Scientific Paper

DECOLORATION OF THE DIAZO DYE REACTIVE BLACK 5 BY IMMOBILISED *Bjerkandera adusta* IN A STIRRED TANK BIOREACTOR

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Received 27-09-2004

Abstract

Decoloration of the synthetic textile dye Reactive Black 5 by the cultivated fungi, *Bjerkandera adusta*, *Irpex lacteus,* and *Hypoxylon fragiforme* was studied. The most effective fungus in shaken flask experiments was *B. adusta*, which was able to decolorise the dye from black-blue to a yellow color in less than 10 days. This fungus was chosen for cultivation in a 5 L stirred tank bioreactor with immobilised mycelium. Here decoloration of the dye in an initial concentration of 0.2 g/L from black-blue to intense yellow was reached in 20 days. Considering the spectral absorption coefficients at three different wavelengths, the final liquid was decolorised to an almost ecologically acceptable degree. Measurement of activities of the oxidative enzymes laccase, lignin peroxidase, manganese dependent- and manganese independent peroxidases showed that initially lignin peroxidases and subsequently manganese dependent peroxidases are presumably responsible for the decoloration of the diazo dye Reactive Black 5 by *B. adusta*.

Key words: diazo dye, Reactive Black 5, decoloration, ligninolytic fungi, *Bjerkandera adusta*, peroxidases, stirred tank bioreactor, immobilised biomass, bioremediation

Introduction

Synthetic dyes are produced in large amounts and are used in different industrial branches including the textile industry. After the dyeing process, the excess of dye remains in the effluent, which must be treated before release into the environment.[1] Conventional treatment systems for color removal are not effective due to their complex structure and synthetic origin. Specific treatment is needed based on either chemical, physical or biological methods. Currently, dyes are removed by physico-chemical means, mostly by oxidation or adsorption. However, alternative methods involving bioremediation have increasingly been sought.[2]

Dyes usually have a complex aromatic molecular structure, which makes them stable and resistant to biodegradation.[3] The chromophore groups in dyes are generally organic compounds with conjugated double bonds. The largest class of commercially

produced dyes are azo dyes in which the chromophores are azo groups.[2–4] The azo linkage can be split either by reduction, the reaction used by some bacterial consortia under anaerobic conditions, or by oxidation, the reaction used by ligninolytic white rot fungi.[1] Since reduction of azo dyes can result in accumulation of toxic aromatic amines, oxidative decoloration is attracting much more interest. The white rot fungus *Phanerochaete chrysosporium* was already reported to decolorise azo dyes in 1990.[4] Since then a number of reports studying the ability of different white rot fungi for decoloration of various dyes have been increasing.[5–10] Several types of bioreactors have been developed for this purpose and it was found that the most efficient decoloration has been achieved with immobilised mycelium.[11]

In our study three fungal species were chosen on the basis of literature data and were cultivated in a medium containing a selected diazo dye, Reactive Black 5, as a model substrate for decoloration. With the most promising strain decoloration was performed in a stirred tank bioreactor using immobilised mycelium. Involvement of specific ligninolytic enzymes in the process was also studied.

Experimental

Microorganisms

Bjerkandera adusta (MZKI G-84) and *Hypoxylon fragiforme* (MZKI G-61) were taken from the Microbiological Culture Collection of the National Institute of Chemistry, Slovenia, while *Irpex lacteus* (CCBAS238) was obtained from the Culture Collection of Basidiomycetes of the Academy of Sciences, Prague, Czech Republic. All the strains were maintained on potato dextrose agar slants at 4 °C.

