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ORIGINAL ARTICLE

Biodegradation of Reactive Orange 16 azo dye by simultaneous action of *Pleurotus ostreatus* and the yeast *Candida zeylanoides*

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Abstract

The purpose was to investigate a simultaneous biodegradation of the recalcitrant monoazo dye Reactive Orange 16 (RO16) in a mixed culture consisting of a biofilm of *Pleurotus ostreatus*–colonizing polyamide carrier and a suspension of the yeast *Candida zeylanoides* to see their biological interactions and possible synergistic action during degradation. Decolorization in the mixed culture was more effective than in the fungal monoculture, the respective decolorizations reaching 87.5% and 70% on day 11. The proliferation of yeast was reduced compared with the *C. zeylanoides* monoculture but enabled the yeast to participate in decolorization. The interaction of *P. ostreatus* with the yeast resulted in a gradual decrease of fungal manganese-dependent peroxidase (MnP) and laccase activities. Gas chromatography-mass spectrometry (GC-MS) analysis of the degradation products brought evidence that *P. ostreatus* split the dye molecule asymmetrically to provide 4-(ethenylsulfonyl) benzene whose concentration was much decreased in the mixed culture suggesting its increased metabolization in the presence of the yeast. In contrast, *C. zeylanoides* split the azo bond symmetrically producing the metabolites 4-(ethenylsulfonyl) aniline and α-hydroxybenzenepropanoic acid. Those metabolites were rapidly degraded in the mixed culture. A novel aspect is represented by the evidence of a mutual cooperative action of the fungal and yeast microorganisms in the mixed culture resulting in rapid decolorization and degradation of the dye.

Introduction

Microbial consortia and mixed cultures of different microorganisms are able to degrade organic pollutants, including recalcitrant synthetic dyes, and often exhibit better performance than single strains. Microbial interactions in such co-cultures consist of concomittant mechanisms ranging from positive to negative effects, but our knowledge of these mechanisms, that may include mutual microbial cooperation, is rather poor (Mikesková et al. 2012; Wang et al. 2014; Wang et al. 2019).

Broad biodegradation potential of ligninolytic fungi (LF) has been established with *Pleurotus ostreatus* serving as one of model organisms, and its biochemical and physiological behavior under various conditions was well documented (Gadd 2008; Svobodová et al. 2016; Ceci et al. 2019). In this group of microorganisms, extracellular lignin-modifying enzymes are implicated in oxidative degradation of

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organopollutants such as industrial dyes (Knapp et al. 2008). Yeasts are another large group of microorganisms that exhibit important biodegradation capacities, e.g., those able to decolorize recalcitrant azo dyes (Yang et al. 2005; Tan et al. 2016). Various reductases, such as azoreductase or NADH-dichlorophenolindophenol reductase, are implicated in reductive cleavage of the azo bond of the dye-producing metabolites that are further degraded to aliphatic amines by the action of oxidative enzymes (Ramalho et al. 2002, 2005; Jadhav et al. 2007; Saratale et al. 2009).

Biodegradation potential of defined consortia of fungi with other microorganisms has been documented, e.g., a fungal-bacterial consortium comprising *Penicillium* sp. QQ strain in azo dye degradation (Gou et al. 2009); consortium consisting of *Aspergillus ochraceus* and *Pseudomonas* sp. in degradation of textile dyes (Kadam et al. 2011); co-cultures of *P. ostreatus* with bacteria *Pseudomonas fluorescens* or *Bacillus licheniformis* degrading anthraquinone dye Remazol Brilliant Blue R (Válková et al. 2017); or fungal consortia of *Aspergillus lentulus*, *A. terreus* and *Rhizopus oryzae*, or *Dichotomomyces cejpii* and *Phoma tropica* used for decolorization of various azo dyes (Mishra and Malik 2014; Krishnamoorthy et al. 2018). Both positive and negative effects of the other organisms on biodegradation by fungal cultures have been reported, the factors generally involving pH, extracellular enzymes, and competition for nutrients (Libra et al. 2003; Spina et al. 2014; Li et al. 2015).

Levels of extracellular enzymes, namely peroxidases and laccase that are involved in degradation of organopollutants in LF, can be affected by interaction with other microorganisms resulting in an increase of laccase activity as observed in *Trametes versicolor* and *P. ostreatus* by Hiscox et al. (2010) and Válková et al. (2017), respectively. Such an increase, however, did not occur in the case of MnP (Válková et al. 2017). The increased enzyme activity may or may not result in higher biodegradation rates (Baldrian 2004; Novotný et al. 2004; Hiscox et al. 2010; Válková et al. 2017).

