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

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ABSTRACT

The azoreductase PpAzoR from *Pseudomonas putida* shows a broader specificity for decolourization of azo dyes than CotA-laccase from *Bacillus subtilis*. However, the final products of PpAzoR activity exhibited in most cases a 2 to 3-fold higher toxicity than intact dyes themselves. We show that addition of CotA-laccase to PpAzoR reaction mixtures lead to a significant drop in the final toxicity. A sequential enzymatic process was validated through the use of 18 representative azo dyes and three model wastewaters that mimic real dye-containing effluents. A heterologous *Escherichia coli* strain was successfully constructed co-expressing the genes coding for both PpAzoR and CotA. Whole-cell assays of recombinant strain for the treatment of model dye wastewater resulted in decolourization levels above 80% and detoxification levels up to 50%. The high attributes of this strain, make it a promising candidate for the biological treatment of industrial dye containing effluents.

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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 bioadsorption 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 Renganahathan, 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

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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 ecotoxycological 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., 2008). Whole microbial cell processes are the most appropriated bio-systems for biodegradative processes as they allow the lowering of costs associated to enzyme purification or cofactor supply. Therefore, an *Escherichia coli* strain co-expressing *ppAzoR* and *cotA* genes was constructed where the sequential action of PpAzoR and CotA enzymes could be tuned by aeration conditions. The development and optimization of a whole cell process for model dye baths treatment lead to significantly high levels of dye decolourization and detoxification.

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 AY49, AB62, AR266, AB210, and AB194 dyes at 0.1 g L^{-1} each and 2 g L^{-1} of Na_2SO_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_2SO_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 tyndallization (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′ GCAGGGAATTGCATATGAAACTGTTGCACATCGATTCG 3′) and PpaR (5′ CCATAACCTAGGTCAGGCAGCCGCAAACAGCTCGCTGGC 3′). The resulting 612-bp DNA fragment was purified, digested with *Nde*I and *Avr*II and cloned between the same restriction sites of the plasmid pETDuet-1™ (Novagen) to produce pAIF-1. Similarly, for the amplification of *cotA* gene oligonucleotides CotA159D (5′ CCAGACAAGGACTAGTAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGACACTTGAAAAATTTGTGGATGC 3′) and CotA1892R (5′ CGCGGATCCTTTATGGGGATCAGTTATATCC 3′) were used. The product of the reaction (1539-bp) was digested with *Spe*I and *BamH*I purified and cloned into pAIF-1 previously digested with *Xba*I and *BamH*I, 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 T7*lac* 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-β-D-thiogalactopyranoside (IPTG) and 0.25 mM $CuCl_2$ were added to the culture medium and the

Table 1
Color index (C.I.) generic names, C.I registration numbers, absorption maxima, purity and calculated molar extinction coefficients of dyes used in this study [a].

C.I. generic name	C.I. constitution number	Dye content (%)	Absorption max (nm)
Direct Blue 1 (DB1)	24,410	80	610
Direct Red R (DRR)	22,120	91	530
Direct Black 38 (DB38)	30,235	50	600
Direct Blue 71 (DB71)	34,140	NS[b]	565
Direct Red 80 (DR80)	35,780	NS	555
Direct Yellow 106 (DY106)	1332	50	420
Reactive Red 4 (RR4)	18,105	50	530
Reactive Black 5 (RB5)	20,505	55	600
Reactive Yellow 145 (RY145)	[c]	50	420
Reactive Blue 222 (RB222)	[c]	NS	600
Reactive Red 195 (RR195)	[c]	30	550
Acid Red 299 (AR299)	[c]	NS	440
Acid Black 210 (AB210)	300,285	30	600
Acid Yellow 49 (AY49)	18,640	50	390
Acid Black 194 (AB194)	[c]	60	570
Acid Red 266 (AR266)	17,101	30	470
Acid Blue 62 (AB62)	62,045	100	600
Acid Orange 7 (AO7)	15,510	20–30	480
Sudan Orange G (SOG)	11,920	98	430
Methyl Red (MR)	11,920	95	430
Mordant Black 3 (MB3)	14,640	30	550
Mordant Black 9 (MB9)	14,855	60–85	550
Mordant Black 17 (MB17)	15,705	50	530

[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 (Montlinglen, 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.

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