Dye

The diazo dye C.I. Reactive Black 5 (RB5), was used. The starting concentration in the medium was 200 mg/L. The chemical structure is represented in Figure 1.[6]

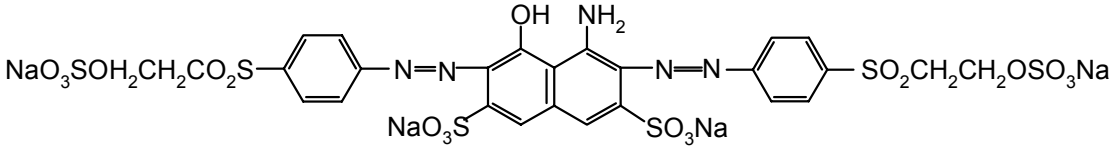

Figure 1. Structural formula of Reactive Black 5.

Medium

A nitrogen limited medium according to Tomaževič and Perdih,[12] with slight modifications, was used for cultivation of fungi in submerged cultures. The composition was as follows: 10 g/L glucose, 0.2 g/L yeast extract, 0.07 g/L veratryl alcohol, 3.0 g/L tartaric acid, 1 g/L Tween 80, 0.2 g/L KH_2PO_4, 0.146 g/L $CaCl_2 \cdot 2H_2O$, 0.157 g/L $(NH_4)_2HPO_4$, 0.05 g/L $MgSO_4 \cdot 7H_2O$, 42.5 mg/L $ZnSO_4 \cdot 7H_2O$, 33.8 mg/L $MnSO_4 \cdot H_2O$, 7 mg/L $CoCl_2 \cdot 6H_2O$, 7 mg/L $CuCl_2 \cdot 2H_2O$, 0.54 mg/L $FeCl_3$, 0.9 mg/L NaCl, and 200 mg/L RB5. All the constituents were dissolved in distilled water. The pH of the medium was adjusted to 4.5 with NaOH.

Shaken flask experiments

For decoloration experiments in submerged shaken cultures 100 mL aliquots of the medium were poured into 500 mL flasks and autoclaved at 121 °C, and 121.6 kPa for 20 min. The inoculum was prepared from fungal cultures cultivated on agar slant at 30 °C for 10 days. The mycelium from one slant was suspended in 25 mL of sterile water. The suspension in aliquots of 5 ml was used to inoculate 100 mL of the medium. The flasks were incubated on a rotary shaker (New Brunswick Scientific) at 30 °C and 100 rpm. Experiments were performed in triplicate.

During fermentation samples of the broth were taken for analysis in aliquots of 1 mL. Aliquots from parallel flasks were combined and stored at –20 °C. Before analysis the samples were thawed and filtered through "black ribbon" filter paper.

Bioreactor experiments

Decoloration of RB5 was performed in an Infors ISF-100 stirred bioreactor with 5-litre working volume. To immobilise the fungal mycelium, autoclavable plastic net in the form of cylinder was attached on the inner side of the vessel. The medium was poured into the bioreactor vessel and autoclaved at 121 °C, and 121.6 kPa for 20 min. A mycelial suspension prepared from three agar slants of *B. adusta* was used for inoculation. Fermentation was performed at 30 °C, with aeration of 1 vvm. The agitation rate was reduced from the initial 250 rpm to 160 rpm on the 5th day of fermentation.

Samples of the broth were collected daily and frozen. For analysis the aliquots were thawed and filtered through a "black ribbon" filter paper.

Measurement of decoloration

Decoloration of the dye was followed during fermentation by measurement of the absorbance of the filtrates. The whole absorption spectra of the samples diluted 3-times with deionised water were recorded in the wavelength range from 350 to 700 nm by a spectrophotometer (Beckmann, Fullerton, CA, USA). From the spectra the values at the wavelength of 598 nm, the absorption maximum for RB5, were taken for evaluation of decoloration. Similarly, readings at wavelengths 436, 525, and 620 nm were used for calculating the spectral absorption coefficient α according to equation 1.

$$\alpha(\lambda) = \frac{A}{d * f} \qquad (1)$$

α (λ)…….. spectral absorption coefficient (m^{-1})
A……... absorbance at wavelength λ
d…… optical path (mm)
f…… conversion factor

Enzyme activities

Ligninolytic enzyme activities of the filtrates were measured at 30 °C. Manganese dependent peroxidase (MnP) and manganese independent peroxidase (MIP) activities were measured on 20 mM 2,6-dimethoxyphenol (DMP) in malonate buffer pH 4.5.[13] The enzyme/substrate mixture contained 1 mM $MnSO_4$ for the MnP or 1 mM EDTA for the MIP activity test. The reaction was started by the addition of H_2O_2 to a final concentration of 0.4 mM and the synthesis of the reaction product was continuously recorded at 468 nm during the first 120 seconds.