Both positive and adverse effects of microorganisms on the degradation process by LF have been reported, for instance, fixed-film cultures of *Phanerochaete chrysosporium* exhibited a high and stable degradation efficiency in the presence of bacteria (Gao et al. 2008) or the efficiency of decolorization of recalcitrant Remazol Brilliant Blue R (RBBR) dye by mature biofilms of *P. ostreatus* was not restrained by populations of *P. fluorescens* or *B. licheniformis*. On the other hand, degradation of polycyclic aromatic hydrocarbons by various LF in soil and in submerged cultures was negatively affected by the presence of bacteria (Borchert and Libra 2001; Borràs et al. 2010). Yeasts were reported to be responsible for failures of bioremediation realized by LF as they exhibited good growth at low pH and high growth rates in carbohydrate-based media (Boekhout and Robert 2003; Knapp et al. 2008). However, little is known about the behavior of yeasts in mixed cultures with LF and how the partners influence the biodegradation process of the other partner fungal microorganism. White rot fungi and yeasts have been reported to use different degradation pathways when decomposing azo dyes (Erkurt et al. 2010; Jafari et al. 2014).

Our aim was to estimate the simultaneous action of the two different biodegradation processes carried out by *P. ostreatus* and *C. zeylanoides* during decolorization of the model recalcitrant azo dye RO16 in their mixed culture where the yeast suspension was added to a preformed biofilm of *P. ostreatus* immobilized on a plastic carrier. In the mixed culture, the growth of both organisms was followed, the activities of extracellular enzymes involved in degradation were monitored, and the analysis of biodegradation products using GC-MS was carried out to check whether both partners contributed to decolorization.

Materials and methods

Microorganisms

P. ostreatus was obtained from the Culture Collection of Basidiomycetes of ASCR, Prague, Czech Republic. The yeast *Candida zeylanoides* was acquired from the Spanish Type Culture Collection, University of Valencia, Spain. The fungus was maintained on malt extract-glucose (MEG) agar (malt extract 5 g/L, glucose 10 g/L, agar powder 20 g/L), grown at 28 °C for 7 days and stored at 4 °C. The yeast was preserved on complex medium plates containing glucose 40 g/L, mycopeptone 10 g/L, and agar 15 g/L (GMA). The yeast was grown at 28 °C for 2 days and then stored at 4 °C.

Chemicals

2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,6 dimethoxyphenol (DMP), 96% 3,4-dimethoxyphenol, malonic acid, EDTA, and Reactive Orange 16 were purchased from Sigma-Aldrich. All chemicals were of analytical grade.

Culture conditions

The fungal inoculum was prepared by transferring ten agar plugs of *P. ostreatus* grown on MEG agar into 250-mL flasks containing 100 mL of liquid MEG medium and incubated at 28 °C for 7 days. Then the cultures were homogenized (Ultra-Turrax T25, IKA) and used as the inoculum (10% *V*/V) to inoculate 250-mL flasks containing a liquid growth medium and polyamide mesh carrier (wire wool: fiber thickness, 2 mm; mesh size, 3 mm) to prepare immobilized fungal cultures. A growth medium (glucose 20 g/L, $(NH_4)_2SO_4$ 2.5 g/L, yeast extract g/L, KH_2PO_4 5 g/L, $MgSO_4{\cdot}7H_2O$ 0.5 g/L, $CaCl_2{\cdot}2H_2O$ 0.13 g/L) was used for this purpose.The fungal

cultures were then grown under static conditions for 7 days to form biofilm on the polyamide carrier and were further used as preformed biofilms in the mixed cultures and control fungal cultures.

A yeast inoculum culture was prepared by growing the microorganism overnight in MEG medium on a rotary shaker (DOS-20L, ELMI) (80 rpm). The final concentration of the yeast in the mixed culture and in the control yeast monoculture was adjusted to achieve a final value of 10^6 colony forming units (CFU) per mL.

RO16 azo dye was added to the mixed or control cultures at a final concentration of 150 mg/L to start the decolorization that took place at 28 °C under shaking (80 rpm) to ensure a sufficient aeration for the dye degradation by the yeast. Samples of the liquid medium were removed in time to determine the dye removal, extracellular enzyme activities, and, where applicable, the yeast cell counts.

The control monocultures of the fungus and the yeast were prepared in a similar way but without the presence of the other microbial partner and were used for decolorization of RO16.

All experiments were carried out in triplicates.

Decolorization and enzymatic assay

Decolorization of RO16 was measured spectrophotometrically at 494 nm using a microplate method (Epoch Microplate Spectrophotometer, Bio-Tek, USA; Program Gen5). Yeasts, bacteria, and fungal fragments were removed by centrifugation before the measurement.

The activities of extracellular enzymes implicated in dye degradation were measured spectrophotometrically: manganese-dependent peroxidase (MnP) using the oxidation of DMP (De Jong et al. 1994), lignin peroxidase (LiP) with veratryl alcohol as the substrate (Tien and Kirk 1988), and laccase using the oxidation of ABTS (Matsumura et al. 1986). One unit of enzyme activity (U) was defined as an amount of the enzyme oxidizing 1 μmol of substrate per min.

