Decolorization of an azo dye, *Reactive Black 5* and MnP production by yeast isolate: *Debaryomyces polymorphus*

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Received 15 September 2004; accepted 25 December 2004

Abstract

The optimum conditions for decolorization of an azo dye, *C.I. Reactive Black 5* (RB5) and the kinetic characteristics of manganese-dependent peroxidase (MnP) production by yeast isolate, *Debaryomyces polymorphus*, were investigated. *D. polymorphus* could completely degrade 200 mg l⁻¹ of non-hydrolyzed and hydrolyzed *C.I. Reactive Black 5* within 24 h of cultivation at an inoculum size of 1.4 g l⁻¹ wet cells in 50 ml medium consisting of 5 g l⁻¹ glucose and 0.5–1.0 g l⁻¹ ammonium sulphate (pH 5–7). In addition, the MnP activities during the cultivation were evaluated in the absence and presence of 200 mg l⁻¹ *C.I. Reactive Black 5*. Maximum activity of MnP (1555.6 U l⁻¹) was detected at 24 h cultivation in the presence of the dye, and a significant reduction of the enzyme activity was observed thereafter. The presence of *C.I. Reactive Black 5* in the culture was found to be indispensable to the production of MnP by *D. polymorphus*. A good correlation was found between the dye degradation and the enzyme production.

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Keywords: Decolorization; Yeast; Manganese-dependent peroxidase; Azo dye; *C.I. Reactive Black 5*

1. Introduction

Azo dyes are the most commonly used dyes in textile dyeing/finishing and also in food, paper and cosmetic industries. Wastewater from these industries is highly colored and the residual azo reactive dyes in it are generally resistant to microbial degradation. Anaerobic reduction and decolorization often generates aryl amines that can be transformed to highly reactive electrophiles and form covalent adducts with DNA, thereby posing a health risk [1,2]. Existing physical and chemical technologies (e.g., membrane technologies, coagulation and flocculation technologies) are expensive and might often produce large amounts of solid wastes [3].

Numerous studies have been reported on decolorization of various dyes using white rot fungi, since Tien and Kirk [4]

first discovered the lignin peroxidase (LiP) from these fungi. White rot fungi could mineralize many types of different synthetic dyes through their highly oxidative and non-specific ligninolytic enzyme system mainly including lignin peroxidase, manganese-dependent peroxidase (MnP) and laccase [5–7]. Most of the white rot fungi used in the research of wastewater treatment process were *Phanerochaete chrysosporium*, *Trametes versicolor* and *Coriolus versicolor*, and several types of bioreactors using these strains have been proposed in a laboratory scale [3]. However, the aging of fungal mycelium and the risk of contamination by bacteria under non-sterile conditions have hindered the application of white rot fungi in wastewater treatment [3]. On the other hand, yeast, another kind of fungi, has been successfully applied to treat industrial effluents from food, molasses, and oil manufacturing wastewater as reported by Japanese scientists [8,9]. Although several yeasts could also remove dyes through the mechanism of biosorption [10,11], there have

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been few reports on decolorization by yeast through the above ligninolytic enzyme system [12].

In a recent study, we reported about the ability of two yeast isolates, *Debaryomyces polymorphus* and *Candida tropicalis*, to produce MnP and effectively decolorize six different reactive dyes through biodegradation [13]. In this study, the decolorization conditions and MnP production by *D. polymorphus* were investigated in detail using a widely used azo dye, *C.I. Reactive Black 5* (RB5).

2. Materials and methods

2.1. Dye

C.I. Reactive Black 5 (Color Index) was obtained from Dystar (Germany) as dye formulation Remazol Schwarz B with 75% purity. The stock solution (10 g l^{-1}) was neutralized by 0.1 M HCl and diluted before use. Hydrolyzation of the dye was accomplished by dissolving the dye in distilled water, adjusting the pH to 10 with 1N NaOH and boiling at 95°C for 3 h under reflux conditions to simulate the dye-house processes. The solutions were sterilized by membrane filtration (pore size, $0.45 \mu\text{m}$).

2.2. Microorganism

D. polymorphus (Y1-0813) was isolated from a municipal wastewater treatment plant in Munich, Germany and preserved in the China General Microbiological Culture Collection Center (CGMCC). The yeast was cultivated at 28°C , 140 rpm in the following medium: glucose 5 g, KH_2PO_4 1 g, $(\text{NH}_4)_2\text{SO}_4$ 1 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 500 mg, yeast extract 200 mg, tap water 1000 ml, pH 5.0–6.0.