Lignin peroxidase (LiP) activity was determined on 2 mM veratryl alcohol in tartaric buffer pH 2.5.[13] The reaction was started by the addition of H_2O_2 (0.4 mM) and the development of the product was traced at 310 nm for up to 100 seconds.

Laccase activity was detected by monitoring the oxidation of 0.5 mM 2,2'-azino-bis-[3-ethylthiazoline-6-sulfonate] (ABTS) at 420 nm for 120 seconds.[14]

One unit of each enzymatic activity was defined as one μmol of the product formed per minute.

Results and discussion

In shaken cultures the fungi grew in the form of pellets. The fungal activity was observed as color change and increased transparency of the medium. Decoloration of the

dye Reactive Black 5 by the selected fungi is shown in Figure 2. Color change was first seen in the cultures of *B. adusta* that turned from black-blue to dark red on the 5th day and proceeded through light red and orange into yellow within 10 days. After an initial lag phase the original dye concentration decreased rapidly and nearly disappeared after 7 days. The fungus *I. lacteus* was less effective regarding decoloration, which started after the 7th day. The color changed from black-blue to only violet within 14 days. There was no change in color in the cultures of *H. fragiforme* and the slight decrease in the dye concentration could be due to adsorbance of RB5 on the mycelium. A decoloration effect by biosorption on living or dead cells was observed with several fungi.[3]

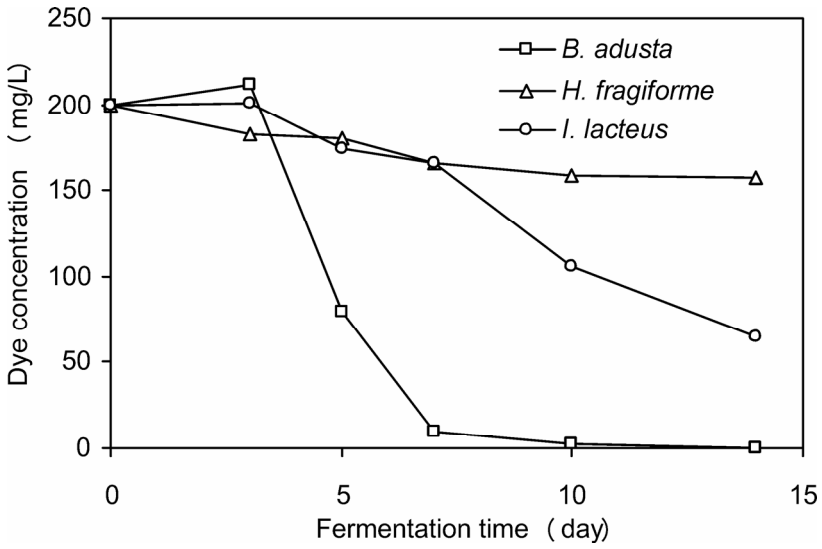

Figure 2. Decoloration of Reactive Black 5 in culture broths of selected fungi.

The wavelength of maximal absorbance in the culture of *B. adusta* changed with fermentation time as shown in the absorption spectrum (Figure 3a). The value at the initial λ_{max} of 598 nm greatly decreased, and the maximal absorbance shifted towards lower wavelengths according to the change in color. This effect can be attributed to the decrease of the number of conjugated double bonds and possibly also to splitting of the dye molecule. In the case of *I. lacteus* the absorbance was partly reduced only at the maximal absorbance wavelength, while no shift could be observed (Figure 3b). We assume that decoloration with this fungus is much slower in the conditions applied, or its mechanism is not the same as with *B. adusta*.

