Journal of Hazardous Materials 164 (2009) 296–300

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

Simultaneous production of laccase and decolouration of the diazo dye Reactive Black 5 in a fixed-bed bioreactor

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ARTICLE INFO

Article history:
Received 30 June 2008
Received in revised form 31 July 2008
Accepted 1 August 2008
Available online 20 August 2008

Keywords:
Fixed-bed reactor
Laccase
Reactive Black 5
Stainless steel sponges
Trametes pubescens

ABSTRACT

In this paper the production of laccase and the decolouration of the recalcitrant diazo dye Reactive Black 5 (RB5) by the white-rot fungus *Trametes pubescens* immobilised on stainless steel sponges in a fixed-bed reactor were studied. Laccase production was increased by 10-fold in the presence of RB5 and reached a maximum value of 1025 U/l. Enhanced laccase production in the presence of RB5 in this fungus is an added advantage during biodegradation of RB5-containing effluents. The decolouration of RB5 was due to two processes: dye adsorption onto the fungal mycelium and dye degradation by the laccase enzymes produced by the fungus. RB5 decolouration was performed during four successive batches obtaining high decolouration percentages (74%, 43% and 52% in 24 h for the first, third and four batch, respectively) without addition of redox mediators. Also, the *in vitro* decolouration of RB5 by the concentrated culture extract, containing mainly laccase, produced in the above bioreactor was studied. The decolouration percentages obtained were considerably lower (around 20% in 24 h) than that attained with the whole culture.

1. Introduction

Considering both volume discharged and effluent composition, wastewater generated by the textile industry is rated as the most polluting among all the industrial sectors [1]. The presence of very low concentrations of dyes in effluents is highly visible and undesirable [2]. There are more than 10^5 commercially available dyes with over 7×10^5 ton of dyestuff produced annually world-wide [3]. Among these available dyes, azo dyes constitute up to 70% of all the known commercial dyes produced [4]. Azo dyes are mostly used in textile dyeing due to their superior fastness to the applied fabric, high stability to light and resistance to microbial attack. However, azo dyes are particularly problematic, because they exhibit low levels of binding to the fabric and about 10–20% of the total dye used in the dyeing process is lost in wastewater together with accessory chemicals [5]. Azo dyes are characterised by the presence of one or more azo bonds (–N=N–) and are prepared by coupling a diazotised aromatic amine with a phenol or aromatic amine. Out of the 2000 dyes synthesised so far, more than 500 are based on carcinogenic amines. In fact, several of these aromatic amines have been classified by the International Agency for Research on Cancer (IARC) as human carcinogens [6]. Hence, the majority of azo dyes are also mutagenic and carcinogenic to humans as well as to other animals [7]. Therefore, there is a need to remove these dyes before the effluent is discharged into receiving water bodies.

Azo dyes are not easily degraded by the conventional existing technologies. Furthermore, under anaerobic conditions azo-reductases cleave azo dyes into the corresponding amines, most of which are mutagenic and/or carcinogenic [8]. Moreover, azo-reductases have been shown to be very specific enzymes, cleaving only azo bonds of selected dyes [9]. Altogether it underlines the need for non-specific processes for the treatment of such kind of dyes.

Recently, biological processes have received more attention as they are cost-effective and environmentally friendly. Colour removal processes with active microorganisms have two different simultaneous steps: an adsorption of dyes on the surface of the organisms and a degradation of dyes by the enzymes produced by these organisms [10–12].

The aim of the present paper was to develop an efficient and relatively simple bioprocess based on the immobilisation of a white-rot fungus for decolourising the recalcitrant diazo dye Reactive Black 5.

2. Materials and methods

2.1. Microorganism

Trametes pubescens MB 89 (CBS 696.94; Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) was obtained from

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doi:10.1016/j.jhazmat.2008.08.032

