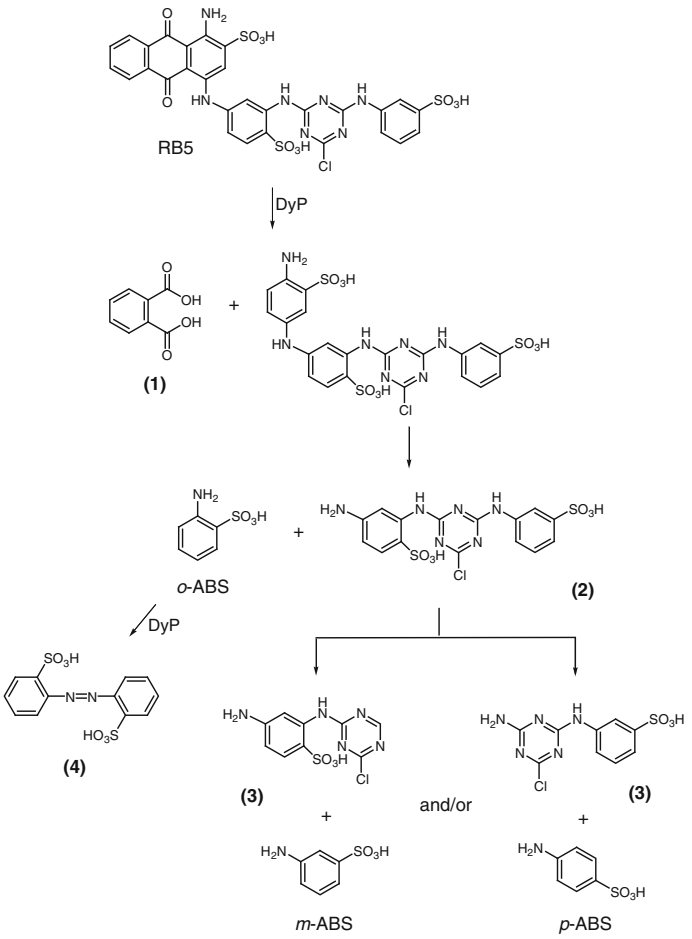

Fig. 16 Proposed pathway for the biotransformation of reactive blue 5 by DyPs. The presence of products (1) and (2) was consistent with an oxidative ring-opening of the anthraquinone frame generated by DyP, which appears in this case to behave as a hydrolase or oxygenase rather than a peroxidase, although H_2O_2 was indispensable for the reaction. The formation of compound (4) can be explained by a reaction mechanism of a typical peroxidase leading to the formation of a radical from *o*-ABS, which will be further involved in a spontaneous chemical reaction. Product (4) was characterized by NMR and ESI-MS techniques and the formation of products (1), (2) and (3) was supported by ESI-MS analysis of the final reaction mixtures (adapted from Sugano et al. 2009)

aromatic amines that represent good oxidative substrates (Sousa et al. 2013). Therefore, azo dyes were reduced by PpAzoR under anaerobic conditions and after 24 h of reaction, CotA-laccase was added with agitation. Interestingly, this sequential enzymatic procedure resulted not only in 100 % decolorization of all azo dyes tested, but also in 50–95 % detoxification of dye-products that exhibited the highest initial toxicity (Fig. 17) (Mendes et al. 2011a).

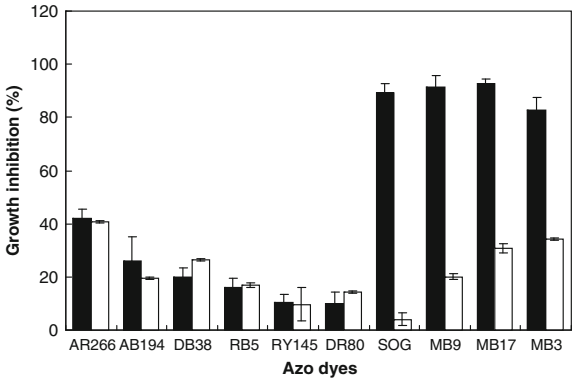

Fig. 17 Inhibitory effects of intact dyes over *Saccharomyces cerevisiae* (*black bars*) and upon the stepwise treatment with PpAzoR followed by CotA (*white bars*) (adapted from Mendes et al. 2011a)