2.3. Determination of optimum conditions for decolorization of RB5

RB5 of 200 mg l^{-1} was used throughout the decolorization experiments. Color removal efficiency was determined using the same method as reported previously [18]. To find a suitable amount of inoculum for the effective dye biodegradation by *D. polymorphus*, experiments were performed in 50 ml medium inoculated with three different inoculum sizes (1.4 ; 4.0 and 17.0 g l^{-1} wet yeast cells). To detect the effect of initial pH on decolorization of RB5, the medium was adjusted to pH 3–10 before 1.4 g l^{-1} wet yeast cells were inoculated. The optimal carbon sources were determined among glucose,

maltose, sucrose and starch at the concentration of 5 g l^{-1} , respectively. The effects of initial concentrations of carbon and nitrogen sources on decolorization were examined in a series of dye-bearing medium with different concentrations of glucose (0 – 5 g l^{-1}) and ammonium sulphate (0 – 0.5 g l^{-1}), respectively.

2.4. Effect of initial dye concentration on decolorization

Effects of initial dye concentrations on RB5 decolorization were evaluated in 50 ml medium containing 5 g l^{-1} glucose, 0.5 g l^{-1} ammonium sulphate (pH 5) by inoculating 1.4 g l^{-1} wet yeast cells. Four different initial dye concentrations of 100, 200, 300, 400 and 1000 mg l^{-1} RB5 were used.

2.5. Assay of manganese-dependent peroxidase

MnP activity was detected using 3-di methyl amino benzoic acid/3-methyl-2-benzo-thiazlinoe-hydrazone (DMAB/MBTH) as described by Lang et al. [14]. One unit (U) of the enzyme activity was defined as the amount of enzyme required to produce $1 \mu\text{mol}$ of product per minute.

3. Results

3.1. Determination of optimum conditions for decolorization

Complete color removal was obtained within 24 h cultivation under the three inoculum sizes. The amount of the inoculum of the yeast was thus set at 1.4 g l^{-1} wet weight in the following experiments.

The effect of initial pH values on color removal of *C.I. Reactive Black 5* by *D. polymorphus* and the corresponding final pH values after 48 h cultivation are given in Table 1. Maximum color removal was obtained in a pH range of 5–7. A significant pH decrease was observed at all the pH conditions after 48 h cultivation. Maximum pH reduction was observed at the optimum decolorization and a pH range of 5–7. The decolorization of RB5 was accompanied by the production of acids suggesting that SO_3 groups were released from the structure of RB5 during the decolorization process.

The effect of carbon source types on decolorization was also investigated (Fig. 1). Except for starch, *D. polymorphus* could grow in other three carbon sources and effectively decolorize the dye in the medium.

Table 1
Effect of initial pH value on decolorization efficiency of *C.I. Reactive Black 5* by *D. polymorphus* and the final pH value of the cultures after 48 h cultivation

	Initial pH							
	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0
Color removal (%)	86.4	87.4	97.9	98.6	98.9	76.7	65.6	27.7
Final pH	1.52	1.52	1.55	1.78	2.16	5.03	5.89	9.25

Fig. 1. Effect of various kinds of carbon sources on decolorization of *C.I. Reactive Black 5* by *D. polymorphus* at the initial concentrations of 200 mg l⁻¹ for dye and 5 g l⁻¹ for carbon sources: (▲) sucrose; (◇) glucose; (■) maltose; (●) starch.

The decolorization efficiencies by *D. polymorphus* at different glucose and ammonium sulphate concentrations are shown in Table 2. The highest decolorization efficiency (95–98%) was obtained under the condition of 5 g l⁻¹ glucose and 0.5–1.0 g l⁻¹ (NH₄)₂SO₄. It was clear that the decolorization depended strongly on the presence of glucose in the medium.

Dyes in effluents from the textile dyeing and finishing industry are often in hydrolyzed forms [15]. Therefore, a decolorization test of 200 mg l⁻¹ hydrolyzed RB5 by *D. polymorphus* was performed. 98% color removal was obtained within 24 h. No significant difference was observed between the decolorization of hydrolyzed and non-hydrolyzed RB5 by *D. polymorphus*.

3.2. Effect of initial dye concentration on the decolorization process

The color removals of *C.I. Reactive Black 5* over 66 h were monitored, and extensive decolorization by *D. polymorphus* was observed at all the initial dye concentrations (Fig. 2). The decolorization process could be divided into two stages: a rapid degradation stage within the first 16 h and a slow decolorization stage thereafter. The dye was removed mainly by biodegradation during the first stage because the cells remained colorless even under high dye concentrations. Biosorption of this azo reactive dye might have occurred along with progress of cultivation time, especially at high initial dye concentrations. The second stage contributed 2.7–25% of the color removal.

Fig. 2. Decolorization of *C.I. Reactive Black 5* by *D. polymorphus* at various initial dye concentrations: (◇) 100 mg l⁻¹; (■) 200 mg l⁻¹; (▲) 300 mg l⁻¹; (◇) 400 mg l⁻¹; (●) 1000 mg l⁻¹.