Fungal biomass and yeast cell counts measurements

Fungal biomass colonizing the polyamide carrier was determined gravimetrically as dry biomass at the end of experiments. The polyamide carrier covered with the colonizing fungal biofilm was removed from the cultivation flask, gently washed with distilled water, and dried at 105 °C until constant weight. Then the pre-weighed mass of the carrier was subtracted to obtain the dry biofilm mass. Yeast cell counts were determined by plating on GMA medium.

Gas chromatography-mass spectrometry analysis

The culture medium was filtered (cellulose wadding), centrifuged (2000 rpm, 5 min, laboratory temperature), and directly extracted with dichloromethane (pesticide grade). The acid-base liquid-liquid extraction at pH 2 and 12 was used. When measured before extraction, all samples had pH values of approximately 6.5. A volume of 100 mL of each sample was extracted in separatory funnels (200 mL) using 5 mL of dichloromethane three times. First, extraction was performed using any sample as-received (pH neutral). Then, the same sample was serially extracted with dichloromethane at pH 12 and, subsequently, at pH 2. All extracts were mixed together, dried using Na_2SO_4, and concentrated to a volume of 1 mL under nitrogen. Finally, the extracts were analyzed by GC-MS (7890N/5975C, Agilent Technologies, USA). GC-MS was equipped with a capillary column DB-XLB (30 m × 0.25 mm × 0.25 μm). The operating conditions for screening analysis were the injection port was maintained at 290 °C and the dichloromethane extracts were injected in splitless mode. The program column temperature started at 40 °C for 2 min, then increased by 5 K/min to 300 °C, and was held for 10 min at 300 °C. The ion source of MS detector was operated at 230 °C. The scan mode was employed, and the experimental data were measured in the range of 50–550 amu. The mass spectra library NIST011 was used for evaluation of the mass spectra obtained. Only compounds with a high or moderately high confidence in structure were identified, for which an excellent (RSI > 900) or good (RSI = 800–900) match between the mass spectrum and that of electron ionization mass spectra library was found (Plachá et al. 2017).

Results and discussion

Growth and decolorization in mixed cultures of *P. ostreatus* and *C. zeylanoides*

In order to investigate the process of decolorization of RO16 dye (150 mg/L) in the mixed culture of *P. ostreatus* and *C. zeylanoides*,7-day-old cultures of the immobilized *P. ostreatus* were exposed to a yeast suspension and the decolorization compared with that of the control monocultures (Fig. 1). The decolorization in the mixed culture was more rapid than in the fungal monoculture, especially between days 5 and 11, the decolorization reaching a value of 87.5% on day 11, whereas in the fungal monoculture the decolorization was only 70%. Similar positive effect of the partner organism was observed on decolorization of the anthraquinone RBBR dye in mixed cultures of immobilized *P. ostreatus* with *Rhodococcus erythropolis* or activated sludge (Svobodová et al. 2016) or in mixed cultures of *Trametes* sp. SQ01 and *Chaetomium* sp. R01 degrading triphenylmethane dyes (Yang et al. 2011).

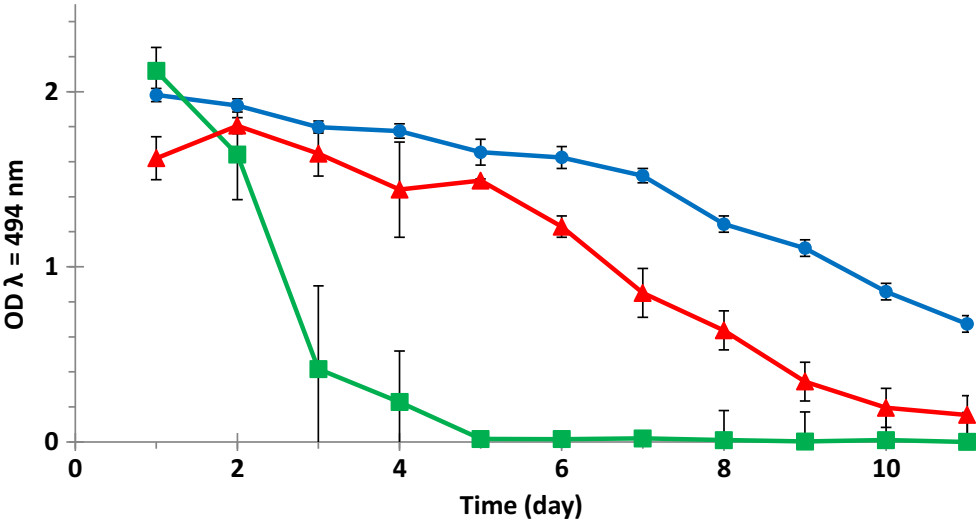

Fig. 1 Effect of *C. zeylanoides* on decolorization of RO16 dye by immobilized *P. ostreatus* at 28 °C under shaking. Mixed culture of *P. ostreatus* and *C. zeylanoides* (▲), monoculture of *P. ostreatus* (●), monoculture of *C. zeylanoides* (■). Starting yeast concentration of 10^6 CFU/mL, the dye was used at 150 mg/L

Evidently, preformed fungal biofilms are rather resistant to adverse effects of other microorganisms as was also confirmed by works where biofilms of various white rot fungi were exposed to other microorganisms such as Gram-negative or Gram-positive bacteria or the yeast *Saccharomyces cerevisiae* without influencing the fungal degrading capacity (Gao et al. 2008; Válková et al. 2017; Šlosarčíková et al. 2017).