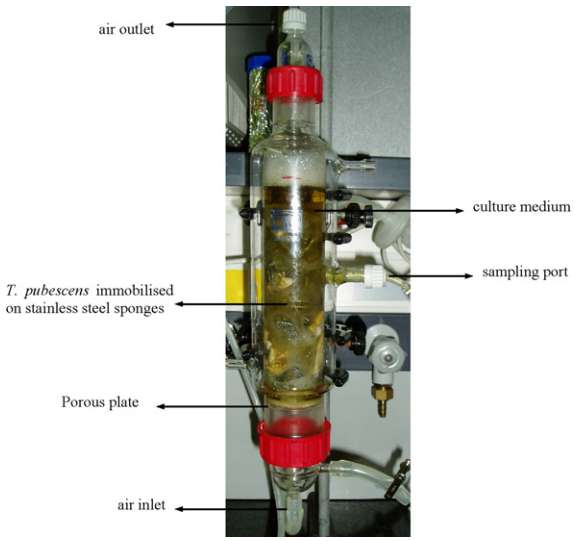

Fig. 1. Photograph of the bioreactor used.

the Institute of Applied Microbiology, University of Agricultural Science (Vienna, Austria) and was maintained on malt extract agar (MEA) plates at 4 °C and sub-cultured every 3 months.

2.2. Support

Stainless steel sponges (Mapa Spontex Ibérica SA, Spain,) were used as immobilisation supports. The sponges were pre-treated by boiling for 10 min and washing thoroughly three times with distilled water. Thereafter, they were dried overnight at room temperature. Prior to use, the sponges were autoclaved at 121 °C for 20 min.

2.3. Pre-cultivation of the fungus in Erlenmeyer flasks

The composition of the culture medium was as follows: 10 g/l glucose, 20 g/l peptone from casein, 0.9 g/l $(NH_4)_2SO_4$, 2 g/l KH_2PO_4, 0.5 g/l $MgSO_4{\cdot}7H_2O$, 0.1 g/l $CaCl_2{\cdot}2H_2O$, 0.5 g/l KCl, 0.5 g/l thiamine in citrate–phosphate buffer (pH 4.5). The medium was sterilised at 121 °C for 20 min. After cooling, the thiamine previously sterilised by filtration (0.22 μm) was added to the medium.

The cultures were performed in cotton-plugged Erlenmeyer flasks (250 ml) containing one stainless steel sponge (weight: 4.0 g; diameter: 4.5 cm) and 100 ml of culture medium. Inoculation was carried out directly in the Erlenmeyer flasks. Three agar plugs (diameter: 7 mm), from an actively growing fungus on MEA, per Erlenmeyer were used as inoculum. The Erlenmeyer flasks were statically incubated under an air atmosphere at 30 °C in complete darkness, for 7 days. After that time, 6 stainless steel sponges colonised by the fungus were transferred to the bioreactor, this being the starting time for the experiment.

2.4. Bioreactor configuration and operation conditions

2.4.1. Fixed-bed tubular bioreactor

It consisted of a glass column with dimensions of 20 cm height and 4.5 cm in internal diameter (working volume of 200 ml). The bioreactor was filled with six stainless steel sponges colonised by *T. pubescens* (Fig. 1). The composition of the culture medium was the same as that used in the pre-cultivation of the fungus but supplemented with 0.5 mM Cu^{2+} to stimulate laccase production. Air was supplied in a continuous way at 1 vvm, occasioning a gentle agitation of the culture medium.

Samples from the middle part of the bioreactor were collected once a day, centrifuged (8000 × *g*, 10 min) and analysed. Duplicate experiments were run for comparison and samples were analysed twice. The values in the figures correspond to mean values with a standard deviation lower than 15%.

2.5. Determination of laccase activity

Laccase activity was determined spectrophotometrically as described by Niku-Paavola et al. [13] with ABTS as a substrate. One activity unit was defined as the amount of enzyme that oxidised 1 μmol of ABTS per min. The activities were expressed in U/l.

2.6. Decolouration of RB5 at bioreactor scale

The decolouration of the diazo dye Reactive Black 5 (RB5) by *T. pubescens* grown on the above bioreactor was performed. The dye was purchased from Sigma–Aldrich (St. Louis, MO, USA). The dye was added after six operation days, when laccase reached values around 100 U/l. According to previous experiments (unpublished results) two dye concentrations were tested: 30 and 60 mg/l. Samples, taken at convenient intervals from the middle part of the bioreactor, were centrifuged (8000 × *g*, 10 min) to eliminate suspended particles. The residual dye concentration was spectrophotometrically measured from 450 to 700 nm and associated with the decrease in the area under the plot. Dye decolouration was calculated by means of the formula:

$$D = \frac{100(A_{ini} - A_{obs})}{A_{ini}}$$

D is the decolouration (in %), A_{ini} is the area under the curve of the absorption spectrum from 450 to 700 nm at the initial time, and A_{obs} is the area under the curve of the absorption spectrum from 450 to 700 nm at a determined time.

