Scientific Paper

Biodegradation of Azo Dye RO16 in Different Reactors by Immobilized *Irpex lacteus*†

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† Dedicated to the memory of Prof. Dr. Davorin Dolar

Abstract

Irpex lacteus is a white rot fungus known to decolorize various synthetic dyes. Decolorization of the azo dye Reactive Orange 16 by immobilized cultures of *I. lacteus* was compared in three different reactor systems of laboratory size: small and large trickle-bed reactors and a rotating-disc reactor. The highest dye decolorization efficiency (90% in 3 days) was observed in the small trickle bed reactor. The production of extracellular ligninolytic enzymes during dye decolorization was measured and the involvement of mycelium-associated activities in the decolorization process assessed. A repeated batch performance test demonstrated the potential of immobilized fungal cultures to decolorize synthetic dyes over long time periods.

Keywords: *Irpex lacteus*, dye decolorization, trickle bed reactor, rotating disc reactor, laccase, manganese-dependent peroxidase

1. Introduction

Contamination of surface water with dyes from the textile- and dyestuff industry represents a serious ecological problem. Besides conventional chemical and physical methods, the possibility of using white rot fungi for dye decolorization has attracted significant attention in the last decade.[1] Biodegradation studies using liquid fungal cultures and purified ligninolytic enzymes, e.g. laccases, manganese-dependent peroxidases (MnP), and lignin peroxidases (LiP), proved the capacity of white rot fungi to degrade synthetic dyes as well as other types of organic pollutants.[2,3,4,5]

The encouraging results obtained in laboratory studies resulted in the construction of reactors for fungal cultivation and continuous treatment of organopollutants. Most of the experiments have been carried out with *Phanerochaete chrysosporium* immobilized on a nylon net [6], in a fixed bed reactor with pulsating flow[7,8,9], and in a rotating tube reactor[10]. These research studies demonstrated that shear effects, limitation of oxygen transfer and excessive fungus growth have to be avoided to obtain good reactor performance.

Similarly, the white rot fungus *Irpex lacteus* was shown to efficiently decolorize various synthetic dyes in static and submerged liquid cultures[11,12] and the involvement of its ligninolytic enzyme activities in the dye decolorization process was discussed. Kasinath et al.[13] reported decolorization of an anthraquinonic dye Remazol Brilliant Blue R by the fungus immobilized on polyurethane foam or pine wood in a fixed-bed reactor. However, it is difficult to compare the dye decolorization efficiencies of the above reactor systems because of differences in the dyes, fungi, and conditions used.

White rot fungi have been demonstrated to have a great potential for removal of synthetic dyes and decolorization of colored effluents but this potential has not so far been adequately exploited in bioremediation.[14,15] The aim of this work was to investigate different reactor systems utilizing immobilized biomass of *Irpex lacteus* and to compare their performance during decolorization of the recalcitrant azo dye Reactive Orange 16. The decolorization rate as the function of dye concentration, as well as the activities of MnP and laccase were also evaluated to get better insight into the decolorization process.

2. Materials and methods

2.1 Organism and Chemicals

The fungus *Irpex lacteus* 617/93 was obtained from the CCBAS culture collection (Academy of Sciences of the Czech Republic, Prague). For culture maintenance,

the strain was cultivated on agar plates with 0.5% (W/V) malt extract and 1% (W/V) glucose at 28 °C for 7 days and stored at 4 °C.

Azo dye Reactive Orange 16 and the substrates for enzyme activity assays, 2,2´-azinobis (3-ethylbenzthiazolinone-6-sulfonate) (ABTS), 3-dimethyl-aminobenzoic acid (DMAB), and 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH), were purchased from Sigma (USA). All the chemicals used were of analytical grade.

2.2 Culture Conditions

For dye decolorization studies, precultures of *I. lacteus* in liquid N-limited mineral medium[16] (250-ml conical flasks, 20 mL medium) inoculated from stock agar plates were grown stationary for 7 days at 28 °C and homogenized using an Ultra-Turrax T25 (Janke & Kunkel, IKA-Labortechnik, Germany) at 9000 rpm for 1 minute under sterile conditions. A volume of 1 mL of the culture homogenate was transferred to 10 mL of the fresh mineral medium in a 100 mL flask and the dye was added after 7 days of cultivation at a final concentration of 0.05 – 0.75 g/L. The cultures were further incubated at 28 °C and the dye decolorization was followed. Heat-inactivated (55 °C, overnight) fungal cultures served as controls for dye sorption.