4.4 Construction of an E. coli *Strain Producing Both Azoreductase and Laccase*

The use of whole cell catalysis is considered one of the most appropriate systems for biodegradative processes. It allows the lowering of the costs associated with enzyme purification and co-factors supply and also providing protection to the biocatalysts from harsh process environment. Therefore, a host strain co-expressing the genes coding for both enzymes of interest, PpAzoR and CotA, was constructed and a whole cell system was tested for the decolorization of dyes (Mendes et al. 2011a).

Three model dye-containing wastewaters were designed to mimic textile effluents produced during cotton or wool textile dyeing processes containing other additives and salts (30–90 % of the total weight) in addition to dyes (Prigione et al. 2008; Mendes et al. 2011b). These dyes are representative of different structural dye types and are widely applied in the textile industry. A step-wise sequential process was set-up, where the sequential action of PpAzoR and CotA enzymes could be tuned by aeration conditions. Whole cells remained in anaerobic conditions for 24 h, appropriate for PpAzoR degradation of azo dyes to aromatic amines, followed by a second 24 h period where with appropriate shaking, CotA aerobically oxidized the aromatic amines, and also the anthraquinonic dyes present in the model dyes (Mendes et al. 2011a). This procedure resulted in almost 100 % decolorization levels for the acid dye bath and around 80 % for both the reactive and direct dye baths (Fig. 18a). After this sequential treatment the toxicity levels of the final products was reduced for both *S. cerevisiae* model growth or for *C. elegans* reproduction (Fig. 18b).

Taken together, the results showed that the genetically engineered *E. coli* strain expressing the gene coding for azoreductase and laccase is able to decolorize and detoxify to a significant level the 3 model wastewaters tested, highlighting its

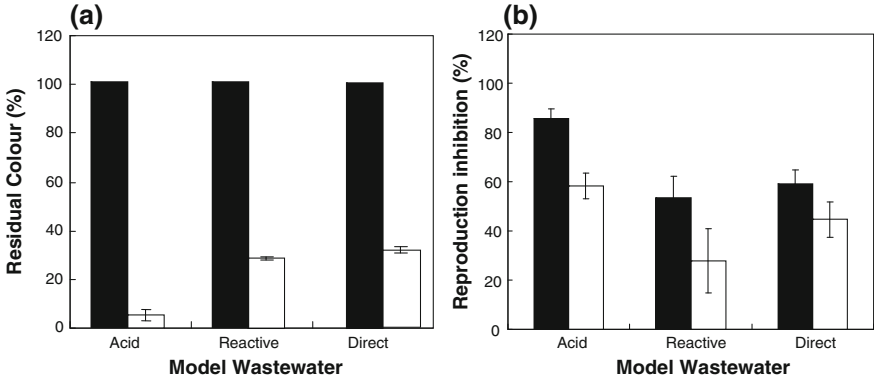

Fig. 18 **a** Residual color after the stepwise sequential process using whole cells co-producing PpAzoR and CotA (*white bars*). **b** Toxicity over *Caenorhabditis elegans* reproduction of intact model wastewater (*black bars*) and after the stepwise sequential process using whole cells co-producing PpAzoR and CotA (*white bars*) (Mendes et al. 2011a)

potential as a degradative and detoxifying bio-system for the treatment of real dye-containing effluents, without the costs associated with enzyme purification and cofactors addition.