The oxidative enzymatic system of white rot ligninolytic fungi is regarded as being responsible for degradation of several resistant compounds, including synthetic textile dyes. The fungus *Phanerochaete chrysosporium* was manly studied,[15–20] but lately some other species have shown superior activity.[21] In our experiments *B. adusta* proved to be the most efficient in dye decoloration. The same species was also successfully applied by some other researchers.[5–7,22–23] On the other hand, *I. lacteus* that was the fungus of choice in some investigations,[9–10,24] was not very effective in our experiments. The reason could lie in the use of shaken instead of static cultivation.

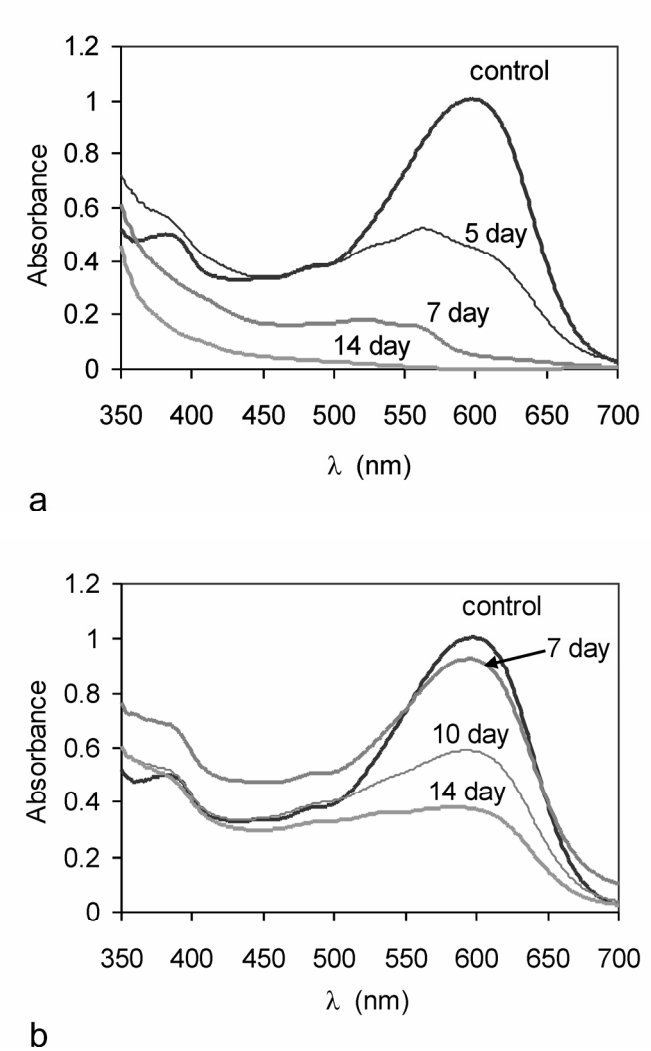

Figure 3. Changes in the absorption spectra of culture media of *B. adusta* (a), and *I. lacteus* (b).

B. adusta was selected for the experiments on dye decoloration in a stirred tank bioreactor. Since in our preliminary experiments with free mycelium the fungus tended

to attach itself to the surface and knowing that ligninolytic enzyme production is sensitive to shear stress,[25–26] the net was mounted into the bioreactor vessel. After inoculation, self-immobilisation occurred and the mycelium gradually overgrew the support in the form of a thin layer. Decoloration began after the 4th day, and after 8 days the color of the fermentation broth shifted from black-blue to red. In the following days the color continued to lighten through different nuances of red into bright orange-yellow, becoming more and more transparent. Changes in color of the filtrates taken on even days from 0 to 20 days are shown in the photo included in Graphical Contents.