Fig. 3. MnP activities and residual concentrations of *C.I. Reactive Black 5* in *D. polymorphus* cultures: (◆) MnP activities in the culture without dye added; (■) MnP activities in the culture with 200 mg l⁻¹ RB5 added; (◇) residual concentrations of RB5.

3.3. Kinetics of MnP production

MnP activities in *D. polymorphus* cultures with 200 mg l⁻¹ RB5 and without the azo reactive dye are given in Fig. 3. MnP activity could be detected after 6 h cultivation and reached the maximum value of 1555 U l⁻¹ at 24 h under the presence of RB5. Afterwards, the MnP activity decreased gradually. The MnP activity in the culture without dye was very low over the whole cultivation period compared to the dye-bearing culture. The presence of *C.I. Reactive Black 5* was indispensable to the MnP production.

The residual concentrations of RB5 during cultivation time were detected and compared with corresponding MnP activity as given in Fig. 3. The rapid drop of residual RB5 concentration within 24 h matched well with the increase of MnP activity. The enzyme of MnP could not be further produced by *D. polymorphus* when the residual dye was reduced to a very low level.

4. Discussion

D. polymorphus needed barely 24 h to decolorize the culture medium containing 200 mg l⁻¹ RB5, which was much shorter than 7–20 days over 90% color removal using white rot fungi (*Phanerochaete chrysosporium* and *Pleurotus sajor-caju*) as reported by Chagas and Durrant [5]. Young and Yu [16] compared the decolorization efficiencies of eight different synthetic dyes including RB5 by LiP, *P. chrysosporium* and *T. versicolor*, respectively. Their results indicated that

Table 2
Decolorization (%) of *C.I. Reactive Black 5* by *D. polymorphus* at various carbon and nitrogen concentrations (48 h)

(NH ₄) ₂ SO ₄ (g l ⁻¹)	Glucose (g l ⁻¹)			
	5.0	2.5	1.0	0
1.0	98.6	70.0	63.5	1.5
0.5	98.4	68.2	–	–
0.1	62.4	–	59.4	–
0	57.4	–	–	4.1

(–) Not determined.

final decolorization of 50 mg l⁻¹ RB5 after 9 days of cultivation was 91.4, 11.3 and 15.6%, respectively. The decolorization efficiency by *D. polymorphus* in the present study was significantly high. Another yeast, *Kluyveromyces marxianus* IBM3 reported by Meehan et al. (2000) could also almost completely decolorize 200 mg l⁻¹ Remazol Black-B (98% decolorization) within 24 h but mainly through physical adsorption of the dye to cellular biomass, not by enzymatic degradation [11].

According to Wang and Yu's report on *Trametes versicolor*, a rapid process of dye adsorption was first observed on the cells and then gradual biodegradation occurred through enzymatic reactions [17]. The same mechanism was not observed in this study of dye decolorization by *D. polymorphus*. It was possible that biodegradation of RB5 by *D. polymorphus* was too fast to observe biosorption phenomenon during the first decolorization stage.

In this study, *D. polymorphus* could not use the dye as a sole carbon source for the cell growth and for enzyme production under the experimental conditions. The dependence of decolorization by *D. polymorphus* on an additional carbon source implies a cometabolic pathway of degradation of aromatic compounds, which was also reported for decolorization of synthesized azo dyes by white rot fungi [18,19]. Dye decolorization by *P. chrysosporium* occurred only after N depletion and was poor in N-rich cultures [20]. However, the decolorization efficiency of *D. polymorphus* depended on a sufficient nitrogen source, which was quite different from most of the cases in white rot fungi [17,21,22].

Our results showed that the presence of RB5 was a necessary condition for MnP production. In our previous experiments, at least four other dyes, *C.I. Reactive Red M-3BE*, *Procion Scharlach H-E3G*, *Procion Marine H-EXL* (BASF) and *C.I. Reactive Brilliant Red K-2BP*, could also induce the enzyme production [13]. It was obvious that MnP was an important enzyme responsible for degradation of different kinds of dyes. However, it seemed that the presence of only MnP was not enough for color removal of RB5. From Fig. 3, we can see that a small amount of residual color could not be removed after 24 h cultivation even though the MnP activity was still high. Results of our further experiments indicated that the decolorization process ceased immediately after the glucose was depleted even MnP activity remained high in the culture. A condition of slow depletion of glucose, such as using yeast cells preserved in the refrigerator in advance for several days as inoculum, or increasing the concentration of glucose will be helpful for complete color removal. Further research is needed to clarify the mechanism of dye decolorization by *D. polymorphus*.

Acknowledgements

This work was conducted at the GSF Institute of Ecological Chemistry funded by the German Federal Min-

istry of Education, Science, Research and Technology (BMBF), and by the National Natural Science Foundation of China (Grants 50278095) and the Natural Science Foundation of Henan Province, China (Grants number: 0411032400).

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