5 Conclusions and Future Perspectives

Synthetic dyes impart an intense color to wastewater effluents from the textile, leather or other dye manipulating industries leading to environmental, medical and aesthetic problems. The diversity and complexity of dyes present in these effluents are designed to resist fading on exposure to light or chemical attack, pose serious problems on the design of technically feasible and cost-effective treatment methods. There are a relatively low number of known enzymes that are efficiently involved in the degradation of synthetic dyes in natural systems. Therefore, the characterization of enzymes, that make a discernible contribution to the degradation of synthetic dyes, paves the way for the improvement of multi-enzymatic systems, through protein engineering strategies, to maximize their biodegradation, biotransformation and valorization potential. In this review, we have described the properties, enzymatic mechanisms and products toxicity of three different types of bacterial enzymatic systems. The CotA-laccase from *B. subtilis* is a promising enzyme for the oxidative degradation of both antraquinonic and azo dyes in addition to their efficiency in the biotransformation of toxic aromatic amines (the degradation products of azoreductases). The azoreductase PpAzoR from *P. putida* MET94 is an enzyme that uses a broad range of azo dyes as substrates leading to high levels of decolorization. The major drawback associated with the use of

azoreductases is their requirement for expensive co-factors and the toxicity of the aromatic compounds produced. This can be overcome using whole cells systems of recombinant cells overproducing azoreductases and laccases as described for the decolorization and detoxification of model wastewater baths. The bacterial dye decolorizing peroxidases are new biocatalysts with a high potential for the set-up of bioprocesses considering the enlarged substrate spectrum exhibited, in particular their high efficiency for the biotransformation of anthraquinonic dyes. Both enzymes from *P. putida* and *B. subtilis* are interesting biocatalysts showing higher rates of decolorization as compared to azoreductases and the laccases tested. Moreover, we have shown the benefits of genetic engineering and evolutionary approaches to modify enzymes and microorganisms with enhanced stability and catalytic performance towards an efficient enzymatic treatment of dye-containing wastewaters.

Acknowledgments The authors are especially thankful to Dr. Luciana Pereira for her contribution in the establishment of this line of research in the laboratory. Funding is acknowledged from the project grants SOPHIED-FP6-NMP2-CT-2004-505899 and BIORENEW-FP6-2004-NMP-NI-4/026456 from the European Commission and POCI/AMB/56039/2004, PTDC/AMB/64230/2006, PTDC/BIO/72108/2006, PTDC/AGR-CFL/103840/2008 and Pest-OE/EQB/LA0004/2011 from Fundação para a Ciência e Tecnologia, Portugal.

References

Abadulla E, Tzanov T, Costa S, Robra KH, Cavaco-Paulo A, Gubitz GM (2000) Decolorization and detoxification of textile dyes with a laccase from *Trametes hirsuta*. Appl Environ Microbiol 66:3357–3362

Ahmad M, Roberts JN, Hardiman EM, Singh R, Eltis LD, Bugg TD (2011) Identification of DypB from *Rhodococcus jostii* RHA1 as a lignin peroxidase. Biochemistry 50:5096–5107

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

Anjaneyulu Y, Chary N, Raj D (2005) Decolorization of industrial effluents—available methods and emerging technologies—a review. Rev. Environ. Sci. Bio/Technology 4:245–273

Bertrand T, Jolivalt C, Briozzo P, Caminade E, Joly N, Madzak C, Mougin C (2002) Crystal structure of a four-copper laccase complexed with an arylamine: insights into substrate recognition and correlation with kinetics. Biochemistry 41:7325–7333

Böttcher D, Bornscheuer UT (2010) Protein engineering of microbial enzymes. Curr Opin Microbiol 13:274–282

Bourbonnais R, Paice MG (1990) Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. FEBS Lett 267:99–102

Brissos V, Goncalves N, Melo EP, Martins LO (2014) Improving kinetic or thermodynamic stability of an azoreductase by directed evolution. PLoS One 9:e87209

Brown ME, Barros T, Chang MC (2012) Identification and characterization of a multifunctional dye peroxidase from a lignin-reactive bacterium. ACS Chem Biol 7:2074–2081

Camarero S, Ibarra D, Martinez MJ, Martinez AT (2005) Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. Appl Environ Microbiol 71:1775–1784

Chacko J, Subramaniam K (2011) Enzymatic degradation of azo dyes: a review. Int J Environ Sci 1:1250–1260

