Synergistic action of azoreductase and laccase leads to maximal decolourization and detoxification of model dye-containing wastewaters

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1. Introduction

Azo dyes are widely used for colouration of textiles, paper, leather or plastics. These compounds represent the largest and most versatile group of synthetic dyes. The textile industry accounts for two thirds of the total dyestuff market and during textile processing as much as 2–50% of applied dyestuff may be lost to wastewater that is ultimately released into the environment (Dos Santos et al., 2007; Forgacs et al., 2004). Many of the azo dyes and/or their breakdown products have been shown to be toxic, potentially carcinogenic and can lead to the formation of bladder cancer in humans, tumours, allergies, nuclear anomalies in experimental animals, and chromosomal aberrations in mammalian cells (Chen, 2006; Golka et al., 2004; Pinheiro et al., 2004; Schneider et al., 2004; van der Zee and Villaverde, 2005). Traditional physicochemical treatment processes, such as coagulation, adsorption and oxidation with ozone, can generate large volumes of sludge and usually requires the addition of environmental hazardous chemical additives (Chen, 2006; Forgacs et al., 2004). Biodegradation methods are attractive alternatives as they can be less expensive and can selectively provide a complete degradation of the organic pollutants without collateral destruction of either the site material or its flora and fauna, and can be used in situ (Dos Santos et al., 2007; Kandelbauer and Guebitz, 2005). Many microorganisms proved their ability to decolourize dyes by biosorption or enzymatic degradation (Cervantes and Dos Santos, 2011; Rai et al., 2005). Among the few redox-active enzymes showing azo dyes degradative activity, azoreductases are particularly effective since they act on the reduction of the azo linkage which is the chromophoric group of the coloured compounds (Kandelbauer and Guebitz, 2005; Rodriguez-Couto, 2009). However, azoreductases require the addition of expensive cofactors such as NAD(P)H as electron donors for the reductive reaction and the products released are aromatic amines which are potentially toxic. In contrast, laccases are oxidoreductases that have a great potential in various biotechnological processes mainly due to their high non-specific oxidation, the lack of a requirement for cofactors, and the use of readily available oxygen as an electron acceptor capacity (Kandelbauer and Guebitz, 2005; Rodriguez-Couto, 2009). Laccase dye degradative processes are considered environmental friendly since the oxidation occurs through a highly non-specific free radical mechanism, forming phenolic type compounds and thus, avoiding the formation of toxic aromatic amines (Chivukula and Renganathan, 1995; Zille et al., 2005b; Pereira et al., 2009a). The objective of the present study was to assess both the enzymatic degradation of azo dyes and the final toxicity of the reaction mixtures in order to contribute for the set-up of an eco-friendly biological treatment.
for wastewater containing dyes. The enzymatic degradation of an array of 18 azo dyes and of three model dye baths was tested with two enzymes with proven ability to degrade synthetic dyes, recombinant FMN-dependent NADPH azoreductase (PpAzoR) from *Pseudomonas putida* MET94 (Mendes et al., 2011) and recombinant CoTA-laccase from the bacterium *Bacillus subtilis* (Martins et al., 2002; Pereira et al., 2009a,b). These enzymes show different catalytic mechanisms and lead to structurally different degradation products (Mendes et al., 2011; Pereira et al., 2009a). The toxicity of dyes and reaction products was measured by using yeast- and nematode-based bioassays (Papaefthimiou et al., 2004; Anderson et al., 2001). *Saccharomyces cerevisiae* is an important microbial eukaryotic model and the nematode *Caenorhabditis elegans* has recognized relevance as a test organism for soil and aquatic ecotoxicological studies. The worm model complements the data obtained with the yeast model, by comprising effects on reproduction and development, neurotoxicity and xenometabolism (Leung et al., 2001).

### 2. Methods

#### 2.1. Chemicals

All chemicals were of the highest grade available commercially. The dyes tested are listed in Table 1. Three different model wastewaters, designed in the frame of the European Commission (EC) project SOPHIED, to mimic effluents produced during wool and cotton textile dyeing processes were prepared as follows: acid bath (for wool) with YA94, AB62, AR266, AB210, and AB194 dyes at 0.1 g L\(^{-1}\) each and 2 g L\(^{-1}\) of Na\(_2\)SO\(_4\) pH 5, reactive bath (for cotton) with RB222, RR195, RY145, and RB5 dyes at 1.25 g L\(^{-1}\) each and 70 g L\(^{-1}\) of Na\(_2\)SO\(_4\) pH 10 and direct bath (for cotton) with DB71, DR80, and DY106 dyes at 1 g L\(^{-1}\) each and 5 g L\(^{-1}\) of NaCl, pH 9 (Prigione et al., 2008a). All solutions were sterilized by tyn-dallization (three 1 h-cycles at 80 °C with 24 h interval between cycles at room temperature) before use.

