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Effect of yeasts on biodegradation potential of immobilized cultures of white rot fungi

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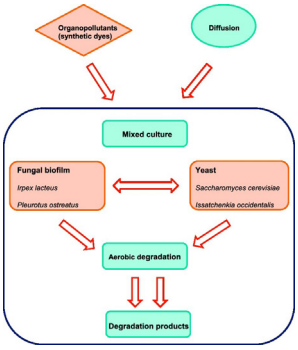
HIGHLIGHTS

- Two different fungi and two yeasts were included in the study.
- No negative effect of yeasts on fungal biofilm structure was observed.
- Degradation rate of fungal biofilms was not decreased by yeasts.
- Yeasts well survived in mixed cultures with fungi.
- No simultaneous degradation of azo dye by the fungus and yeast was achieved.

GRAPHICAL ABSTRACT



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ABSTRACT

The aim was to investigate the effect of yeast organisms on the degradation process by immobilized cultures of ligninolytic fungi. Immobilization was accomplished by 7-day colonization of polyamide mesh with mycelial fragments. *Irpex lacteus* decolorized >90% of the initial concentration of 150 mg l^{-1} of anthraquinone Remazol Brilliant Blue R dye in three subsequent decolorization cycles and the degradation capacity was not negatively affected by the presence of 10^6 *Saccharomyces cerevisiae* cells per ml in the mixed culture. The yeast was not able to degrade the dye. *I. lacteus* biofilm was also resistant to bacterial infection with *E. coli*. Inoculation of the yeast to pre-formed *I. lacteus* biofilm culture resulted in a reduction of fungal biomass by 27%. Levels of LiP, MnP and laccase of *I. lacteus* were not much influenced by *S. cerevisiae* or *E. coli*. Similar resilience of *P. ostreatus* biofilms was observed after exposure to yeast *Issatchenkia occidentalis* when the fungal degradation capacity measured with Reactive Orange 16 azo dye was maintained over two decolorization cycles. *I. occidentalis* did not degrade the dye under the conditions used. Formation of densely packed fungal biofilms with abundant extracellular polysaccharide was not impeded by the yeast. Increase of MnP and laccase levels attributable to the presence of *I. occidentalis* was observed.

Abbreviations: ABTS, 2,2′ Azinobis(3-ethylbenzothiazoline-6-sulfonic acid); CW, calcofluor white; DMP, 2,6 dimethoxyphenol; LiP, lignin peroxidase; MEG, malt extract-glucose medium; MnP, manganese-dependent peroxidase; NA, Nutrient Agar; NB, Nutrient Broth; NDM, Normal Dextrose Medium; RBBR, Remazol Brilliant Blue R; RO16, Reactive Orange 16; SAD, Sabouraud Agar with Dextrose; SEM, scanning electron microscopy; U, unit of enzyme activity; VeOH, 3,4-dimethoxyphenol.

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1. Introduction

Contamination of water with hazardous chemicals is a serious ecological problem where environmental biotechnologies can be very helpful. Among microorganisms that can be used in bioremediation processes, white rot fungi represent a promising group widely applicable in biodegradation of recalcitrant pollutants (Gadd, 2008). Fungi immobilized on solid supports have been used in various types of reactors in the form of biofilters for bioremediation of polluted water (e.g. Rodríguez Couto, 2009).

Most studies were carried out under aseptic conditions in the laboratory and thus the fungal performance under non-sterile conditions is poorly understood. Various studies documented both positive and negative effects of exogenous contaminating microorganisms on the biodegradation efficiency of fungal cultures in bioreactors (Li et al., 2015; Spina et al., 2014; etc.). Generally, the factors involved in these microbial interactions that affect fungal biodegradation include pH, level of extracellular enzymes engaged in the biodegradation process, and competition for nutrients. A low pH or limitation of nitrogen source available that were thought to be the measures offering a long-term protection against contamination were found not to be very effective as bacteria can adapt to a low pH and the aging and lysis of fungal hyphae can provide additional nitrogen for bacterial growth (Libra et al., 2003). The secreted fungal enzymes, peroxidases and laccase, are attacked by proteases produced by contaminating microorganisms that rapidly decrease their activity (Libra et al., 2003). Negative effects of contamination can be weakened by growing the fungus on a natural lignocellulosic substrate that serves as a less efficient carbon source for other microorganisms, favors the production of high fungal enzyme levels and may contribute to maintaining stable enzyme levels in the culture (Libra et al., 2003; Rodríguez Couto et al., 2000). The compact extracellular polysaccharide matrix that develops in the course of aging, covers the fungal biofilm and also serves as a natural carrier where extracellular enzymes are immobilized. It can offer the fungal organism a certain protection against the attack by contaminating microorganisms (Evans et al., 1991; Maziero et al., 1999).

Notwithstanding the negative effects of competing microorganisms on biodegradation capacity of fungal biofilms, examples of consortia of fungi with bacteria and other fungi that exhibit a significant degradation potential were reported (Gou et al., 2009; Kadam et al., 2011, etc.). In some of them the biodegradation effectivity exceeded that of the corresponding monocultures, e.g. consortia of *Aspergillus lentulus, A. terreus* and *Rhizopus oryzae* used for decolorization of Pigment Orange 34 and Acid Blue 161 dyes (Mishra and Malik, 2014) and of *Trametes* sp. SQ01 and *Chaetomimum* sp. R01 used for decolorization of triphenylmethane dyes (Yang et al., 2011).