Chen H (2006) Recent advances in azo dye degrading enzyme research. Curr Protein Pept Sci 7:101–111
Chengalroyen MD, Dabbs ER (2013) The microbial degradation of azo dyes: minireview. World J Microbiol Biotechnol 29:389–399
Chivukula M, Renganathan 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
Colpa DI, Fraaije MW, van Bloois E (2013) DyP-type peroxidases: a promising and versatile class of enzymes. J Ind Microbiol Biotechnol 41:1–7
Correia B, Chen Z, Mendes S, Martins LO, Bento I (2011) Crystallization and preliminary X-ray diffraction analysis of the azoreductase PpAzoR from *Pseudomonas putida* MET94. Acta Crystallogr Sect F Struct Biol Cryst Commun 67:121–123
Deller S, Macheroux P, Sollner S (2008) Flavin-dependent quinone reductases. Cell Mol Life Sci 65:141–160
dos Santos AB, Cervantes FJ, van Lier JB (2007) Review paper on current technologies for decolorization of textile wastewaters: perspectives for anaerobic biotechnology. Bioresour Technol 98:2369–2385
Durão P, Bento I, Fernandes AT, Melo EP, Lindley PF, Martins LO (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
Forgacs E, Cserhati T, Oros G (2004) Removal of synthetic dyes from wastewaters: a review. Environ Int 30:953–971
Giardina P, Faraco V, Pezzella C, Piscitelli A, Vanhulle S, Sannia G (2010) Laccases: a never-ending story. Cell Mol Life Sci 67:369–385
Golka K, Kopps S, Myslak ZW (2004) Carcinogenicity of azo colorants: influence of solubility and bioavailability. Toxicol Lett 151:203–210
Gonçalves AM, Mendes S, de Sanctis D, Martins LO, Bento I (2013) The crystal structure of *Pseudomonas putida* azoreductase—the active site revisited. FEBS J 280:6643–6657
Guan ZB, Zhang N, Song CM, Zhou W, Zhou LX, Zhao H, Xu CW, Cai YJ, Liao XR (2013) Molecular cloning, characterization, and dye-decolorizing ability of a temperature- and pH-stable laccase from *Bacillus subtilis* X1. Appl Biochem Biotechnol 172:1147–1157
Haritash AK, Kaushik CP (2009) Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. J Hazard Mater 169:1–15
Hofrichter M, Ullrich R, Pecyna MJ, Liers C, Lundell T (2010) New and classic families of secreted fungal heme peroxidases. Appl Microbiol Biotechnol 87:871–897
Husain Q (2006) Potential applications of the oxidoreductive enzymes in the decolorization and detoxification of textile and other synthetic dyes from polluted water: a review. Crit Rev Biotechnol 26:201–221
Kallio JP, Auer S, Janis J, Andberg M, Kruus K, Rouvinen J, Koivula A, Hakulinen N (2009) Structure-function studies of a *Melanocarpus albomyces* laccase suggest a pathway for oxidation of phenolic compounds. J Mol Biol 392:895–909
Kandelbauer A, Guebitz GM (2005) Bioremediation for the decolorization of textile dyes—a review. In: Environmental Chemistry. Springer, Berlin, pp 269-288
Kaur J, Sharma R (2006) Directed evolution: an approach to engineer enzymes. Crit Rev Biotechnol 26:165–199
Khan R, Bhawana P, Fulekar MH (2013) Microbial decolorization and degradation of synthetic dyes: a review. Rev Environ Sci Biotechnol 12:75–97
Khersonsky O, Tawfik DS (2010) Enzyme promiscuity: a mechanistic and evolutionary perspective. Annu Rev Biochem 79:471–505
Kim SJ, Shoda M (1999) Purification and characterization of a novel peroxidase from dec 1 involved in decolorization of dyes. Appl Environ Microbiol 65:1029–1035
Klonowska A, Gaudin C, Asso M, Fournel A (2005) LAC3, a new low redox potential laccase from *Trametes* sp. strain C30 obtained as a recombinant protein in yeast. Enzyme Microbial Technol 36:34–41