#### 2.2. Construction of an *E. coli* strain co-expressing *cotA* and *ppAzoR*

The *ppAzoR* gene was PCR amplified from chromosomal DNA of strain *P. putida* MET94 using the primers PpaF (5’ GGAGAGAATTCAATTAGAAACTGTTGCACATGATTCG 3’) and PpaR (5’ CCATACCTTACGGGATCCGCAGC 3’). The resulting 612-bp DNA fragment was purified, digested with *Nde*I and *Avr*I and cloned between the same restriction sites of the plasmid pETDuet-1TM (Novagen) to produce pAIF-1. Similarly, for the amplification of *cotA* gene oligonucleotides CotA159D (5’ GCACACTGGCTGGCTGATTGC 3’) and CotA1892R (5’ CCAGGATCTTATGAGGATCAGTCGTC 3’) were used. The product of the reaction (1539-bp) was digested with *Sfi*I and *Bam*HI purified and cloned into pAIF-1 previously digested with *Bst*EII and *Bam*HI to yield pAIF-2. The correct sequence of the inserts was confirmed by sequencing. The plasmid pAIF-2 was introduced into the host expression strain *E. coli* BL21star (DE3), producing the strain LOM529, in which both genes were expressed under the control of the T7lac promoter.

#### 2.3. Overproduction of enzymes in heterologous host

The recombinant strain LOM529 was grown in Luria Bertani (LB) medium supplemented with ampicillin (100 g mL\(^{-1}\)) at 37 °C. Growth was followed until the midlog phase (OD\(_{600}\) = 0.6), at which time 0.1 mM isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG) and 0.25 mM CuCl\(_2\) were added to the culture medium and the

### Table 1

<table>
<thead>
<tr>
<th>C.I. generic name</th>
<th>C.I. constitution number</th>
<th>Dye content (%)</th>
<th>Absorption max (nm)</th>
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<tr>
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<tr>
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<td>600</td>
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<td>480</td>
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<tr>
<td>Acid Orange 7 (AO7)</td>
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<td>95</td>
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<td>Mordant Black 9 (MB9)</td>
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</tr>
<tr>
<td>Mordant Black 17 (MB17)</td>
<td>15,705</td>
<td>50</td>
<td>530</td>
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</table>

\(^a\) The dyes were purchased from Sigma–Aldrich (St. Louis, MO, USA), Merck (Darmstadt, Germany), Town End (Leeds, UK), DyStar Textilfarben (Germany), Yorkshire Europe (Belgium) and Bezema AG (Montlingen, Switzerland). The absorption maxima were determined in BR buffer (pH 7). If the dye purity was not indicated by the supplier, it was assumed that the preparations consisted of pure dye.

\(^b\) NS, dye purity not specified by the supplier.

\(^c\) Confidential.

temperature lowered to 25 °C. Incubation and agitation continued for a further 4 h, after which the agitation was stopped and the growth remained static overnight at 25 °C. Cells were harvested by centrifugation (8000 × g, 10 min, 4 °C). To measure the enzymatic activity in crude extracts, cell suspensions were prepared in 20 mM phosphate buffer, pH 8, containing DNase I (10 µg mL⁻¹ extract), MgCl₂ (5 mM), and a mixture of protease inhibitors, antipain and leupeptin (2 µg mL⁻¹ extract). Cells were disrupted in a French Press and the cell debris was removed by centrifugation (18000 × g, 2 h, 4 °C). Supernatants were used for SDS–PAGE analysis and enzymatic assays. Laccase activity was determined using ABTS as substrate (Martins et al., 2002). Azoreductase activity was measured using NADPH and reactive black 5 azo dye as substrates as described before (Mendes et al., 2011).