In nature, ligninolytic fungi are often found growing with other microorganisms including yeasts (Dill and Kraepelin, 1986). Yeasts have often been reported as the contaminating microorganisms responsible for failures of bioremediation processes realized by white rot fungi because of their good growth at pH 5–6 in carbohydrate-based media (Knapp et al., 2008) and higher growth rates compared with filamentous fungi (Boekhout and Robert, 2003; Trinci, 1971). On the other hand, yeast organisms may exhibit important biodegradation capacities, for instance, those able to decolorize recalcitrant azo dyes (Yang et al., 2005; Tan et al., 2016). The enzymes involved in these biodegradations belong to the class of azoreductases that catalyze reductive cleavage of azo bonds by using reduction equivalents (NADH or NADPH) generated from the metabolism of organic compounds (Shah and Madamwar, 2013; Imran et al., 2016). This biodegradation mechanism is different from that of ligninolytic fungi that use extracellular oxidative enzymes, MnP, laccase and LiP, which oxidize azo dyes using a highly nonspecific free radical mechanism producing phenolic compounds (Svobodová et al., 2007). The yeast *I. occidentalis* used in our study is capable of degradation of azo dyes using azoreductase (Ramalho et al., 2004). It was used to both investigate the interactions with *P. ostreatus* but also test whether the two biodegradation processes, the fungal and the yeast one, can work simultaneously.

Our investigation focused on the effect of massive inoculation of fungal biofilms of *I. lacteus* and *P. ostreatus* immobilized on polyamide mesh with *S. cerevisiae* and *I. occidentalis* yeasts, respectively, to measure the effect on stability and sustenance of the fungal biofilms and on their capacity of biodegradation of recalcitrant anthraquinone- and azo dyes. In order to characterize the mixed fungal/yeast cultures, MnP, LiP and laccase were estimated, electron microscopy was used to determine the structure of fungal biofilms, yeast counts were measured to see survival and growth of yeast populations, and fluorescence microscopy was used to detect viability of both organisms.

2. Materials and methods

2.1. Microorganisms

The fungi *Irpex lacteus* and *P. ostreatus* were obtained from the Culture Collection of Basidiomycetes of ASCR, Prague, Czech Republic. The yeast *Issatchenkia occidentalis* was acquired from the Spanish Type Culture Collection, University of Valencia, Spain. The yeast *Saccharomyces cerevisiae* CCM 8191 and the bacterium *Escherichia coli* CCM 3988 were purchased from the Czech Collection of Microorganisms, Masaryk University, Brno, Czech republic. The fungi were maintained on MEG agar (malt extract 5 g l^{-1}, glucose 10 g l^{-1}, agar powder 20 g l^{-1}), grown at 28 °C for 7 days and stored at 4 °C. The yeasts were preserved on SAD plates (glucose 40 g l^{-1}, mycopeptone 10 g l^{-1}, agar powder 15 g l^{-1}). Both yeasts were grown at 28 °C for 2 days and then stored at 4 °C. The bacterium was maintained on NA medium (HiMedia).

2.2. Chemicals

2,2′ Azinobis(3-ethylbenzothiazoline-6-sulfonic acid, 2,6 dimethoxyphenol, 96% 3,4-dimethoxyphenol, malonic acid, ethylenediaminetetraacetic acid, Remazol Brilliant Blue R and Reactive Orange 16 were purchased from Sigma-Aldrich. All chemicals were of analytical grade.

2.3. Culture conditions

The fungal inocula were prepared by transferring ten agar plugs of *I. lacteus* or *P. ostreatus* grown on MEG agar into 250-ml cotton-plugged flasks containing 100 ml of liquid MEG medium and incubated at 28 °C for 7 days. After this period of growth, the cultures were homogenized and 15 ml of inoculum (10% V/V) was added into each 250-ml cotton-plugged flask with growth medium that also contained polyamide mesh carrier (wire wool: fiber thickness, 2 mm; mesh size, 3 mm) to prepare immobilized fungal cultures. A mineral medium (Tien and Kirk, 1988) was used for this purpose. The fungal cultures were then grown under static conditions or shaken conditions (80 rpm) for 7 days to form biofilm on the polyamide carrier and were further used as pre-formed biofilms in mixed cultures.

The *S. cerevisise* and *E. coli* inocula were prepared by growing the microorganisms overnight in MEG or NB media, respectively, on a rotary shaker (80 rpm), the final concentration of the microorganism was adjusted so that the final value of 10^6 CFU per ml was achieved in the mixed cultures. RBBR anthraquinone dye or RO16 azo dye were added to mixed cultures at a final concentration of 150 mg l^{-1} to start decolorization. A new portion of the dye was added at the beginning of a new decolorization cycle. If the effect of *E. coli* on the formation of fungal biofilm was studied, the bacterium was added simultaneously with the fungal inoculum to a flask containing the medium and the carrier; this situation is referred to as a nascent biofilm.

The experiments with *P. ostreatus* and *I. occidentalis* followed the same protocol as above but sometimes the fungal biofilm was allowed to form under shaking conditions (80 rpm) to prevent the yeast to

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