Klonowska A, Gaudin C, Fournel A, Asso M, Le Petit J, Giorgi M, Tron T (2002) Characterization of a low redox potential laccase from the *basidiomycete* C30. Eur J Biochem 269:6119–6125

Leelakriangsak M, Huyen NT, Towe S, van Duy N, Becher D, Hecker M, Antelmann H, Zuber P (2008) Regulation of quinone detoxification by the thiol stress sensing DUF24/MarR-like repressor, YodB in *Bacillus subtilis*. Mol Microbiol 67:1108–1124

Li J, Liu C, Li B, Yuan H, Yang J, Zheng B (2011) Identification and molecular characterization of a novel DyP-type peroxidase from *Pseudomonas aeruginosa* PKE117. Appl Biochem Biotechnol 166:774–785

Liers C, Bobeth C, Pecyna M, Ullrich R, Hofrichter M (2010) DyP-like peroxidases of the jelly fungus *Auricularia auricula-judae* oxidize nonphenolic lignin model compounds and high-redox potential dyes. Appl Microbiol Biotechnol 85:1869–1879

Liers C, Ullrich R, Hofrichter M, Minibayeva FV, Beckett RP (2011) A heme peroxidase of the *ascomyceteous lichen Leptogium saturninum* oxidizes high-redox potential substrates. Fungal Genet Biol 48:1139–1145

Liu G, Zhou J, Fu Q, Wang J (2009) The *Escherichia coli* AzoR is involved in resistance to thiol specific stress caused by electrophilic quinones. J Bacteriol 191:6394–6400

Liu X, Du Q, Wang Z, Zhu D, Huang Y, Li N, Wei T, Xu S, Gu L (2011) Crystal structure and biochemical features of EfeB/YcdB from *Escherichia coli* O157: ASP235 plays divergent roles in different enzyme-catalyzed processes. J Biol Chem 286:14922–14931

Madhavi V, Lele SS (2009) Laccase: properties and applications. Bioresources 4(4):1694–1717

Madzak C, Mimmi MC, Caminade E, Brault A, Baumberger S, Briozzo P, Mougin C, Jolivalt C (2006) Shifting the optimal pH of activity for a laccase from the fungus *Trametes versicolor* by structure-based mutagenesis. Protein Eng Des Sel 19:77–84

Maier J, Kandelbauer A, Erlacher A, Cavaco-Paulo A, Gubitz GM (2004) A new alkali-thermostable azoreductase from *Bacillus* sp. strain SF. Appl Environ Microbiol 70:837–844

Martinez AT, Ruiz-Duenas FJ, Martinez MJ, Del Rio JC, Gutierrez A (2009) Enzymatic delignification of plant cell wall: from nature to mill. Curr Opin Biotechnol 20:348–357

Mendes S, Farinha A, Ramos CG, Leitao JH, Viegas CA, Martins LO (2011a) Synergistic action of azoreductase and laccase leads to maximal decolorization and detoxification of model dye-containing wastewaters. Bioresour Technol 102:9852–9859

Mendes S, Pereira L, Batista C, Martins LO (2011b) Molecular determinants of azo reduction activity in the strain *Pseudomonas putida* MET94. App Microbiol Biotechnol 92:393–405

Mikolasch A, Schauer F (2009) Fungal laccases as tools for the synthesis of new hybrid molecules and biomaterials. Appl Microbiol Biotechnol 82:605–624

Molina-Guijarro JM, Perez J, Munoz-Dorado J, Guillen F, Moya R, Hernandez M, Arias ME (2009) Detoxification of azo dyes by a novel pH-versatile, salt-resistant laccase from *Streptomyces ipomoea*. Int Microbiol 12:13–21

Morozova OV, Shumakovich GP, Gorbacheva MA, Shleev SV, Yaropolov AI (2007) "Blue" laccases. Biochemistry 72:1136–1150