2.4. Enzyme assays using purified enzymes

The recombinant PpAzoR from P. putida and the recombinant CotA-laccase from B. subtilis were produced and purified as previously described (Martins et al., 2002; Mendes et al., 2011). For the PpAzoR reactions serum bottles contained 0.25 mM NADPH and 3 mM of dye (or 0.5 mM for toxicity studies) in Britton Robinson (BR) buffer (0.1 M phosphoric acid, 0.1 M boric acid, 0.1 M acetate acid titrated to the desired pH with 0.5 M NaOH) pH 7, at 30 °C. The bottles were sealed with rubber stoppers and made anaerobic by argon bubbling. Reactions were initiated by the injection of anoxic-made purified enzyme through the use of a syringe. The CotA-laccase reactions were assessed using 3 mM of dye (or 0.5 mM for toxicity studies) in BR buffer pH 8, 37 °C. Supernatants were used for SDS–PAGE analysis and enzymatic assays. Laccase activity was determined using ABTS as substrate (Martins et al., 2002). Azoreductase activity was measured using NADPH and reactive black 5 azo dye as substrates as described before (Mendes et al., 2011).

2.5. Enzyme assays using recombinant whole cells

The recombinant E. coli cells overproducing PpAzoR and CotA were harvested at the late-exponential phase of growth by centrifugation (8000 × g, 10 min, 4 °C) and washed twice with 0.9% (w/v) NaCl solution. Cells were permeabilized before use, by shaking at 250 rpm 30 °C for 30 min upon addition of toluene (0.1% v/v) or Triton X-100 (0.1% v/v). The permeabilization level was assessed by measuring protein content and activity in cells supernatants. For the enzymatic assays, the washed pellet was suspended in 20 mM phosphate buffer, pH 8 to an OD₆₀₀ = 20, unless otherwise stated. The cells were added to a sealed serum bottle containing 5 mL of each model wastewater bath, or 5 mL of 0.5 mM of RB5 in phosphate buffer, pH 8, at 30 °C. The reaction mixtures were previously degassed with purified argon, providing the appropriate anoxic conditions for PpAzoR catalysis. Samples were withdrawn at different times and the decolourization was measured spectrophotometrically at three different wavelengths (400, 500 and 600 nm) after appropriate dilution for the model wastewater baths, or at 600 nm for reactive black 5. After this period of time the reaction mixtures were made aerobic through shaking at 150 rpm and the temperature was increased to 37 °C; these aerobic conditions inhibited PpAzoR activity while promoting CotA laccase oxidative action over dyestuff.

2.6. Toxicity analysis

Toxicity assays were performed based on the inhibitory effects of dyes and products of enzymatic degradation on the growth of the yeast S. cerevisiae BY4741 (Papaefthimiou et al., 2004; Pereira et al., 2009a,b), or on the reproduction of the nematode C. elegans wild type strain Bristol N2 (Anderson et al., 2001). Briefly, the yeast cell suspension used as inoculum was prepared from a mid-exponential phase culture, which was centrifuged and suspended to OD₆₀₀ = 0.15 in a triple strength minimal growth medium that contained (per litre): 1.7 g YNB without aminoacids or NH₄Cl (Difco, Detroit, USA), 20 g glucose (Merck, Germany), 2.65 g (NH₄)₂SO₄ (Merck), 20 mg methionine, 20 mg histidine, 60 mg leucine and 20 mg uracil (Sigma, USA). For the toxicity tests, 501 of the standardized yeast cell suspension were mixed with 1001 of test or control solutions in 96-well polystyrene microplates (Greiner Bio-one) that were sealed and incubated at 30 °C for 16 h with constant agitation. Growth of the yeast cell population was assessed by measuring the optical density (OD₇₅₀nm, which did not interfere with any of the maximal absorption wavelength of the dyes tested) attained after 16 h of incubation. The toxicity was estimated based on the percentage of yeast growth inhibition defined as: 100×(ODₓ – OD₀)/OD₀, where OD₀ is the absorbance values attained by the yeast cell population in the presence and in the absence of each test solution, respectively. For the reproduction assays with C. elegans Bristol N2, experiments were performed in 48-well polystyrene microplates (Greiner Bio-one) by adding a single worm from age-synchronized cultures at the L4 larval stage, to 50 l of 10 × K medium (Anderson et al., 2001). 50 l of E. coli OP50 suspension with an OD₆₀₀ = 1.5 and 400 l of test or control solutions. The plates were incubated at 20 °C in a FITOCULTURA S600 (Arab) incubator. After incubation for 96 h, the number of offspring at all beyond the eggs was scored, using a Zeiss Stemi 2000-C stereomicroscope (50 × magnification). Data reported are average values with standard deviations of results from at least two independent toxicity experiments performed in quadruplicate.

