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The use of the fungus *Dichomitus squalens* for degradation in rotating biological contactor conditions

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ABSTRACT

Biodegradation potential of *Dichomitus squalens* in biofilm cultures and rotating biological contactor (RBC) was investigated. The fungus formed thick biofilms on inert and lignocellulosic supports and exhibited stable activities of laccase and manganese peroxidase to reach 40–62 and 25–32% decolorization of anthraquinone Remazol Brilliant Blue R and heterocyclic phthalocyanine dyes, respectively. The decolorization ceased when glucose concentration dropped to 1 mmol l^{-1}. In RBC reactor, respective decolorizations of Remazol Brilliant Blue R and heterocyclic Methylene Blue and Azure B dyes (50 mg l^{-1}) attained 99%, 93%, and 59% within 7, 40 and 200 h. The fungus exhibited tolerance to coliform and non-coliform bacteria on rich organic media, the inhibition occurred only on media containing tryptone and NaCl. The degradation efficiency in RBC reactor, capability to decolorize a wide range of dye structures and tolerance to bacterial stress make *D. squalens* an organism applicable to remediation of textile wastewaters.

1. Introduction

White-rot fungi have been recognized as microorganisms with important degradation potential that can be exploited for bioremediation of industrial effluents in the form of biofilms immobilized on solid surfaces applicable in various types of reactors (Knapp et al., 2008; Rodríguez Couto, 2009). A general need for new robust strains with a broad degradation range, resistant to toxic and bacterial effects and usable in fungal bioreactors in a long-term regime exists (Singh, 2006). Our study focused on *Dichomitus squalens* whose significant biodegradation potential has been shown (Gill et al., 2002, etc.) but has not so far been tested in chemically-engineered bioreactors (e.g. Rodríguez Couto, 2009; Singh, 2006).

D. squalens belongs to WRF that express predominantly MnP and laccase but not LiP (Hatakka, 1994), its liquid and soil cultures were shown to degrade a range of pollutants, PAHs, synthetic dyes and endocrine-disrupting compounds (Cajthaml et al., 2009; Covino et al., 2010; Eichlerová et al., 2006). Tolerance to bacterial stress is important if a fungal organism is to be used for bioremediation as most processes have to be run under nonsterile conditions. The tolerance of *D. squalens* is not known. The fungus was found not to be able to colonize nonsterile soil and the syntheses of laccase and MnP were decreased in the presence of soil organisms, but its biochemical activity measured by decomposition of straw continued under those conditions (Lang et al., 2000).

The conditions in RBC reactors in both repeated-batch and continuous mode are generally suitable for the use of WRF for biodegradation, RBCs advantages include simple construction, great surface area per unit volume, low power requirement and no flow clogging (e.g. Alleman et al., 1995; Nilsson et al., 2006). Several WRF, for instance *Phanerochaete chrysosporium*, *Trametes versicolor* and *Bjerkandera* sp., have been shown to degrade chlorophenols, PAHs and synthetic dyes as well as to decolorize and detoxify Kraft and bleach plant effluents when used in RBC (e.g. Alleman et al., 1995; Axelsson et al., 2006). Plastic, metal-mesh and wooden disks are normally used as carriers in RBC, hydraulic retention times being 2–3 d and dye concentrations typically not exceeding 200 mg l^{-1} (Kapdan and Kargi, 2002; Nilsson et al., 2006). To our knowledge, the biodegradation potential of *D. squalens* has so far not been tested under the conditions of a RBC-type reactor.

Abbreviations: AB, Azure B; BPB, Bromophenol Blue; CFU, colony forming units; CR, Congo Red; CuP, Cu-phthalocyanine; DB3, Disperse Blue 3; LiP, lignin peroxidase; MEG, malt extract-glucose medium; MnP, manganese-dependent peroxidase; MB, Methylene Blue; MM, mineral medium; NBB, Naphtol Blue Black; PAHs, polycyclic aromatic hydrocarbons; PDA, potato dextrose agar; RBC, rotating biological contactor; RB5, Reactive Black 5; RBBR, Remazol Brilliant Blue R; RO16, Reactive Orange 16; WRF, white rot fungi.