For the decolouration the medium composition was: 2 g/l glucose, dye (60 mg/l), 0.1 mM acetate buffer (pH 4.5) and copper (0.5 mM). The medium was added to the bioreactor under non-sterilised conditions.

2.7. In vitro decolouration of RB5

Laccase was produced as indicated in Section 2.4. Culture broth was collected at the maximum laccase activity, filtered and the supernatant was clarified by centrifugation at 8000 × *g* for 15 min. After that, the supernatant was ultra-filtrated in an Amicon stirred cell apparatus (YM 10 membrane). The resulting concentrated extract was used to perform the *in vitro* decolourising experiments.

The reaction mixture for RB5 decolouration consisted of an aqueous solution of RB5 (60 mg/l, final concentration), laccase extract (final concentration: 100, 300, 500 and 1000 U/l) in citrate phosphate buffer (pH 4.5) in a final volume of 1.5 ml. All the reactions were incubated at room temperature, without shaking and in complete darkness. Decolouration was measured as indicated above. A control test containing the same amount of a heat-denatured laccase was performed in parallel. The assays were done twice, the experimental error being below 3%.

2.8. Polyacrylamide gel electrophoresis under denaturing conditions

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the concentrated culture extract, obtained as

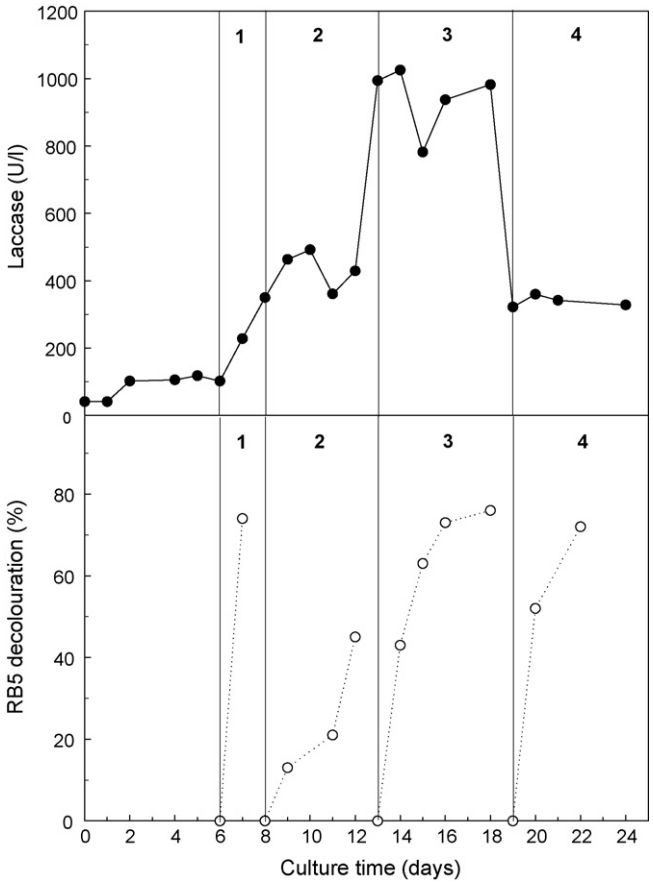

Fig. 2. Laccase production and percentage of RB5 decolouration obtained by *T. pubescens* immobilised on stainless steel sponges in a fixed-bed bioreactor along four successive batches.

indicated above, was performed on a 10% resolving gel and a 4% stacking gel according to the method of Laemmli [14]. Prior to gel application, the samples were precipitated with trichloro acetic acid (TCA). Protein bands were visualised by staining with Coomassie Brilliant Blue (Sigma–Aldrich, St. Louis, MO, USA) and compared with standard molecular weight markers (Bio-Rad, Richmond, USA).