For the experiments in trickle-bed reactors, immobilized cultures of *I. lacteus* were prepared in 1-L Erlenmayer flasks containing 200 mL of the mineral liquid medium and 3 g of polyurethane foam (PUF) cut into 1- and 2-cm cubes. The cultures were inoculated from agar plates and cultivated as static cultures at 28 °C for 10 days.

2.3 Small Trickle Bed Reactor

Two sizes of trickle-bed reactors were used. The small one (STBR) was made of a 20 cm long glass cylinder of 4 cm ID with a round bottom with a liquid exit port. A gas inlet and an exit were mounted at the side of the reactor at the bottom and the top, respectively. A perforated teflon plate was used to support 70 1-cm PUF cubes. A liquid distributor made of teflon of 3.5 cm OD provided uniform liquid distribution over the immobilized microbial biomass through 7 stainless steel needles. It was mounted on a glass tube and inserted through the silicone rubber stopper at the top of the reactor. A 500-ml round bottom flask with 100 mL of mineral medium was used as a liquid reservoir. The liquid was pumped with a masterflex peristaltic pump (1.8 L/h) to the top of the reactor and sprinkled through the liquid distributor onto the immobilized biomass and collected in the liquid reservoir. Aeration at 10 minute time intervals was provided by a Trixie aquarium membrane air pump (50 L/h) through sterile filters so that the average aeration rate was 0.1 L/min.

Fungal biomass from one *I. lacteus* culture colonizing PUF cubes as mentioned above was aseptically transfered into the reactor. The experiments were carried out in cycles with one batch of biomass and four batches of 100 mL dye solution, and the last one with synthetic sewage medium under nonsterile conditions. Batches with initial dye concentrations were used as follows: 0.15, 0.3, 0.6 and 0.6 g/L. The respective cycles were 7, 12, 10 and 8 days with one free day between the cycles when the culture was exposed to a batch of dye-free medium in order to recover.

2.4 Synthetic Sewage Medium

The synthetic sewage medium contained (per L of tap water) 160 mg peptone, 30 mg urea, 4 mg K_2HPO_4, 7 mg NaCl, 4 mg $CaCl_2 .2H_2O$, and 2 mg $MgSO_4 .7H_2O$.

2.5 Large Trickle Bed Reactor

A large trickle-bed reactor (LTBR) was constructed using a 30 cm long and 10 cm ID glass cylinder, a glass round-bottom cylinder with a liquid exit and a stainless steel top lid with several ports. In this case, a teflon perforated plate supported 80 pieces of 2-cm PUF cubes and a teflon distributor with 19 stainless steel needles was used to uniformly distribute the liquid over the culture surface from the top of the reactor. A 2-L Erlenmeyer

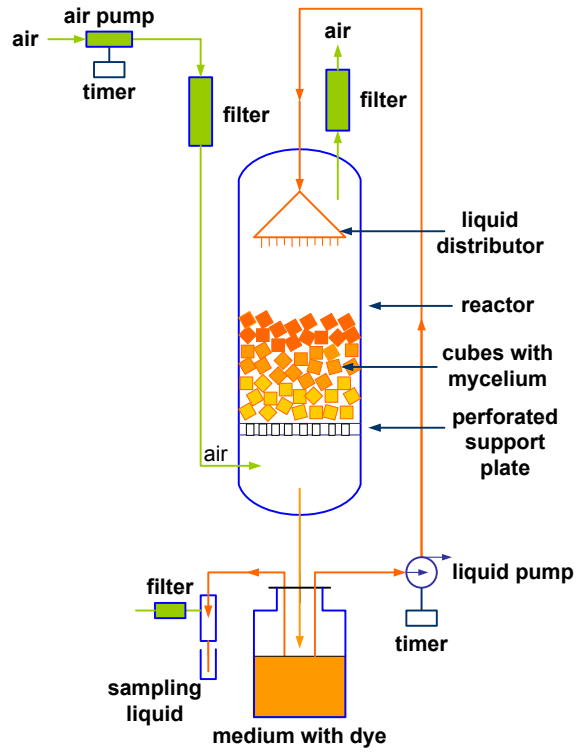

Figure 1. Schematic drawing of a large trickle bed reactor

flask was used as a reservoir containing 1L of the medium, which circulated in the reactor at a flow rate of 3.5 L/h. Aeration at 10 minute time intervals was provided by a Trixie aquarium membrane air pump (50 L/h) through sterile filters so that the average aeration rate was 0.1 L/min. The reactor was inoculated with 10-day-old fungal biomass grown on PUF obtained from 5 cultures grown as described above. The initial dye concentration was 0.3 g/L.