The decolorization by the shaken submerged culture of *C. zeylanoides* was more efficient than that of the fungal culture and reached 100% decolorization within 5 days (Fig. 1). However, the decolorization by the yeast was strongly inhibited by the presence of the fungus even though the yeast was still able to significantly contribute to the total decolorization capacity of the mixed culture by increasing the decolorization rate and the total percentage of the decolorized dye, compared with the fungal monoculture (Fig. 1). A relative shortage of nutrients in the mixed culture resulting from the consumption by the two organisms could be responsible for both the lower yeast cell counts observed (Table 1) and reduced efficiency of decolorization by the yeast (Knapp et al. 2008).

The inoculation of the fungal culture immobilized on the solid support with a massive suspension of *C. zeylanoides* followed by further incubation of the mixed culture at 28 °C for 11 days resulted only in a negligible decrease of the fungal dry biomass (Table 1) which was in agreement with the observations of the effects of bacteria *P. fluorescens* or *B. licheniformis* and of *S. cerevisiae* on biofilms of *P. ostreatus* and *I. lacteus*, respectively (Válková et al. 2017; Šlosarčíková et al. 2017).

In the control yeast monoculture, the yeast cell counts inoculated to the level of 1.10^6 CFU increased by two orders of magnitude within 9 days and the yeast growth was not affected by the presence of the dye (Table 1). When the yeast was added to the culture of *P. ostreatus*, the yeast cell numbers increased only about five times within 9 days, probably due to the competition for nutrients in the culture. This behavior was similar to that of the yeast *S. cerevisiae* when in coexistence with biofilms of *I. lacteus* (Šlosarčíková et al. 2017) but was in contrast to the behavior of *P. fluorescens*, *B. licheniformis*, and activated sludge bacteria whose CFU values were decreasing when added to preformed *P. ostreatus* biofilms (Svobodová et al. 2016; Válková et al. 2017).

Table 1 Dry biomass yields of *P. ostreatus* and growth of *C. zeylanoides* in mixed cultures compared with the monoculture controls during decolorization of RO16

Type of culture	*P. ostreatus* biomass yield Day 9, g	Yeast counts (CFU/mL)			
		Day 0	Day3	Day 6	Day 9
P. ostreatus	1.19 ± 0.00	-	-	-	-
P. ostreatus + RO16	1.16 ± 0.04	-	-	-	-
P. ostreatus + yeast	1.09 ± 0.00	1.0E + 06	2.4 + 06	6.6 + 06	6.4 + 06
P. ostreatus + yeast + RO16	0.97 ± 0.07	1.0E + 06	3.3 + 06	4.1 + 06	5.2 + 06
Yeast	-	1.0E + 06	7.9 + 07	1.1 + 08	1.1 + 08
Yeast + RO16	-	1.0E + 06	1.0 + 08	1.1 + 08	1.1 + 08

Manganese-dependent peroxidase and laccase activities during decolorization of RO16

MnP and laccase activities have been shown to be involved in degradation of synthetic dyes by white rot fungi (Knapp et al. 2008). In the mixed culture of *P. ostreatus* and *C. zeylanoides* containing RO16, the initial level of laccase of about 30 U/L was maintained for 4 days and then the enzyme activity gradually decreased to attain a level of about 10 U/L at the end of the decolorization experiment (Fig. 2). A similar behavior was observed also in the absence of RO16 dye in the mixed culture. In comparison, the levels of laccase in *P. ostreatus* monocultures were increasing during the experiment, two-fold in the monoculture with RO16 and four-fold in the monoculture without RO16 (Fig. 2). This result clearly demonstrated a negative effect of the presence of the yeast on the fungal laccase activity in the mixed culture. This is in contrast to the observations of other studies that often reported an increase in the level of laccase as a result of the interaction with other microorganisms (Hiscox et al. 2010; Svobodová et al. 2016; Válková et al. 2017). The fact that the effect appeared in later phases of the experiment might suggest that lack of nutrients caused by a higher demand of the two organisms in the mixed culture could be responsible for this behavior (Knapp et al. 1997; Zhang et al. 1999). The results also demonstrated a negative effect of RO16 on the synthesis of laccase by *P. ostreatus* (Fig. 2).