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Our aim was to characterize degradation performance of biofilm cultures of a selected *D. squalens* strain and check the efficiency and behavior of the fungus under the conditions of RBC reactor. Biodegradation characteristics of biofilm cultures using various types of carriers were measured with model dyes, the corresponding activities of MnP and laccase were determined and their involvement in dye degradation shown, the biodegradation efficiency of a beechwood-disks-operating RBC reactor was characterized, and tolerance of the fungus to bacterial stress was documented.

2. Methods

2.1. Microorganisms

The fungal strains used: *Armillaria mellea* 330; *Agaricus bisporus* 308; *Aurantioporus croceus* 422/93; *Bjerkandera adusta* 606/93; *Ceriporia metamorphosa* 193/93; *Coriolopsis polyzona* 740; *Daedaleopsis confragosa* 491/93; *Daedalea quercina* 528; *Dichomitus squalens* 750; *Flammulina velutipes* 366. *Phanerochaete chrysosporium* 854, *Phanerochaete magnoliae, Irpex lacteus* 931, *Pleurotus ostreatus* 106, *Trametes versicolor* 167/93, *Stereum rugosum* 210/93. All strains originated from Culture Collection of Basidiomycetes, Institute of Microbiology ASCR, Prague and were maintained on MEG medium (per liter: 5 g malt extract, 10 g glucose, pH 4.5) agar slants at 4 °C.

The bacterial strains used: *Bacillus subtilis* CCM 1999, *Citrobacter koseri* CCM 2535, *Serratia marcescens* CCM 303, *Escherichia coli* CCM 3988, *Corynebacterium glutamicum* (type strain ATCC13032), *Pseudomonas aeruginosa* CCM 1960, *Rhodococus erythropolis* CCM 2595. Except for *C. glutamicum,* all other bacterial strains were obtained from Czech Collection of Microorganisms, Masaryk University, Brno and were maintained on meat peptone agar (pH 7) at 4 °C.

2.2. Chemicals

Model dyes: Reactive Black 5 (Sigma–Aldrich, Czech Rep.), Congo Red (Merck, Czech Rep.), Reactive Orange 16 (Sigma–Aldrich, Czech Rep.), Naphtol Blue Black (Sigma–Aldrich, Czech.Rep.) (all azo dyes), Disperse Blue 3 (Sigma–Aldrich, Czech Rep.) (anthraquinone dye), Cu(II) phthalocyanine (Sigma–Aldrich, Czech Rep.) (phthalocyanine dye), Methylene Blue (Sigma–Aldrich, Czech Rep.), Azure B (Sigma–Aldrich, Czech Rep.), (both phenothiazine dyes), Bromophenol Blue (Sigma–Aldrich, Czech Rep.) (triphenyl methane dye). Textile dyes: C.I. Reactive Black 5, 70–80% (CAS No. 17095-24-8, reactive, azo dye, Ciba Spec. Chem. Inc., Australia), Yoracron Grün K395 (Yorkshire Group, UK, information on the dye type and CAS No. not found), C.I. Reactive Blue (CAS No. 400-510-7, reactive, azo dye, Sandoz Chem. Corp., USA), C.I. Disperse Blue 56 (CAS No. 12217-79-7, disperse, anthraquinone dye, Chemapol a.s., Czech Rep.), C.I. Acid Black 172 (CAS No. 57693-14-8, acid, azo dye, Yorkshire Group, UK), C.I. Reactive Orange 7 (reactive, azo dye, Hoechst AG, Germany), C.I. Reactive Red 158 (CAS No. 64104-00-3, reactive, azo dye, Bayer AG, Germany), C.I. Disperse Blue (CAS No. 416-860-9, disperse, azo dye, ICI Spec., UK), and C.I. Reactive Blue 19 (Remazol Brilliant Blue R, RBBR, CAS No. 2580-78-1, reactive, anthraquinone dye, Hoechst AG, Germany).

All other chemicals were of analytical grade. Bacto™Tryptone was purchased from Becton, Dickinson and Co. (USA), malt extract, yeast extract, PDA and Lab Lamco Beef Extract medium from Oxoid (UK).