2.6 Rotating Discs Reactor

The body of the rotating discs reactor (RDR) was made of a 'T' glass element of 10 cm ID, which was placed horizontally on a metal support so that the top opening could be used for sampling, while both side openings were covered with a stainless steel lid. From one side, a driving axis with six 1-cm thick and 8-cm OD PUF plates was inserted centrally into the reactor. Air flow as well as liquid sampling was provided through the side ports. The liquid volume in the reactor was 1 L so that approximately 40% of the PUF plate volume was always immersed in the liquid. The PUF plates rotated at a speed of 2 revolutions per min.

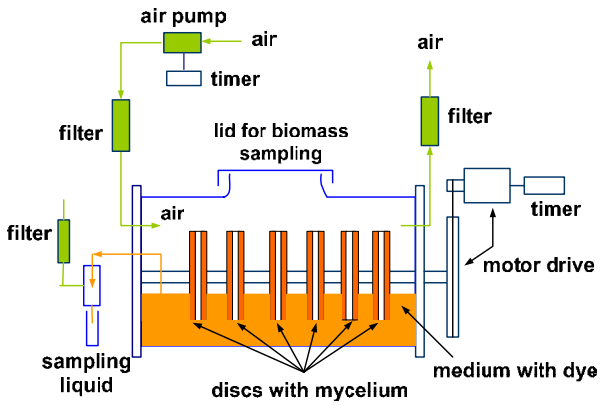

Figure 2. Schematic drawing of a rotating discs reactor

The experiments were performed aseptically in two steps. First, the discs in the reactor were inoculated with 15 mL of culture homogenate, prepared in the same way as the inoculum for dye decolorization experiments in liquid cultures. After 10 days of cultivation, when the fungus colonized the discs, the culture liquid in the reactor was replaced with 1 L of a fresh medium containing 0.3 g/L of the dye. Aeration at 10 minute time intervals was provided by a Trixie aquarium membrane air pump (50 L/h) through sterile filters so that the average aeration rate was 0.1 L/min or 0.1 vvm. All experiments in reactors were performed at a temperature of 25 °C. Sterile liquid sampling was done on a daily basis and the fungal biomass was estimated gravimetrically at the end of the experiment as dry biomass.

Enzyme assays

Laccase activity was determined by oxidation of 5 mM ABTS.[17] LiP and MnP activities were measured with veratryl alcohol and DMAB/MBTH, respectively.[16,18] One unit of activity was defined as the amount of enzyme oxidizing 1 μmol of enzyme substrate per min. An amount of 20 mg of fresh fungal biomass suspended in a final volume of 1 mL of the reaction mixture was used for the detection of mycelium-bound enzyme activities. The samples of mycelium were incubated for 1 min at room temperature under shaking, the mycelium was subsequently separated by centrifugation and absorption at the corresponding wavelength maximum was measured. The enzyme activities were expressed as units per mg of dry weight (DW) of fungal mycelium.

2.7 Dye Decolorization

Dye decolorization was measured spectrophotometrically at the wavelength of 494 nm using a Varian spectrophotometer, type Cary 50 Probe.

3. Results and Discussion

I. lacteus is capable of efficient decolorization of azo-, anthraquinone-, triphenyl methane- and phthalocyanine dyes.[14] In this study the biodegradation capacity of different types of reactors based on immobilized biomass of the fungus was investigated by following the decolorization of the Reactive Orange RO 16.

Initially, a series of decolorization experiments in liquid fungal culture were made to evaluate the dye decolorization rate at different initial dye concentrations. The dye concentrations (C, g/L) were measured during the time (t,h) course of the experiment and the decolorization rate (-r, g/L/h) was evaluated as $(-r) = \Delta C/\Delta t$ for the first two hours of decolorization and plotted against the initial dye concentration (Fig. 3). The decolorization rate was proportional to the initial dye concentration up to a concentration of 0.45 g/L. For higher initial dye concentrations the decolorization rate remained constant, probably due to saturation of the enzyme.

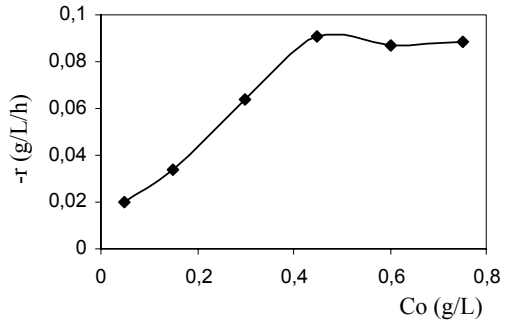

Figure 3. Decolorization rate at different initial dye concentrations observed in *I. lacteus* liquid cultures.