MnP activity in the *P. ostreatus* monocultures was steadily increasing or fluctuating in the range of 1–14 U/L, whereas in the presence of the yeast in the mixed cultures, the initial level of MnP was slowly decreasing to reach a negligible activity after day 7 (Fig. 3). Comparable results from other studies obtained with LF when confronted with other microorganisms are varied, ranging from a decrease of MnP (*Rhodococcus erythropolis*, Svobodová et al. 2016), no significant effect (activated sludge, Svobodová et al. 2016; *P. fluorescens*, *B. licheniformis*, Válková et al. 2017; *S. cerevisiae*, Šlosarčíková et al. 2017) to a five-fold increase (*Chaetomium* sp., Yang et al. 2011). The effect was depending on the culture medium (Svobodová et al. 2016) or the physical properties of the carrier material (Gao et al. 2008) where nonsterile conditions resulted either in an increase (polyurethane foam) or decrease (reticulated material) of the MnP level. An increase in MnP activity was also observed in majority of interactions of *T. versicolor* with other basidiomycetes on a solid medium (Hiscox et al. 2010). Similar to laccase, a negative effect of RO16 on the synthesis of MnP by *P. ostreatus* was demonstrated (Fig. 3).

The *C. zeylanoides* monoculture growing in the presence of RO16 was checked for the production of LiP, MnP, and laccase, but no activities were detected. No LiP activity was found in *P. ostreatus* cultures either. In spite of the decrease of the MnP and laccase activities during decolorization in the mixed culture, our results showed that both laccase and MnP activities were present during a significant part of the decolorization experiment with the mixed culture of *P. ostreatus* and *C. zeylanoides*, suggesting that they could take part in the degradation process.

GC-MS analysis of RO16 degradation products

The degradation of the dye RO16 by the fungus *P. ostreatus* and the yeast *C. zeylanoides* were followed by the analysis of degradation products by GC-MS. The characteristics of the

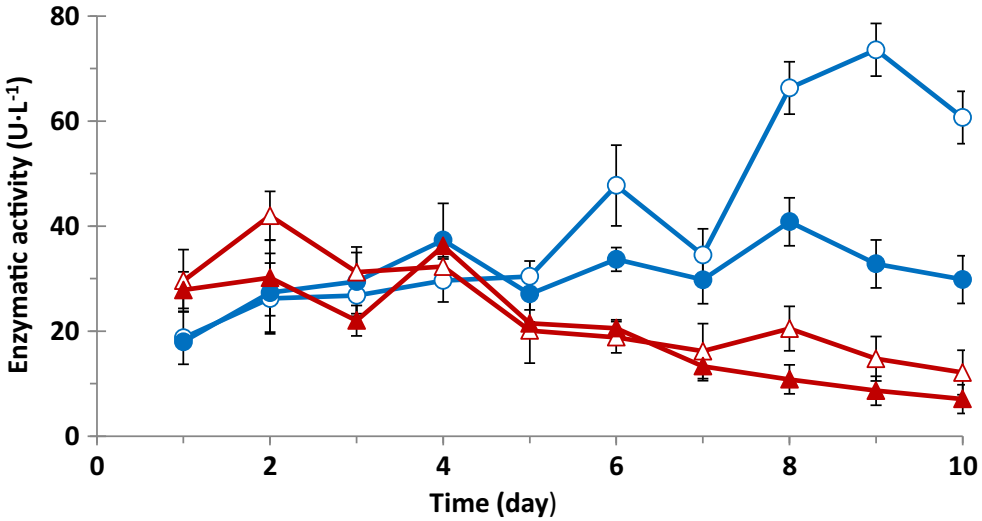

Fig. 2 Laccase activities in the mixed culture of *P. ostreatus* and *C. zeylanoides* and in the *P. ostreatus* monoculture during RO16 decolorization compared with the corresponding control cultures without the dye. Mixed culture of *P. ostreatus* and *C. zeylanoides* with RO16, ▲; mixed culture of *P. ostreatus* and *C zeylanoides* without RO16, △; *P. ostreatus* monoculture with RO16, ●; *P. ostreatus* monoculture without RO16 ○