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

Ogola HJ, Kamiike T, Hashimoto N, Ashida H, Ishikawa T, Shibata H, Sawa Y (2009) Molecular characterization of a novel peroxidase from the cyanobacterium *Anabaena* sp. strain PCC 7120. Appl Environ Microbiol 75:7509–7518

Pereira L, Coelho A, Viegas AC, Ganachaud C, Lacazio G, Tron T, Robalo M, Martins LO (2009a) On the mechanism of biotransformation of the anthraquinonic dye Acid Blue 62 by laccases. Adv Synth Catal 351:1857–1865

Pereira L, Coelho AV, Viegas CA, Santos MM, Robalo MP, Martins LO (2009b) Enzymatic biotransformation of the azo dye Sudan Orange G with bacterial CotA-laccase. J Biotechnol 139:68–77

Pinheiro H, Tourad E, Thomas O (2004) Aromatic amines from azo dye reduction: status review with emphasis on direct UV spectrophotometric detection in textile industry wastewaters. Dyes Pigm 61:121–139

Piontek K, Antorini M, Choinowski T (2002) Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.90 Å resolution containing a full complement of coppers. J Biol Chem 277:37663–37669

Pogni R, Brogioni B, Baratto M, 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 25:269–275

Prigione V, Tigini V, Pezzella C, Anastasi A, Sannia G, Varese G (2008) Decolorization and detoxification of textile effluents by fungal biosorption. Water Res 42:2911–2920

Rai HS, Bhattacharyya MS, Singh J, Vats P, Banerjee UC (2005) Removal of dyes from the effluent of textile and dyestuff manufacturing industry: a review of emerging techniques with reference to biological treatment. Critical Rev Environ Sci Tecnhol 35:219–238

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

Roberts JN, Singh R, Grigg JC, Murphy ME, Bugg TD, Eltis LD (2011) Characterization of dye-decolorizing peroxidases from *Rhodococcus jostii* RHA1. Biochemistry 50:5108–5119

Robinson T, McMullan G, Marchant R, Nigam P (2001) Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative. Bioresour Technol 77:247–255

Rodriguez Couto S (2009a) Enzymatic biotransformation of synthetic dyes. Curr Drug Metab 10:1048–1054

Rodriguez Couto S (2009b) Dye removal by immobilised fungi. Biotechnol Adv 27:227–235

Rodriguez Couto S, Sanroman M, Gubitz GM (2005) Influence of redox mediators and metal ions on synthetic acid dye decolorization by crude laccase from *Trametes hirsuta*. Chemosphere 58:417–422

Rodriguez Couto S, Toca Herrera JL (2006) Industrial and biotechnological applications of laccases: a review. Biotechnol Adv 24:500–513

Rosado T, Bernardo P, Koci K, Coelho AV, Robalo MP, Martins LO (2012) Methyl syringate: an efficient phenolic mediator for bacterial and fungal laccases. Bioresour Technol 124:371–378

Ruiz-Duenas FJ, Martinez AT (2009) Microbial degradation of lignin: how a bulky recalcitrant polymer is efficiently recycled in nature and how we can take advantage of this. Microb Biotechnol 2:164–177

Salvachua D, Prieto A, Martinez AT, Martinez MJ (2013) Characterization of a novel dye-decolorizing peroxidase (DyP)-type enzyme from *Irpex lacteus* and its application in enzymatic hydrolysis of wheat straw. Appl Environ Microbiol 79:4316–4324

Santos A, Mendes S, Brissos V, Martins LO (2014) New dye-decolorizing peroxidases from *Bacillus subtilis* and *Pseudomonas putida* MET94: towards biotechnological applications. Appl Microbiol Biotechnol 98:2053–2065

Saratale RG, Saratale GD, Chang JS, Govindwar SP (2011) Bacterial decolorization and degradation of azo dyes: a review. J Taiwan Inst Chem Eng 42:138–157

Scheibner M, Hulsdau B, Zelena K, Nimtz M, de Boer L, Berger RG, Zorn H (2008) Novel peroxidases of *Marasmius scorodonius* degrade β-carotene. Appl Microbiol Biotechnol 77:1241–1250