3. Results and discussion

3.1. Degradation of dyes using the reductive PpAzoR and the oxidative CotA-laccase enzymes

Two well characterized bacterial enzymatic systems were tested, one reductive, the recombinant FMN-dependent NADPH azoreductase PpAzoR from P. putida MET94 (Mendes et al., 2011) and one oxidative, the recombinant CotA-laccase from the bacterium B. subtilis (Pereira et al., 2009a). PpAzoR is an oxygen-sensitive azoreductase that exhibits a broad range specificity for azo dyes while CotA-laccase is a thermoactive and intrinsically thermostable enzyme, that does not require the addition of redox mediators for the decolourization of a wide range of structurally different dyes. The decolourization levels of 18 structurally different synthetic dyes, representative of the most common classes of dyes (direct, reactive, and acid, Table 1) were measured after 24 h of incubation. The results show that PpAzoR exhibits a broader substrate specificity than CotA-laccase with decolourization levels above 80% for most of the dyes tested (Fig. 1). CotA-laccase, on the other hand, is able to decolourize at a high extent (above 80%) only a few dyes, showing levels of decolourization for the majority of the dyes between 40 and 60%.

3.2. Toxicity of azo dyes over S. cerevisiae growth and C. elegans reproduction

The toxicity of the synthetic dyes and enzymatic products was tested based on the inhibitory effects on the growth of S. cerevisiae and on the reproduction inhibition of C. elegans (Fig. 2). In general, the toxicity of intact dyes correlates well between the two model
eukaryotic organisms tested. Nevertheless, C. elegans seems slightly more susceptible to the presence of intact dyes since some dyes such as DB1, DB38, DR80, AB194, RB5, and RY145 showed more than 2-fold higher inhibition for the nematodes reproduction than for S. cerevisiae growth (Fig. 2A and B). The observed differences between the two systems probably relate to the different complexity of the yeast and the worm cells. Indeed, the assessment of dye toxicity on C. elegans may integrate the contributions of their effects not only at subcellular and cellular, but also at tissue and organ levels, leading to a higher sensitivity for these xenobiotic compounds. A reduction of toxicity in more than 50% was observed after the enzymatic treatment (with either one or the other enzyme) of dyes belonging to the group showing the initial highest levels of toxicity (SOG, Mordant Black dyes, DRR, and MR) (Fig. 2A and B). However, for the majority of the other dyes tested, the enzymatic products present a higher toxicity than intact dyes themselves, especially as assessed by the S. cerevisiae system (Fig. 2A). This is particularly evident for the PpAzoR products of AR266, AB194, DB38, DR80, and reactive dyes RR4, RB5, and RY145, exhibiting 2 to 4-fold higher toxicity than those exhibited by the intact dyes. The results also show that the oxidative products of CotA resulted for a few dyes in a higher toxicity, as reported for other laccases (Champagne and Ramsay, 2010; Moya et al., 2010). The higher toxicity of the azoreductase products is most likely related to the toxic nature of the aromatic amines formed (Pinheiro et al., 2004). Indeed, 1-amino-2-naphthol (predicted reductive product of MB3, MB17, and AB194), benzidine (one of the degradation products of DB38), and

**Fig. 1.** Dye decolourization by the enzymes PpAzoR (open bars) and CotA-laccase (filled bars). Decolourization was measured after 24 h of reaction.

**Fig. 2.** Inhibitory effects of intact dyes (black bars) and of 24 h–reaction mixtures treated with either PpAzoR (light grey bars) or CotA-laccase (dashed bars) over (A) *Saccaromyces cerevisiae* BY4741 growth, or (B) *Caenorhabditis elegans* Bristol N2 reproduction. The results are means of two independent experiments with n = 8.

3.3dimethoxybenzidine (one of the degradation products of DB1) have been reported to be highly toxic to humans and carcinogenic (Golka et al., 2004; Mendez-Paz et al., 2005; Snyderwine et al., 2002). Aniline, one of the degradation products of SOG, has also been described as possibly carcinogenic and genotoxic to aquatic life (Pinheiro et al., 2004). In contrast, N,N-dimethyl-p-phenylenediamine, was described as non-toxic for plants and microorganisms (Parshetti et al., 2010) and consistently, the degradation products of MR, that includes this amine (Mendes et al., 2011) showed one of the lowest level of toxicity for both the yeast and worm models (Fig. 2A and B).