2.3. Growth and decolorization on agar plates and in biofilm cultures

The following agar (20 g l^{-1}) media were used: MEG or MM (Tien and Kirk, 1988), both pH 4.5, containing 200 mg dye l^{-1}. Decolorization and fungal growth on agar plates were estimated as described by Novotný et al. (2001). All cultures were run in duplicate at 28 °C.

Biofilm cultures were grown on chopped wheat straw, polyamide mesh or 1-cm^3 cubes of polyurethane foam, pine wood or beechwood for 25 days. In order to prepare the solid support for fungal colonization, the materials were three times washed with hot distilled water and air dried. Amounts of 3 g of dry polyurethane foam, 15 g of dry wood or 5 g of dry straw were used to set up cultures in 500 ml Erlenmayer flasks. In the case of polyamide mesh, a piece having a size of *ca.* 150 cm^3 was used as the carrier per flask. Volumes of 100 ml of liquid MM were added and the flasks sterilized by autoclaving (120 °C, 20 min). The flasks were inoculated (10% V/V) from static liquid, 7-d pre-cultures grown at 28 °C and homogenized using an Ultra-Turrax T25 (IKA Werk, Germany, 20 s, low speed) before use. Aliquots of culture liquid were collected during growth at 28 °C and used to determine enzyme activities and decolorization value. All experiments were made in triplicates.

2.4. Growth inhibition in the presence of bacteria

Growth inhibition was measured on agar plates simultaneously inoculated with a mycelium-covered disk (diameter 0.9 cm) and with four 10-μl drops of a bacterial culture (10^{10} CFU ml^{-1}) placed 1–1.5 cm from the agar plate edge (Radtke et al., 1994). The bacterial inoculum culture was grown overnight under agitation (37 °C, Lab Lamco beef extract medium pH 7), cells counted by plating. The plates with the fungus and bacteria were grown for 7 d at 28 °C and growth inhibition expressed according to the width of the inhibition zone between the fungal and bacterial colonies (0, fungal and bacterial colonies overlapped; I, 1–3 mm; II, 4–7 mm; III, $\geqslant$8 mm). Six different agar media were used at pH values of 4.5, 5, 6 and 7 (agar 20 g l^{-1}): MEG, PDA (39 g l^{-1}), MM (Tien and Kirk, 1988), LB (tryptone 10 g l^{-1}, yeast extract 5 g l^{-1}, NaCl 10 g l^{-1}; Bertani, 1951), 2×TY (tryptone 16 g l^{-1}, yeast extract 10 g l^{-1}, NaCl 5 g l^{-1}; Elbing and Brent, 2002), phenazine-induction medium (Radtke et al., 1994).

2.5. RBC reactor

The vessel of RBC was made of a, T' glass element (diameter 10 cm) mounted horizontally on a metal support. The top opening was used for filling the reactor with growth medium while two side openings covered with stainless steel lids were used to ensure the air flow (50 l h^{-1}) and sample removal during cultivation. A driving axis with six beechwood plates (diameter 8 cm, thickness 1 cm) was inserted in the reactor. The working volume was 1 l so that about 40% of the wood plate volume was always immersed in the liquid. The rotation speed was 2 rpm.

The experiments were performed aseptically in two steps. The fungus was first cultivated in MEG medium for 10 d to colonize the disks. Then the medium was replaced with 1 l of MM medium containing 50 mg l^{-1} of RBBR, MB or AB dyes.

2.6. Enzyme assays and isolation

The activities of ligninolytic enzymes were determined spectrophotometrically (Perkin Elmer Lambda 25, Perkin Elmer, Inc., USA or SPECTRAmaxPLUS^{384}, Molecular Devices Instruments, USA) using standard methods: laccase by oxidation of 2,2́-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (Matsumura et al., 1986), MnP by reaction with 3-(dimethylamino)benzoic acid/3-methyl-2-benzothiazolinone-hydrazone hydrochloride (Novotný et al., 2001), and LiP by oxidation of veratryl alcohol (Tien and Kirk, 1988). One unit