The absorbances at three different wavelengths were measured in order to estimate the spectral absorption coefficients (α), which are recommended as the criterion for permissible coloration of effluents. Three specific wavelengths of 620 nm, 525 nm, and 436 nm, corresponding to the perceived colors blue, red, and orange, respectively, were chosen. The decrease of spectral absorption coefficients with fermentation time is presented in Figure 4. As the black-blue color vanished after 8 days the α (620 nm) value decreased by 90%. The same trend could be observed for α (525 nm) as the red color disappeared after 18 days.

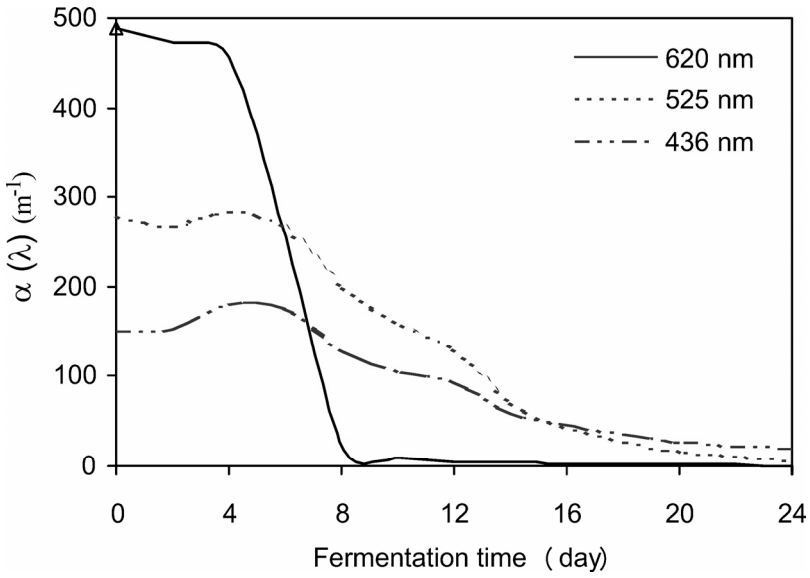

Figure 4. Time course of spectral absorption coefficients $\alpha(\lambda)$ of the filtrate during decoloration of Reactive Black 5 by *Bjerkandera adusta.*

The final values of α (λ) were compared to the values recommended for release of effluents into water bodies (Table 1). Coloration of the broth after 24 days of incubation

with *B. adusta* was near the allowed values, and it is supposed that with a lower starting dye concentration, or by optimising the fermentation conditions, the result could be further improved.

Table 1. Values of spectral absorption coefficient α (λ) at the beginning and at the end of fermentation with *Bjerkandera adusta* in comparison with the recommended ones.

Values of α (λ)	α (436 nm) (m^{-1})	α (525 nm) (m^{-1})	α (620 nm) (m^{-1})
Initial	152.1	279.8	488.5
After 24-day fermentation	20.2	7.4	0.8
Recommended	7	5	3

Four types of oxidative enzymes are known to be synthesised by *B. adusta*.[27] Therefore, they were measured during the bioprocess in order to find which were most responsible for the decoloration effect. According to the observed time course of the enzyme activities, it can be presumed that in the starting phase during the shift from black-blue to red color the effect of LiP prevailed (Figure 5). LiP activity was also present later during decoloration from red to yellow but its amounts were insignificant compared to MnP, which started on the 6th day and prevailed throughout the rest of the fermentation. Laccase and MIP activities seemed not to have significant effects.