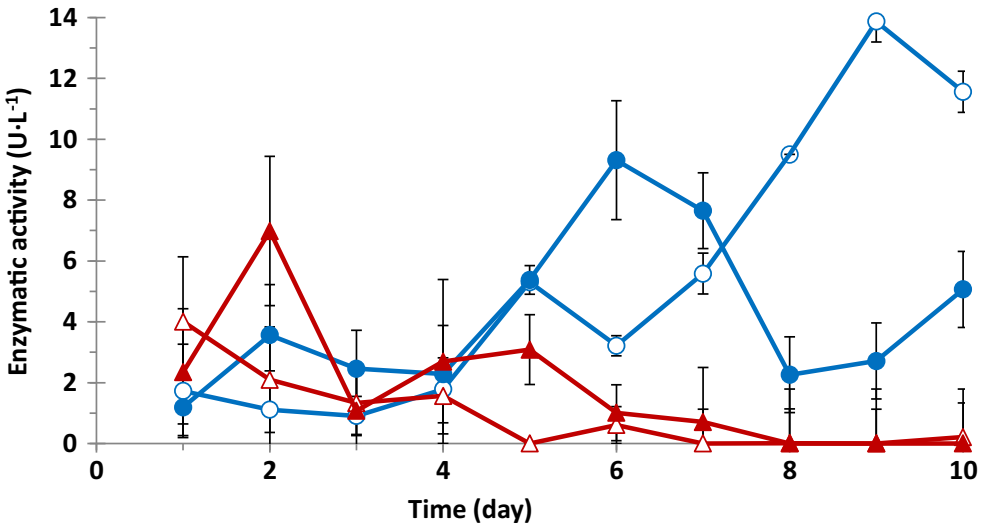

Fig. 3 Manganese-dependent peroxidase activities in the mixed culture of *P. ostreatus* and *C. zeylanoides* and in the *P. ostreatus* monoculture during RO16 decolorization compared with the corresponding control cultures without the dye. Mixed culture of *P. ostreatus* and *C. zeylanoides* with RO16, ▲; mixed culture of *P. ostreatus* and *C. zeylanoides* without RO16, △; *P. ostreatus* monoculture with RO16, ●; *P. ostreatus* monoculture without RO16 ○

products are listed in Table 2. In the monoculture of the fungus, the dye molecule was split into two parts. Three degradation products were found, namely 4-(ethenylsulfonyl) benzene, (methylsulfonyl) benzene, and 2-(phenylsulfonyl) ethanol. Additional data are given in Electronic Supplementary Material (Online Resource 1). The asymmetric cleavage of RO16 molecule and subsequent removal of hydrazine in the form of N_2 probably provided 4-(ethenylsulfonyl) benzene (cf. Svobodová et al. 2007). The asymmetrical cleavage of azo bonds in azo dyes by fungal peroxidase and laccase was described in 1990's (Spadaro and Renganathan 1994; Chivukula and Renganathan 1995). (Methylsulfonyl) benzene and 2-(phenylsulfonyl) ethanol were probably formed by demethylation and hydrolysis of the ethenyl double bond in

Table 2 Major metabolites detected by GC-MS in the mixed culture of *P. ostreatus* and *C. zeylanoides* during degradation of RO16

Compound	m/z	CAS no.	Metabolite characteristic	Reference
Degradation of RO16 dye by *Pleurotus ostreatus*				
4-(Ethenylsulfonyl)benzene	168, 125, 77, 51	005535-48-8	Dye cleavage product by *P. ostreatus*	Spadaro and Renganathan 1994
2-(phenylsulfonyl)ethanol	77, 78, 51	20611-21-6	Dye cleavage product by *P. ostreatus*	Spadaro and Renganathan 1994
Degradation of RO16 dye by *Candida zeylanoides*				
4-(Ethenylsulfonyl)aniline	183, 156, 140, 92	25781-90-2	Dye cleavage product by *C. zeylanoides*	Jafari et al. 2014
α-Hydroxybenzenepropanoic acid	166, 148, 91	20312-36-1	Dye cleavage product by *C. zeylanoides*	Jafari et al. 2014
Other metabolites				
Phenylethyl alcohol	122,91,92	000060-12-8	Yeast volatile product	Mo et al. 2003
1H-indol-3-ethanol	161,13	000100-51-6	Plant auxin hormone precursor produced in yeasts	Mo et al. 2003
4,6-Dimethoxy phthalide	194,165,137, 122	058545-97-4	Fungal antibiotic	Leon et al. 2017; Sazanova et al. 2018
3-Methyl-2(5H)-furanone	98,69,109,124	22122-36-7	Yeast signal molecules; antifungal antibiotic	Colin Slaughter 1999; Li et al. 2019
4-Methoxybenzaldehyde	135,136,77	000123-11-5	Fungal flavor compound	Berger and Zorn 2004
Benzaldehyde	105,106,77	000100-52-7	Fungal flavor compound	Lapadatescu et al. 1997; Lomascolo et al. 2001

4-(ethenylsulfonyl) benzene, respectively (Fig. 5). Demethylase activities were found in various fungi having function in tolerance to toxic plant phytoalexins (Delserone et al. 1999; Coleman et al. 2011). 2-(phenylsulfonyl) ethanol was detected as a product of degradation of the azo dye Remazol Orange 3R by plant consortium of *Aster amellius* and *Glandularia pulchella* using laccase, peroxidase, and oxidase activities (Kabra et al. 2011). The other part of the RO16 molecule including two condensed aromatic rings (cf. Spadaro and Renganathan 1994) was probably rapidly oxidized to small molecules as no relevant degradation product was detected (Table 2). In the monoculture of *C. zeylanoides*, two compounds resulting from dye decolorization were detected. The azo bond was symmetrically split as also observed in studies using *Galactomyces geotrichum* and other yeasts (Jadhav et al. 2008; Jafari et al. 2014), and 4-(ethenylsulfonyl) aniline and α-hydroxybenzenepropanoic acid were detected as degradation products in the samples removed on days 3, 6, and 9.

