Bioresource Technology 101 (2010) 2224–2229

Contents lists available at ScienceDirect

Bioresource Technology

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

Contributions to a better comprehension of redox-mediated decolouration and detoxification of azo dyes by a laccase produced by *Streptomyces cyaneus* CECT 3335

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ARTICLE INFO

Article history:
Received 10 September 2009
Received in revised form 11 November 2009
Accepted 16 November 2009
Available online 16 December 2009

Keywords:
Laccase
Streptomyces cyaneus
Natural mediator
Azo dyes
Toxicity

ABSTRACT

The ability of a laccase (EC 1.10.3.2) produced by *Streptomyces cyaneus* CECT 3335 to decolourise and detoxify azo dyes was assessed. Results showed that a colour loss of 90% was achieved only in the presence of acetosyringone (0.1 mM) acting as a redox mediator for the laccase. Toxicological analysis of the decolourised dyes revealed that there was no direct correlation between decolouration and detoxification; in fact, in the case of the dyes Methyl Orange and Orange II, a significant increase in toxicity was produced after the treatment. In contrast, a significant decrease in toxicity was observed after the decolouration of New Coccine and Chromotrope 2R. Finally, HPLC analysis of the dyes after treatment revealed the complete disappearance of both dyes and mediator and a concomitant appearance of new chromatographic peaks which could be responsible of the residual toxicity detected in some cases.

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1. Introduction

The development of the textile industry has resulted in the generation of large volumes of wastewaters that are released into the environment. In 2000, consumption of dyestuff alone totalled around 6×10^5 MT, with the major uses including India, China and East Europe countries (Wesenberg et al., 2003). The discharge of these industrial effluents into aquatic ecosystems is problematic not only for aesthetic reasons, but also for the alteration of the solubility of gases in water and importantly, because of their toxic and mutagenic effects on the ecosystem (Dos Santos et al., 2007). Of 4000 dyes investigated by ETAD (Ecological and Toxicological Association of the Dyestuffs), 90% were found to be highly toxic (Robinson et al., 2001).

Among all dyestuff, the azo dyes are considered the most important and represent 70% of total dyes produced per year (Hao et al., 2000; Dos Santos et al., 2007). Because of the low rate of fixation of these dyes to the textile fibre, their concentration in wastewaters has been estimated between 5 and 150 ppm (Gottlieb et al., 2003). The environmental hazard of azo dyes lies, more than in its low biodegradability, in their cleavage products which are carcinogenic (Chung and Cerniglia, 1992). The degradation of azo dyes under anaerobic conditions produced aromatic amines such as *o*-toluidine or aminobiphenyl-type compounds, which have been banned in Germany because their high toxicity and carcinogenicity (Tauber et al., 2005). These aromatic amines are bioaccumulated in the adipose tissue of fish reaching the humans throughout the alimentary chain. In humans, these compounds are frequently metabolised to its acyloxyamine derivatives which binds to guanine altering DNA structure (Takahashi and Hashimoto, 2001). Thus, to avoid the generation of aromatic amines caused by the anaerobic reduction of azo dyes, an effective oxidative strategy is required. Several physico-chemical oxidative processes have been developed, although the high economic costs of such techniques represent a serious obstacle for full commercial application. As a potentially cost-effective alternative the use of microorganisms and/or their oxidative enzymes to degrade azo dyes is being considered by a number of researchers. The most promising microorganisms to be investigated are the ligninolytic ones because of their high oxidative potential (Husain, 2006). Recently, several publications describing the degradation of synthetic dyes by ligninolytic fungi and/or their oxidative enzymes have been reported with special emphasis in the use of fungal laccases (Abadulla et al., 2000; Soares et al., 2001a; Camarero et al., 2005; Rodríguez Couto and Toca-Herrera, 2006).

Laccases (EC 1.10.3.2) are multicooper oxidases with a large catalytic versatility due to their low substrate specificity against phenolic compounds and aromatic amines. Moreover, these enzymes, in the presence of redox mediators are able to extend their oxidative action even to non-phenolic compounds (Bourbonnais et al., 1992; Call and Mücke, 1997). These mediators are low

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doi:10.1016/j.biortech.2009.11.061

molecular mass compounds oxidized by the laccase acting as electron carriers between the enzyme and other compounds. Laccase and laccase-mediator systems have been studied in white-rot fungi for a wide range of applications such as pulp delignification (Bourbonnais et al., 1992; Camarero et al., 2007), Polycyclical Aromatic Hydrocarbons (PAHs) and pesticide degradation (Johannes and Majcherczyk, 2000) and in the decolouration of synthetic dyes. However, there are other ligninolytic microorganisms such as *Streptomycetes* that also produced laccases whose application to synthetic azo dyes degradation is also being explored (Molina-Guijarro et al., 2009). The oxidative enzymatic mechanisms of these bacteria involved in lignocellulose degradation have been studied (Berrocal et al., 2000; Antonopoulos et al., 2001; Arias et al., 2003; Molina-Guijarro et al., 2009). Among the enzymes already characterized, a laccase produced by *Streptomyces cyaneus* CECT 3335 has demonstrated its usefulness for biotechnological purposes such as delignification of kraft pulp (Arias et al., 2003). Moreover, the physico-chemical and kinetics characteristics of this laccase suggest its potential application to the degradation of synthetic textile dyes.

The aim of this paper was to assess the capability of the laccase produced by *S. cyaneus* CECT 3335 to decolourise and detoxify a series of synthetic azo dyes commonly used by the textile industry.