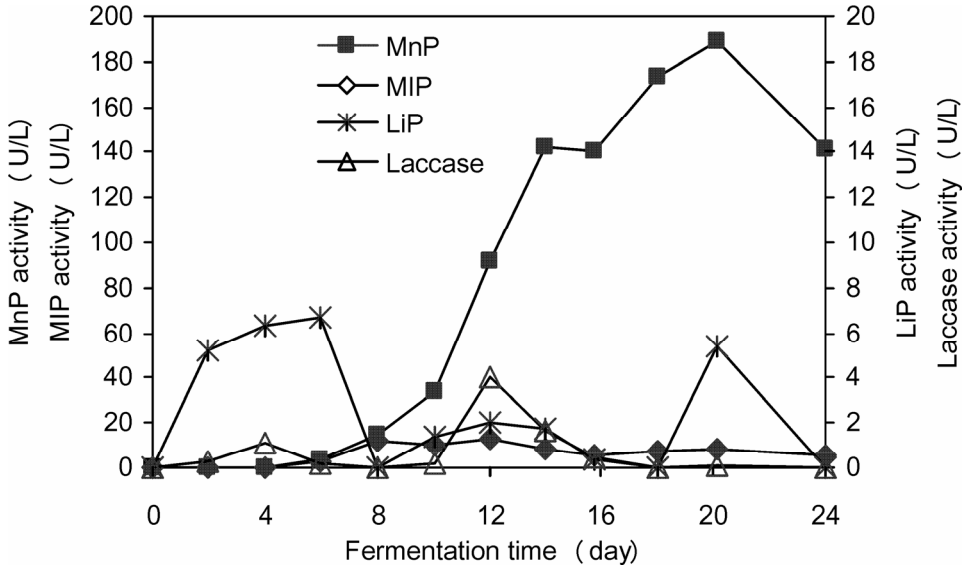

Figure 5. Enzymatic activities during decoloration of Reactive Black 5 by *Bjerkandera adusta.*

Comparing this finding to the results obtained by other researchers, our work confirms the involvement of LiP and MnP.[17,22,28] Laccase was also found to be the

responsible decoloration enzyme by some investigators,[21,28–30] but in our experiments its production by *B. adusta* was low and it was probably not involved in the decoloration of the dye. However, further experiments with purified enzymes should be made to elucidate which enzyme is manly responsible for decoloration and degradation of RB5.

Conclusions

B. adusta proved to be the most effective among the tested fungi in decoloration of the diazo dye Reactive Black 5 in the applied submerged cultivation conditions.

Cultivation of the fungus in a stirred tank bioreactor was possible after immobilisation of the mycelium on plastic net.

After 20 days of cultivation in the bioreactor, decoloration nearly reached the recommended values of spectral absorption coefficients acceptable for wastewater release into water streams.

Among the four oxidative enzymes synthesized by *B. adusta* in the first stage LiP, but later MnP, seemed to be responsible for the decoloration.

Acknowledgements

Financial support of the program via grants P4-0176 and P2-1951 from the Ministry of Education, Science and Sport of the Republic of Slovenia, the Slovenian-Czech bilateral project 2004-2005 and the COST Action D25 “Applied Biocatalysis” are gratefully acknowledged.

The authors wish to thank Prof. Dr. Novotny for kindly supplying the strain *Irpex lacteus* from Culture Collection of Basidiomycetes of the Academy of Sciences, Prague, Czech Republic.

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Povzetek

Med gojenjem gliv *Bjerkandera adusta*, *Irpex lacteus* in *Hypoxylon fragiforme* smo proučevali razbarvanje sintetičnega tekstilnega barvila Reactive Black 5. V stresanih kulturah je bila najučinkovitejša *B. adusta*, ki je razbarvala barvilo iz črno-modre do rumene barve v manj kot 10 dneh. Z imobiliziranim micelijem te glive smo študirali razbarvanje barvila v 5-litrskem bioreaktorju. V 20 dneh je gliva razbarvala barvilo v začetni koncentraciji 0,2 g/L od črno-modre do intenzivno rumene. Upoštevajoč spektralne absorpcijske koeficiente pri treh valovnih dolžinah, je bila izhodiščna tekočina razbarvana skoraj do ekološko dopustne stopnje. Merjenje aktivnosti oksidativnih encimov kot so lakaza, lignin peroksidaza, mangan odvisna in mangan neodvisna peroksidaza, so pokazale, da sta za razbarvanje barvila Reactive Black 5 z glivo *B. adusta* verjetno odgovorni sprva lignin peroksidaza, kasneje pa mangan odvisna peroksidaza.