Schneider K, Hafner C, Jager I (2004) Mutagenicity of textile dye products. J Appl Toxicol 24:83–91

Sezer S, Santos A, Kielb P, Pinto T, Martins LO, Todorovic S (2013) Distinct structural and redox properties of heme active in bacterial DyP-type peroxidases from two subfamilies: resonance Raman and electrochemistry study. Biochemistry 52:3074–3084

Singh R, Grigg JC, Armstrong Z, Murphy ME, Eltis LD (2012) Distal heme pocket residues of B-type dye-decolorizing peroxidase: arginine but not aspartate is essential for peroxidase activity. J Biol Chem 287:10623–10630

Singh R, Grigg JC, Qin W, Kadla JF, Murphy ME, Eltis LD (2013) Improved manganese-oxidizing activity of DypB, a peroxidase from a lignolytic bacterium. ACS Chem Biol 8:700–706

Solís M, Solís A, Pérez H, Manjarrez N, Flores M (2012) Microbial decoloration of azo dyes: a review. Process Biochem 47:1723–1748

Sollner S, Deller S, Macheroux P, Palfey BA (2009) Mechanism of flavin reduction and oxidation in the redox-sensing quinone reductase Lot6p from *Saccharomyces cerevisiae*. Biochemistry 48:8636–8643

Sollner S, Nebauer R, Ehammer H, Prem A, Deller S, Palfey BA, Daum G, Macheroux P (2007) Lot6p from *Saccharomyces cerevisiae* is a FMN-dependent reductase with a potential role in quinone detoxification. FEBS J 274:1328–1339

Solomon EI, Sundaram UM, Machonkin TE (1996) Multicopper oxidases and oxygenases. Chem Rev 96:2563–2606

Sousa AC, Martins LO, Robalo MP (2013) Laccase-catalysed homocoupling of primary aromatic amines towards the biosynthesis of dyes. Adv Synth Catal 355:2908–2917

Stoj C, Kosman D (2005) Copper proteins: oxidases. In: King R (ed) Encyclopedia of inorganic chemistry, vol II. Wiley, Hoboken

Stolz A (2001) Basic and applied aspects in the microbial degradation of azo dyes. Appl Microbiol Biotechnol 56:69–80

Strittmatter E, Liers C, Ullrich R, Wachter S, Hofrichter M, Plattner DA, Piontek K (2012) First Crystal structure of a fungal high-redox potential dye-decolorizing peroxidase: substrate interaction sites and long-range electron transfer. J Biol Chem 288:4095–4102

Sugano Y (2009) DyP-type peroxidases comprise a novel heme peroxidase family. Cell Mol Life Sci 66:1387–1403

Sugano Y, Muramatsu R, Ichiyanagi A, Sato T, Shoda M (2007) DyP, a unique dye-decolorizing peroxidase, represents a novel heme peroxidase family: ASP171 replaces the distal histidine of classical peroxidases. J Biol Chem 282:36652–36658

Sugano Y, Matsushima Y, Tsuchiya K, Aoki H, Hirai M, Shoda M (2009) Degradation pathway of an anthraquinone dye catalyzed by a unique peroxidase DyP from *Thanatephorus cucumeris* Dec 1. Biodegradation 20:433–440

Sugano Y, Nakano R, Sasaki K, Shoda M (2000) Efficient heterologous expression in Aspergillus oryzae of a unique dye-decolorizing peroxidase, DyP, of *Geotrichum candidum* Dec 1. Appl Environ Microbiol 66:1754–1758

Tauber MM, Guebitz GM, Rehorek A (2005) Degradation of azo dyes by laccase and ultrasound treatment. Appl Environ Microbiol 71:2600–2607

Towe S, Leelakriangsak M, Kobayashi K, Van Duy N, Hecker M, Zuber P, Antelmann H (2007) The MarR-type repressor MhqR (YkvE) regulates multiple dioxygenases/glyoxalases and an azoreductase which confer resistance to 2-methylhydroquinone and catechol in *Bacillus subtilis*. Mol Microbiol 66:40–54