### 3.3. Combined sequential enzymatic treatment

Laccases catalyze the oxidation of ortho- or para-substituted phenolic or amine aromatic 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, toxic aromatic amines, are known to represent good oxidative substrates of laccases (Franciscon et al., 2010; Zille et al., 2005a). In order to set-up enzymatic processes that lead to maximal decolorization as well as to maximal detoxification levels, a sequential enzymatic procedure was followed: incubation of dyes with PpAzoR for 24 h, under anaerobic conditions, addition of CotA and incubation for an additional period of 24 h under shaking conditions. Ten dyes were selected among i) the group of the most toxic dyes such as SOG, MB9, MB17, and MB3, and ii) the group of dyes for which the enzymatic azoreductase products exhibited higher toxicity than the dyes themselves (AR266, AB194, DB38, RB5, RY145, and DR80) (Fig. 2). Interestingly, this sequential enzymatic procedure resulted in 100% decolorization of all azo dyes tested (Fig. 3A). More
importantly, the detoxification levels range from 50 to 95% for dyes that exhibited the highest initial toxicity (around 80%), while for the remaining of the dyes, the final products showed a significantly 2.5–5-fold lower toxicity values as compared with the single enzymatic treatments with PpAzoR (Fig. 3B). Considering that auto-oxidation is very likely to occur with aromatic amines bearing hydroxyl substituents (Kudlich et al., 1999; Pinheiro et al., 2004), such as the products of SOG and mordant black dyes bearing 2–3 –OH groups, we have included a control test, incubation under aerobic conditions without addition of CotA-laccase. In some cases a reduced toxicity was observed while in others (e.g. AR266, DB38, and RB5 products, with only 1 hydroxyl substituent) the toxicity of the presumed auto-oxidation products is clearly increased in relation to intact dyes (Fig. 3B).

3.4. Sequential enzymatic treatment of model wastewaters

The major chemical pollutants present in the textile wastewaters belong to the class of reactive (~36%), acid (~25%) and direct (~15%) dyes (Franciscon et al., 2009), designed to be recalcitrant for long-term use and thus resistant to treatment (Hsueh and Chen, 2007). Only a few very studies have been performing using multi-dye solutions simulating industrial effluents (Prigione et al., 2008a,b; Russo et al., 2009). In order to test the appropriateness of the enzymatic PpAzoR/CotA sequential approach in a more realistic context, the decolourization of three model wastewaters was assessed. These were designed to mimic effluents produced during cotton or wool textile dyeing processes containing other additives and salts (30–90% of the total weight) in addition to dyes (Mendes et al., 2011; Prigione et al., 2008a). The dyes selected are representative of commercial important dye types, widely applied in the textile industry. Decolourization was measured spectrophotometrically at 400, 500 and 600 nm after 24 h of a single-enzyme treatment with either PpAzoR or CotA-laccase, or after the combined enzymatic treatment that lasted for 48 h. Decolourization of the three model dyeing baths with PpAzoR is between 80 and 95% while CotA shows a more modest performance with decolourisation levels between 20 and 45% (data not shown). Noteworthy, using the combined sequential treatment the decolourisation values reached almost 100% (as measured at 500 or 600 nm) (Fig. 4A). Moreover, relatively low levels of toxicity (<30% inhibition for most of the treated dye baths) were measured with both bioassays tested (Fig. 4B and C). Therefore, our results show that the combined enzymatic system with both PpAzoR and CotA is quite promising for the simultaneous degradation and detoxification of multi azo-dye mixtures. However, this approach is not economically feasible considering that PpAzoR activity is dependent on the exogenous addition of the co-factor NADPH, which is quite expensive. Therefore the utilization of whole-cell systems rather than isolated enzymes, providing NADPH recycling, would represent a more economically sustainable solution.

3.5. Construction of an E. coli strain co-expressing both ppAzoR and cotA genes

We have decided to construct a host strain co-expressing the genes coding for both enzymes of interest, PpAzoR and CotA. The ppAzoR and cotA genes were cloned into the expression vector pETDuet-1, yielding pAIF-2. This final construct was transformed into E. coli BL21 star (DE3), producing strain LOM529. E. coli is a convenient host for heterologous protein expression. Its advantages include high levels of heterologous gene expression and scalability of experiments, low cost and fast growth. SDS–PAGE analysis of the crude extracts from E. coli LOM529 revealed that the addition of IPTG to the culture medium resulted in the accu-