3. Results and discussion

3.1. Laccase production by T. pubescens in a fixed-bed reactor

During the first cultivation days laccase was at values around 100 U/l (Fig. 2). After addition of the dye (30 mg/l) on day 6, laccase activity appeared to be boosted as 24 h after the dye addition it increased by 2-fold (228 U/l) and 48 h after by 3.5-fold (350 U/l) (Fig. 2). This phenomenon was also observed by Shliepkaher and Lonergan [15], who found a considerably increase in laccase activity 30 min after the addition of the dye Remazol Brilliant Blue R to *Pycnoporus cinnabarinus* cultures. Recently, Malhotra et al. [16] also found that the addition of the dyes Ethidium Bromide, Malachite Green, Phenol Red and Thymol Blue to cultures of γ-proteobacterium JB enhanced laccase production by several folds. More recently, D'Souza et al. [17] reported the simultaneous increased production of laccase and decolouration of different dyes and effluents in cultures of an unidentified marine basidiomycetous fungus.

On day 10, the decolourised medium was poured out and the reactor was filled with fresh dye-containing medium (60 mg/l). Laccase activity increased again showing a maximum value of 994 U/l on the 13th day. Afterwards, the decolourised medium was poured out and the reactor was filled again with fresh dye-containing medium (60 mg/l). Laccase activity was kept at values of about 900 U/l and from day 18 onwards it sharply diminishes showing values around 330 U/l and although the medium was replaced again on day 19, the activity did not increase. This was likely due to the high pH values (around 8–9) found at the last stages of the cultivation [18,19].

3.2. Decolouration of RB5 by T. pubescens in a fixed-bed bioreactor

The diazo dye RB5 was aseptically added to the above-mentioned bioreactor on day 6 as an aqueous solution to a final concentration of 30 mg/l. The dye was decolourised about 68% in only 5 h and 74% after 24 h (Fig. 2). Decolouration was due to laccase enzyme, since no other lignin-degrading enzymes were detected in the fermentation broth. After that, the reactor was poured out and filled with new medium containing 60 mg/l of RB5 under non-sterilised conditions. A decolouration percentage of 13% was reached in 24 h, 21% in 72 h and 45% in 96 h (Fig. 2). Decolouration was slower than in the first batch but also dye concentration was 2-fold higher. Then, the reactor was poured out and filled with new medium containing 60 mg/l of RB5. A decolouration percentage of 43% was reached in 24 h, 63% in 48 h, 73% in 72 h and 76% in 120 h (Fig. 2). After that, the reactor was poured out and filled with new medium containing 60 mg/l of RB5. A decolouration percentage of 52% was reached in 24 h and of 70% in 72 h (Fig. 2). So, despite the low laccase levels produced in batch 4 (around 300 U/l) dye removal was as high as in batch 3.

It is worth mentioning that the fungus remained well attached to the carrier during the whole experiment and the medium remained totally clear. In addition, no operational problems were detected. However, a certain amount of dye was removed from the solution by absorption to fungal mycelium. Fig. 3 shows the dye-containing medium before and after laccase treatment. As it can be observed the dark blue colour of the RB5 was removed.

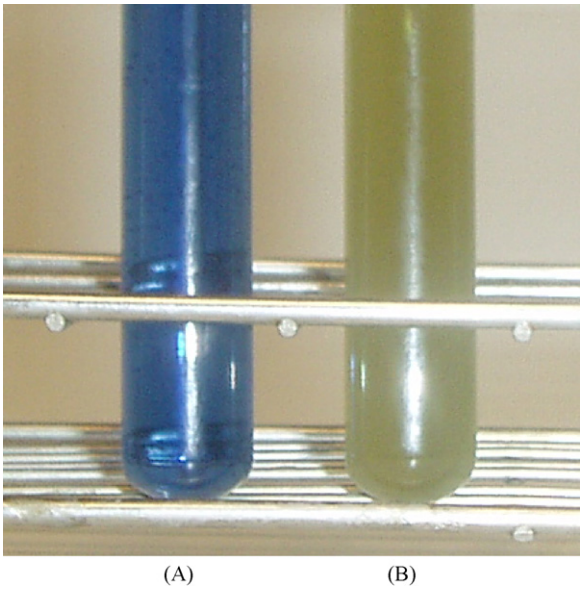

Fig. 3. RB5 solution before (A) and after (B) fungal treatment.