Figure 4 showed that gradual decolorization of RO16 in the *P. ostreatus* monoculture between days 1–11 (Fig. 1) resulted in a transient accumulation of the degradation product 4-(ethenylsulfonyl) benzene that was further metabolized by the fungus using, for instance, hydrolytic or demethylation reactions (cf. Fig. 5).This behavior pointed to a more rapid early reactions producing 4-(ethenylsulfonyl) benzene, compared with those that further oxidized it in the degradation pathway, when working under the conditions of a relative abundance of the dye at the beginning of the decolorization process. Similar transient accumulation was also observed for 4-(ethenylsulfonyl) aniline, the product of degradation of the dye by *C. zeylanoides*. On day 6, when the dye was practically no more present in the medium, as evidenced by spectrophotometry (Fig. 1), the concentration of 4-(ethenylsulfonyl) aniline reached its maximum and was further transformed by the yeast metabolism between days 6–9, as documented by a decreased level of 4-(ethenylsulfonyl) aniline on day 11 (Fig. 4). Figure 4 thus documented that both degradation products were not end-products of degradation of RO16 by the fungal and yeast metabolism.

In the mixed culture of *P. ostreatus* and *C. zeylanoides*, only traces of 4-(ethenylsulfonyl) aniline and 4-(ethenylsulfonyl) benzene and no α-hydroxybenzenepropanoic acid were determined in the samples removed on days 3, 6, and 9, which suggested a more rapid metabolization of the fungal and yeast degradation products in the presence of the two microorganisms, compared with the two individual monocultures. Probably, the respective degradation activities of the partner microorganisms in the mixed culture contributed to a faster disappearance of the above-mentioned fungal and yeast products by working in a mutual cooperative mode. Generally, a fast degradation of RO16 by both microorganisms resulted in the detection of only a few metabolites resulting from the cleavage of the dye molecule. Possible pathways of RO16 biodegradation by the yeast *C. zeylanoides* and the fungus *P. ostreatus* were suggested (Fig. 5).

There were other organic compounds identified by GC-MS after the extraction of the culture liquid samples from the fungal and yeast cultures, i.e., benzenacetaldehyde, 4-methoxybenzaldehyde, benzaldehyde, 3-methyl-2(5H)-furanone, phenylethyl alcohol, 4,6-dimethoxy phthalide, and 1H-indol-3-ethanol. As they were detected also in the cultures where the dye was absent, they represented metabolites produced by the microorganisms in the growth medium used.

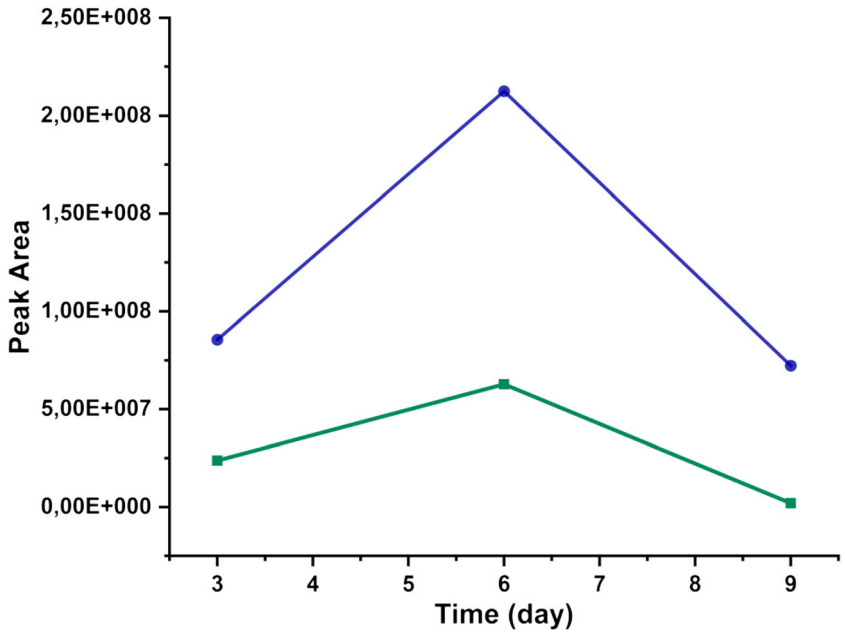

Fig. 4 Respective accumulation kinetics of 4-(ethenylsulfonyl)benzene and 4-(ethenylsulfonyl) aniline in *P. ostreatus* and *C. zeylanoides* monocultures during decolorization of RO16