On the condition that the dye decolorization rate depends on the dye concentration according to the equation:

$$(-r) = dC/dt = kC^n \quad (1)$$

the order of the reaction (n) as well as the decolorization rate constant (k) can be estimated from the logarithmic plot:

$$\log (-r) = \log k + n \log C \quad (2)$$

Consequently, in the range of initial dye concentrations of 0.05 to 0.45 g/L, the reaction order for dye decolorization by *I. lacteus* cultures was n = 0.7, while at higher concentrations, the reaction order became zero.

The ability of fungal cultures to decolorize synthetic dyes can sometimes be correlated with the activities of extracellular peroxidases and laccases present in the culture liquid.[19,20] These enzymes were proven to be involved in *in vitro* decolorization reactions.[21,22] Table 1 compares the extracellular activities of MnP and laccase produced by *I. lacteus* cultures in the column- and disc bioreactors, detected after Reactive Orange 16 was added to the culture medium. Evidently, both enzyme activities were present in the cultures in the course of dye decolorization and thus could participate in the dye removal. That MnP and laccase of *I. lacteus* are implicated in the decolorization of Reactive Orange 16 has been suggested (Svobodova, unpublished). The enzyme activity profiles measured as a function of time were different in the three reactors tested.

Whereas LTBR cultures manifested maximal MnP activities (9.7 U/L) relatively soon after the dye was added, STBR cultures showed a small decrease of MnP on Days 2 and 3 after addition of the dye and then the MnP level steadily increased until the end of the experiment (Table 1). The latter trend of MnP level development was also observed in RDR cultures. In the case of laccase, the enzyme activities in the reactor cultures were rather low (0.1-1.4 U/L) and steady in the course of decolorization (Table 1).

The dye decolorization performance of the three reactors was compared (Fig. 4). In order to explain the performance of the different types of the reactors, both the enzyme activities present and the concentrations of fungal biomass normalized to the volume of the liquid recycled in the particular reactor (cf. Table 1) should be taken into account, since in static cultures of *I. lacteus* as much as an 8000-fold amount of laccase was found associated with the fungal mycelium, compared to the »free« fraction dissolved in the culture liquid (cf. Table 1). In contrast, no mycelium-bound MnP was found in static cultures. It can be hypothesized that the mycelium-associated enzyme will also participate in the decolorization process. The results obtained confirmed this hypothesis as the decolorization rate decreased in the order STBR (11.6 g DW/L) > LTBR (8.3 g DW/L) > RDR (4.9 g DW/L), where 90% decolorization rates were achieved after 3, 4 and 6 days, respectively. The high level of free MnP observed in STBR could also contribute to the high decolorization rate observed in this reactor since this enzyme has been shown to be involved in dye decolorization.[23]

Table 1. Extracellular enzyme activities present in crude culture liquids and amounts of immobilized fungal biomass in three types of reactors measured after the addition of RO16 dye to the medium. The data for static liquid cultures are given for comparison.

Time (days)	1	2	4	6	8	10	11
			STBR (biomass 11.6 g DW /L)[a]				
MnP (U/L)	10.9±0.8	6.2±0.3	14.2±0.5	15.9±0.2	18.8±0.2	24±0.25	32.9±0.12
Lac (U/L)	0.8±0.2	1.3±0.3	2.3±1.3	0.1±0.1	0.15±0.05	1.02±0.46	0.46±0.24
			LTBR (biomass 8.3 g DW /L)[a]				
MnP (U/L)	2.9±0.2	2.85±0.1	9.7±0.5	7.11±0.36	5.63±0.41	4.7±0.53	4.31±0.47
Lac (U/L)	0.5±0.2	0.3±0.8	0.7±0.3	0.51±0.33	0.65±0.53	0.9±0.65	0.54±0.43
			RDR (biomass 4.9 g DW /L)[a]				
MnP (U/L)	0.6±0.3	0.73±0.06	0.84±0.2	2.34±0.31	4.5±0.2	5.8±0.1	6.0±0.5
Lac (U/L)	1.0±0.2	0.97±0.05	0.46±0.6	0.71±0.96	1.3±0.6	1.2±0.5	1.4±0.3
			Static liquid cultures (biomass 18.1 mg DW/flask containing 10ml liquid medium)[a, b]				
MnP (U/L)	0±0	16.3±5.1					
Lac, free (U/L)	6.5±3.1	2.6±1.0					
Lac, mycelium-associated (U/mg DW)	2.44±0.74	11.1±0.22					

[a] Fungal biomass was expressed as dry weight biomass (DW) and, in the case of the reactors, normalized to the recycled volume of the medium for comparison. The dye was added at Day 0 at an initial concentration of 0.3 g/L.
[b] Static cultures represented a nonimmobilized fungal biomass mat floating on the surface of liquid medium.