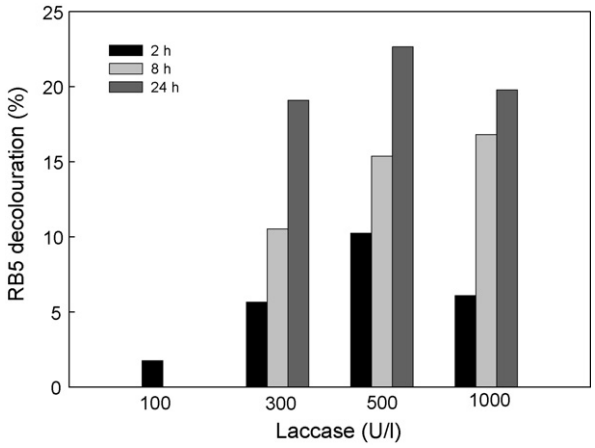

Fig. 4. *In vitro* decolouration of RB5 by laccase.

3.3. *In vitro* decolouration of RB5 by crude laccase from *T. pubescens*

The experiments were performed at pH 4.5 and laccase activities ranging from 100 to 1000 U/l were considered. As observed in Fig. 4 the optimum activity of laccase enzyme for RB5 decolouration was 500 U/l. Thus, a decolouration percentage of 22.7% in 24 h was obtained. This value is much lower than those attained with the whole culture. This indicates that the mycelial biomass may supply other intracellular or mycelial-bound enzymes, or other compounds that favour RB5 decolouration [20,21].

On the other hand, it was observed than from 24 h onwards dye decolouration did not increase (data not shown) which could be due to laccase inhibition by some products generated in the degradation process. The fact that a laccase activity of 1000 U/l led to a lower decolouration percentage could be attributed to the lack of oxygenation as well as to the increasing concentration of degradation products. Rodríguez Couto et al. [18] also found this effect on the *in vitro* decolouration of the leather dye Luganil Green.

As for the experiments with heat-denatured laccase, they did not show any change in their visible spectrum along incubation time. This indicates that dye decolouration was due to laccase enzyme, since no other enzymes were detected in the fermentation broth.

Fig. 5 shows the SDS-PAGE of the concentrated culture extract produced. The main band likely corresponds to laccase, since no other ligninolytic enzymes were detected in the culture extract, with a molecular weight of about 63 kDa as visualised by Coomassie Brilliant Blue staining. This molecular weight is similar to those found for other laccases from fungal species belonging to the genus *Trametes* [22–26].

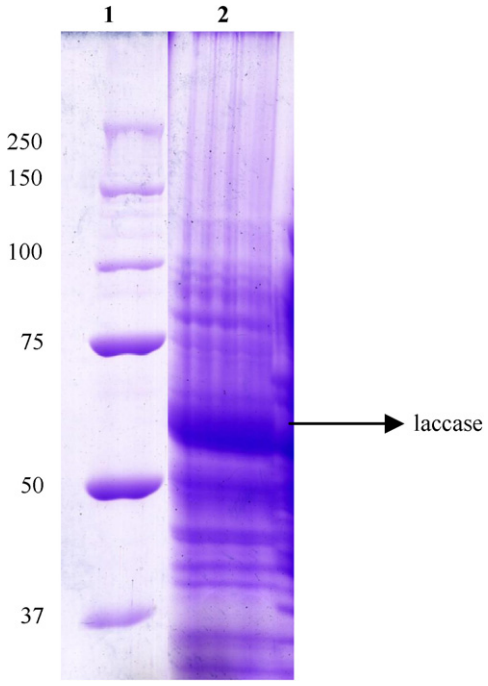

Fig. 5. SDS-PAGE of the concentrated culture extract obtained. Lane 1: standard markers; lane 2: concentrated extract.

4. Conclusions

In view of the results obtained, it can be concluded that the white-rot fungus *T. pubescens* immobilised on stainless steel sponges in a fixed-bed bioreactor decolourised the recalcitrant diazo dye RB5 in successive batches with high efficiency. In addition, no operational problems were detected during cultivation. This makes this system very promising for its application to a continuous operation.

Acknowledgements

This research was financed by the Spanish Ministry of Education and Science (Project CTQ2007-66541). SRC is a *Ramón y Cajal* Senior Research Fellow and gratefully acknowledges the Spanish Ministry of Education and Science for promoting the *Ramón y Cajal Programme* and the European Social Fund for co-financing her contract. KE thanks the University of Tehran (Iran) for financial support.

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