![Fig. 5](image-url)
mulation of two extra bands at ~23 kDa and ~65 kDa, consistent with the overproduction of CotA and PpAzor, respectively (Fig. 5A). Enzymatic assays for laccase activity showed that the levels of expression of cotA in strain LOM529 are half of those present in strain AH3517 (with cotA gene, Martins et al., 2002) while the ppAzor expression is similar to the observed for LOM528 strain (with the ppAzor gene, Mendes et al., 2011) (Table 2). Cells were permeabilized before use, by shaking at 30 °C during 30 min. Addition of toluene (0.1% v/v) or Triton X-100 (0.1% v/v) did not result in increased levels of permeabilization as assessed by protein content and activity measurements in cells supernatants (data not shown). Whole-cell reactions with reactive black 5 showed that reaction rates increase linearly with the biomass concentration following a first order reaction with an optimum at around pH 8 (data not shown). The rates of decolourization provided by the whole-cell LOM528 or AH3517 systems are comparable (2.7 and 2.4 Abs h\(^{-1}\), respectively) as well as the decolourization levels (94 and 89%, respectively) after 24 h (Fig. 5B). The rates of decolourization by LOM529 either at anaerobic (selecting for PpAzor activity) or aerobic (selecting for laccase activity) are in the same order of magnitude as the individual systems, with decolourization levels of almost 100% (Fig. 5B and results not shown). However, the decolourization obtained is not only due to the recombinant enzymatic degradation, but is also due to intrinsic azoreductase activity of the E. coli strain (Nakanishi et al., 2001) (please see Table 2) and biosorption of dye onto the biomass (Fig. 5C).

3.6. Enzymatic treatment of model wastewaters using whole cells co-expressing ppAzor and cotA

The results of decolourization experiments with the three model dye-containing wastewaters using whole cells co-producing PpAzor and CotA are illustrated in Fig. 6A. The decolourization was assessed by following a stepwise sequential process; the anaerobic process (first 24 h) followed by the aerobic treatment (second period of 24 h) resulted in almost 100% decolourization levels for the acid dye bath and around 80% for both the reactive and direct dye baths (Fig. 6A). The toxicity of the final products after this sequential treatment was significantly reduced for both S. cerevisiae model growth (Fig. 6B) or for C. elegans reproduction (Fig. 6C). Taken together, the results showed that the genetically engineered E. coli strain LOM529 is able to decolourize and detoxify to a significant level the three model wastewaters tested, highlighting its 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.

Table 2

<table>
<thead>
<tr>
<th>Strains</th>
<th>Specific activity U mg(^{-1})</th>
<th>Azoreductase(^a)</th>
<th>Laccase(^b)</th>
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<td>0.016 ± 0.0002</td>
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<tr>
<td>E. coli BL21 star (DE3)</td>
<td>0.006 ± 0.0002</td>
<td>0.014 ± 0.0004</td>
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<tr>
<td>LOM528</td>
<td>0.013 ± 0.009</td>
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<td>5.1 ± 0.3</td>
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<tr>
<td>AH3517</td>
<td>-</td>
<td>-</td>
<td>2.9 ± 0.6</td>
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<tr>
<td>LOM529</td>
<td>0.015 ± 0.002</td>
<td>-</td>
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\(^a\) Activities were measured by following the rate of NADPH oxidation in reactions with 1 mM BRS, in BR buffer, pH 7, at 30 °C under anaerobic conditions.

\(^b\) Activities were measured by following the rate of ABTS oxidation in BR buffer, pH 8, at 37 °C under aerobic conditions.

Fig. 6. (A) Whole-cell decolourization using Escherichia coli LOM529 (co-expressing ppAzor and cotA) of model wastewaters measured at 500 nm after 24 h of reaction under anaerobic conditions (black bars) and after more 24 h under aerobic conditions (white bars). Toxicity for yeast cells (B) and Caenorhabditis elegans (C) of intact model wastewater (black bars), after 24 h treatment with PpAzor under anaerobic conditions (grey bars), and after additional 24 h with CotA under aerobic conditions (white bars).

4. Conclusions

Azoreductases are enzymes able to degrade a wider range of azoic substrates as compared with laccases. However, in most cases the aromatic amine products of their reactions shows a 2
to 3-fold higher toxicity than the intact dyes themselves. The addition of laccases to azoreductase treated reaction mixtures resulted in a significant final decreased toxicity. A heterologous E. coli strain was successfully constructed co-expressing the genes coding for both the azoreductase PpAzOR and CotA-laccase. This provided a new system for the detoxification of azo-dye contaminated effluents based on a whole-cell sequential enzymatic degradative process.

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References