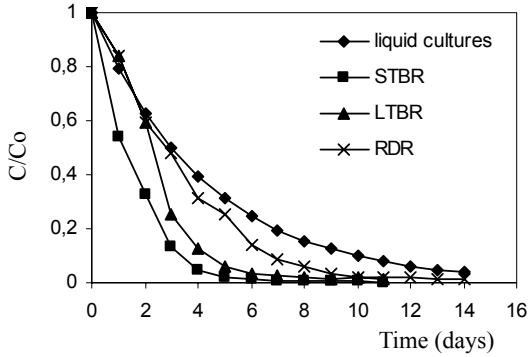

Figure 4. The time course of residual RO16 concentration in three reactors and in static cultures. The initial dye concentration was 150 mg/L.

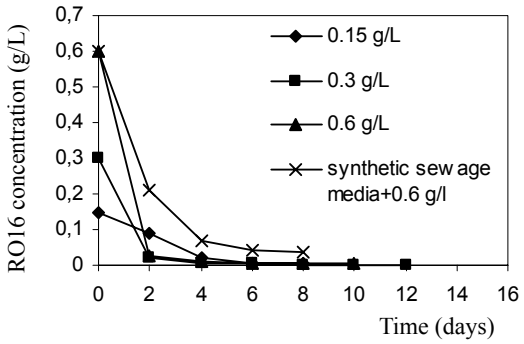

Figure 5. The time course of decolorization in small trickle-bed reactor in four subsequent additions of RO16 to the same batch of microbial biomass.

The decolorization rates obtained were comparable to the dye decolorization capacities of other white rot fungi used in reactors. For instance, *Phanerochaete sordida* decolorized 80% of the phthalocyanine dye Basic Blue 22 in a rotating disc reactor operating with a retention time of 48h.[24] Biodegradation of Grey Lanaset G, a mixture of metal complex dyes, was studied in a reactor with the fungus *Trametes versicolor*, working under conditions of laccase production.[25] Although dye decolorization was highly efficient (90% of the initial dye amount), no direct relationship between the extracellular enzyme activity and dye decolorization capacity was apparent. Similarly, Swamy and Ramsay[26] postulated the involvement of mycelium-associated activities in dye decolorization by *T. versicolor*.

The results of a long-term performance test of the small trickle-bed reactor are shown in Fig. 5. The first cycle was done with the lowest initial dye concentration (150 mg/L) and it took four days to decolorize the batch of dye. From the slope of the decolorization curves it is seen that the slowest decolorization took place in this case, probably due to the lag period necessary for the microorganism to adapt to the new operating conditions in the reactor. In the next two cycles, the decolorization rate rose and it took only two days to decolorize the two liquid batches with increasing initial contents of dye. These experiments were made under sterile conditions. Finally, the dye was added to a batch of chemically-simulated wastewater and the experiment carried out under nonsterile conditions. The decolorization rate was lower compared to the previous cycle, but after four days, the liquid batch was completely decolorized.

Microbial biomass slowly overgrew the PUF cubes in the reactor during the 37 day long experiment in four cycles. Consequently, the permeability of the bed observably decreased so we stopped the experiment due to expected clogging problems.

4. Conslusion

The study clearly demonstrates the potential of ligninolytic fungi growing on a solid phase to degrade recalcitrant xenobiotics and organopollutants dissolved in water media and effluents. When column reactors with immobilized fungus are used for over a time periods of weeks, often the flow is clogged by extensive growth of the fungus in the reactor. For this reason the use of fungal rotating disc reactors can be considered suitable for the degradation of pollutants in spite of the fact that their efficiency is slightly lower. The improvement of their decolorization performance through the optimization of culture conditions and ligninolytic enzyme production needs further investigation.

5. Acknowledgements

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Povzetek

Razbarvanje sintetičnega barvila RO 16 z glivo bele trohnobe *Irpex lacteus* smo proučevali v treh laboratorijskih bioreaktorjih z imobilizirano biomaso: malem in velikem kapalnem reaktorju in reaktorju z rotirajočimi diski. Najbolj učinkovito razbarvanje (90% v treh dneh) je poteklo v malem kapalnem reaktorju kot posledica aktivnosti ekstracelularnih, predvidoma pa tudi na micelij vezanih encimov. Uporaba iste biomase za razbarvanje štirih zaporednih šarž barvila je pokazala na potencialno možnost uporabe imobilizirane kulture v daljšem časovnem obdobju.