2. Methods

2.1. Microorganism and growth conditions

S. cyaneus CECT 3335 was maintained as a suspension of spores and hyphal fragments in 20% (v/v) glycerol at −70 °C. For experiments, the microorganism was routinely cultivated on Soy-Manitol Agar (Hobbs et al., 1989) and incubated at 28 °C until sporulation occurred (4–6 days).

2.2. Laccase production and enzyme assay

Laccase from *S. cyaneus* was produced and purified as previously reported (Arias et al., 2003). Enzyme activity was determined using ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate)] (Sigma–Aldrich) as substrate (Werner et al., 1970). The reaction mixture contained 50 mM acetate buffer pH 4.5 and 5 mM ABTS. The increase in the absorbance was monitored at 436 nm using a Hitachi 2001 spectrophotometer connected to an Ultraterm thermostatic bath and the reaction was carried out at 50 ± 2 °C. Enzymatic activity was calculated using a molar extinction coefficient of 29,300 M^{-1} cm^{-1} for oxidized ABTS. One activity unit was defined as the amount of enzyme that releases 1 μmol of product per min. Proteins in the supernatant were estimated according to the Bradford method (Bradford, 1976).

2.3. Spectrophotometric characterization of azo dyes

To determine the maximum wavelength of absorption of the eight water-soluble azo dyes investigated, [*i.e.* Acid Yellow 17, Chromotrope 2R, Crocein Orange G, Methyl Orange, New Coccine (aka Acid Red 18, Ponceau 4R), Orange II (aka Acid Orange 7), Tartrazine (aka Acid Yellow 23) y Tropaeolin O (aka Acid Orange 6)] spectra between 800 and 200 nm were recorded using a Hitachi 2001 spectrophotometer. All chemicals were purchased from Sigma–Aldrich. Once established the maximum absorption wavelength for the dyes, different concentrations of each one in a range from 0 to 100 μM were prepared in 8 mM phosphate buffer (pH 5) and the molar extinction coefficient (ε) at those wavelengths were calculated according to the Lambert–Beer law. All the assays were carried out by triplicate. Chemical structures of all assayed azo dyes are available at the website of the Sigma home business (www.sigmaaldrich.com).

2.4. Dye decolouration by laccase and laccase-mediator system

Initially, the ability of purified laccase produced by *S. cyaneus* to decolourise the selected azo dyes was evaluated. Reaction mixtures containing 0.4 U laccase mL^{-1} and 50 μM of each dye were incubated in 50 mM acetate buffer pH 4.5 for 3 h at 35 °C.

In order to increase the oxidative potential of the enzyme, screening of natural mediators was carried out using as model dye, Crocein Orange G (λ = 480 nm). Phenolic compounds derived from lignin structure and purchased from Sigma Aldrich [*i.e.* syringic acid (3,5-dimethoxy-4-benzoic acid), 4-hydroxybenzoic acid, 4-hydroxyacetophenone, vanillic acid (3-methoxy-4-hydroxybenzoic acid) and acetosyringone (3,5-dimethoxy-4-hydroxyacetophenone)] were assayed together with laccase in decolouration experiments. For this purpose, three different concentrations of each phenolic compound (0.1, 1 and 10 mM) were tested together with 0.4 U laccase mL^{-1} and 50 μM (175 mg L^{-1}) Crocein Orange G. The reactions were maintained in 50 mM acetate buffer pH 4.5 for 4 h at 35 °C. Decolouration was determined by the decrease in the absorbance at 480 nm compared with controls. Experiments were carried out in duplicate and heat inactivated laccase was used in control reactions.

Once acetosyringone was selected as the best mediator, assays of decolouration of the 8 azo dyes were performed with 0.4 U mL^{-1} laccase and 100 μM acetosyringone in the buffer described above for 4 h at 35 °C. All dyes were assayed at 50 μM which correspond to the following concentrations in ppm: Acid Yellow 17, 275 mg L^{-1}; Chromotrope 2R, 234 mg L^{-1}; Crocein Orange G, 175 mg L^{-1}; Methyl Orange, 163.5 mg L^{-1}; New Coccine, 302 mg L^{-1}; Orange II, 175 mg L^{-1}; Tartrazine, 267 mg L^{-1} and Tropaeolin O, 158 mg L^{-1}. In all cases, decolouration was determined by monitoring the decrease in the absorbance at the maximum absorption visible wavelength of each dye and expressed as concentration (μM) using the molar extinction coefficient of each compound (see Table 1). Experiments were carried out in duplicate and heat inactivated laccase was used in all blank reactions.

2.5. Toxicity assessment

Toxicity of New Coccine, Orange II, Methyl Orange and Chromotrope 2R azo dyes and decolourised reaction mixtures was determined against the bioluminescent organism *Vibrio fischeri* using the Microtox® System (Azur Environmental) according to ISO/TC 147/SC S. All samples were serially diluted in 2% (w/v) NaCl. All the assays were performed in duplicate at pH between 7.0 and 8.0 and at a temperature of 15 °C. Sodium chloride 2% (w/v) was used as the negative control. The sample concentration that inhibited 50% of the light output after 5 min exposure period (EC50) was

Table 1
Maximum absorption wavelengths in the visible spectrum (λ) and molar extinction coefficients (ε) of the azo dyes at pH 5.

Dye	λ (nm)	ε (M^{-1} cm^{-1})
Acid Yellow 17	402	34,114
Chromotrope 2R	505	31,495
Crocein Orange G	480	22,895
Methyl Orange	457	27,933
New Coccine	506	25,746
Orange II	485	23,251
Tartrazine	427	28,524
Tropaeolin O	388	22,276

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