Trovaslet M, Enaud E, Bazes A, Jolivalt C, Van Hove F, Vanhulle S (2007) Laccase-catalyzed azo dye synthesis. Enzyme Microbial Technol 41:368–376

van Bloois E, Torres Pazmino DE, Winter RT, Fraaije MW (2010) A robust and extracellular heme-containing peroxidase from *Thermobifida fusca* as prototype of a bacterial peroxidase superfamily. Appl Microbiol Biotechnol 86:1419–1430

van der Zee FP, Villaverde S (2005) Combined anaerobic-aerobic treatment of azo dyes-a short review of bioreactor studies. Water Res 39:1425–1440

Vanhulle S, Enaud E, Trovaslet M, Billottet L, Kneipe L, Habib Jiwan JL, Corbisier AM, Marchand-Brynaert J, Marchand-Brynaert J (2008a) Coupling occurs before breakdown during biotransformation of Acid Blue 62 by white rot fungi. Chemosphere 70:1097–1107

Vanhulle S, Trovaslet M, Enaud E, Lucas M, Taghavi S, Van der Lelie D, Van Aken B, Foret M, Onderwater RC, Wesenberg D, Agathos SN, Schneider YJ, Corbisier AM (2008b) Decolorization, cytotoxicity, and genotoxicity reduction during a combined ozonation/fungal treatment of dye-contaminated wastewater. Environ Sci Technol 42:584–589

Wang C-J, Hagemeier C, Rahman N, Lowe E, Noble M, Coughtrie M, Sim E, Westwood I (2007) Molecular cloning, characterization and ligand-bound structure of an azoreductase from *Pseudomonas aeruginosa*. J Mol Biol 373:1213–1228

Wang M, Si T, Zhao H (2012) Biocatalyst development by directed evolution. Bioresour Technol 115:117–125

Wesenberg D, Kyriakides I, Agathos SN (2003) White-rot fungi and their enzymes for the treatment of industrial dye effluents. Biotechnol Adv 22:161–187

Wheeldon IR, Gallaway JW, Barton SC, Banta S (2008) Bioelectrocatalytic hydrogels from electron-conducting metallopolypeptides coassembled with bifunctional enzymatic building blocks. Proc Natl Acad Sci U S A 105:15275–15280

Willner I, Yan Y-M, Willner B, Tel-Vered R (2009) Integrated enzyme-based biofuel cells–a review. Fuel Cells 1:7–24

Yoshida T, Tsuge H, Konno H, Hisabori T, Sugano Y (2011) The catalytic mechanism of dye-decolorizing peroxidase DyP may require the swinging movement of an aspartic acid residue. FEBS J 278:2387–2394

Zhang C, Diao H, Lu F, Bie X, Wang Y, Lu Z (2012) Degradation of triphenylmethane dyes using a temperature and pH stable spore laccase from a novel strain of *Bacillus vallismortis*. Bioresour Technol 126:80–86

Zille A, Gornacka B, Rehorek A, Cavaco-Paulo A (2005a) Degradation of azo dyes by *Trametes villosa* laccase over long periods of oxidative conditions. Appl Environ Microbiol 71:6711–6718

Zille A, Munteanu F, Gubitz G, Cavaco-Paulo A (2005b) Laccase kinetics of degradation and coupling reactions. J Mol Catal B Enzym 33:23–28

Zille A, Ramalho P, Tzanov T, Millward R, Aires V, Cardoso MH, Ramalho MT, Gubitz GM, Cavaco-Paulo A (2004) Predicting dye biodegradation from. Biotechnol Prog 20:1588–1592

http://www.springer.com/978-3-319-10941-1

Microbial Degradation of Synthetic Dyes in Wastewaters
Singh, S.N. (Ed.)
2015, XIV, 367 p. 85 illus., 21 illus. in color., Hardcover
ISBN: 978-3-319-10941-1