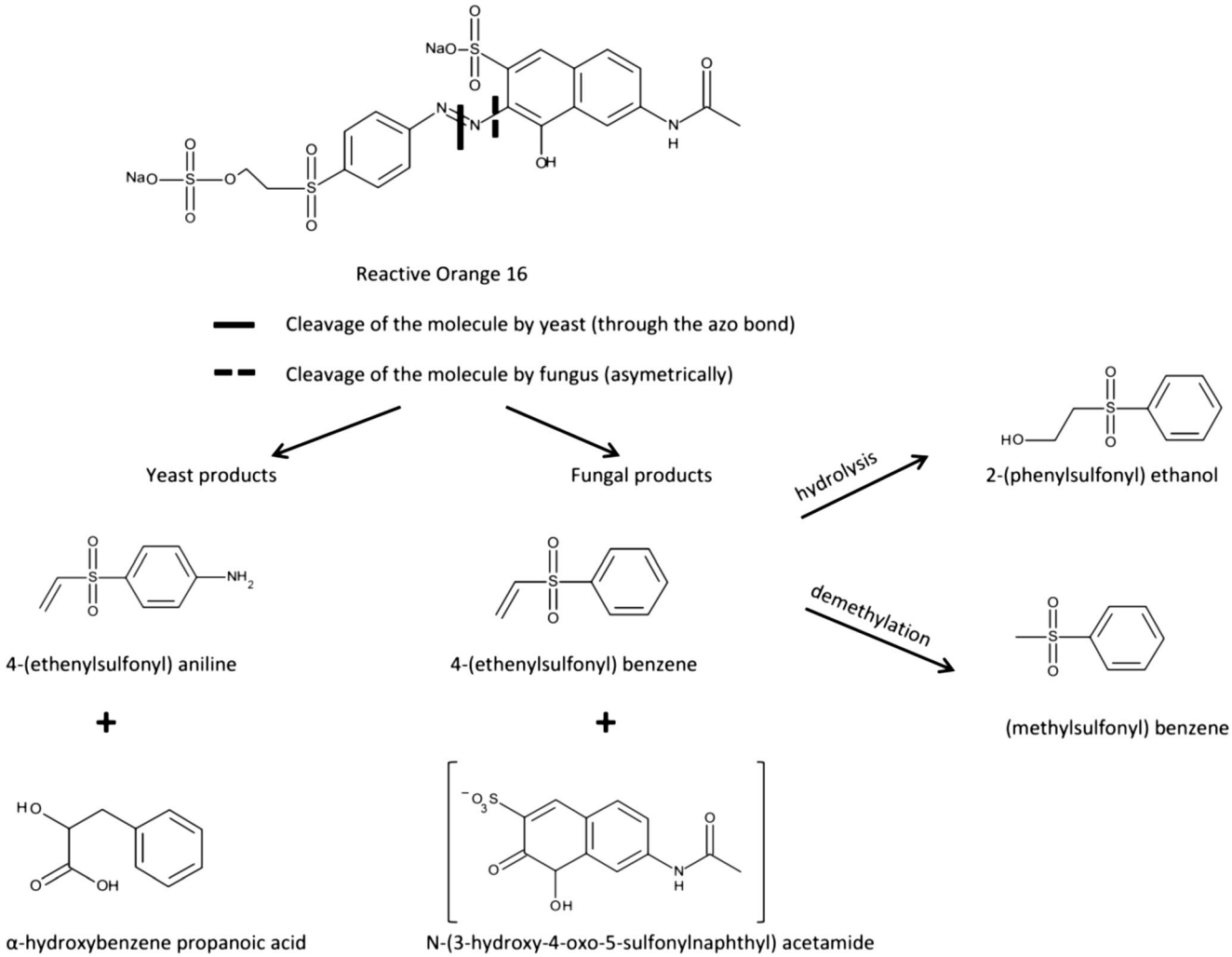

Fig. 5 A suggested pathway of RO16 degradation by the yeast *C. zeylanoides* and the fungus *P. ostreatus*. The compound in brackets was not found; it was expected to be decomposed at the early phase of the degradation process before removing the samples

Phenylethyl alcohol and 1H-indol-3-ethanol were described as volatile products in *Pichia* spp. (Mo et al. 2003), 4,6-dimethoxy phthalide was detected in the agaricomycete *Lignomyces vetlinianus* (Sazanova et al. 2018), fungal furanones are known to have antibiotic effects (Huff et al. 1994; Li et al. 2019), and benzeneacetaldehyde and 4-methoxybenzaldehyde are metabolites found in *Pleurotus* cultures (Kabbaj et al. 1997; Berger and Zorn 2004).

Conclusion

The mixed culture of the immobilized ligninolytic fungus *P. ostreatus* and the yeast *C. zeylanoides* exhibited a higher efficiency of decolorization of the recalcitrant RO16 monoazo dye in comparison with a monoculture of *P. ostreatus*. This finding, together with the products detected by GC-MS, showed that two parallel degradation processes were operating simultaneously, one realized by the fungus and the other by the yeast. A more rapid disappearance of the metabolites in the mixed culture, compared with the monocultures, documented a cooperative action of the two microorganisms probably resulting from their different enzyme activities involved in the dye degradation. The evidence of the cooperative action between the fungus and the yeast represents a novel finding as reports on such mixed cultures are rather rare. The compatibility of the two different biodegradation processes and resilience and stability of the mixed fungal-yeast culture support the concept of combining the activities of various microorganisms in biodegradation technology. The observed reduction of the yeast biodegradation capacity in the mixed culture however accentuated the importance of optimizing the conditions in the mixed culture for all microorganism partners.

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