

Decolorization of Six Synthetic Dyes by Fungi

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Abstract: To find out ability of fourteen basidiomycetes and four ascomycetes strains to grow in the presence of synthetic colour dyes and to degrade them, fungi were cultivated on the malt agar plates containing 0.5 g kg⁻¹ dye, either Remazol Brilliant Blue R, Remazol Brilliant Yellow GL, Remazol Brilliant Orange 3 R, Reactive Blue 4, Remazol Brilliant Red F3B or Reactive Black 5. Fungi representing basidiomycetes were *Phlebia radiata* (FBCC 43), *Tremella encephala* (FBCC 1145), *Dichomitus squalens* (FBCC 312), *Physisporinus rivulosus* (syn. *Obba rivulosa*, FBCC 939), *Cerrena unicolor* (FBCC 387), *Pleurotus abieticola* (FBCC 517), *Phanerochaete velutina* (FBCC 941), *Agrocybe praecox* (FBCC 476), *Trametes pubescens* (FBCC 735), *Pleurotus ostreatus* (FBCC 498), *Fomitopsis pinicola* (FBCC 18), *Postia placenta* (= syn. *Rhodonina placenta*, FBCC 112), *Gloeophyllum trabeum* (FBCC 328) and *Piptoporus betulinus* (FBCC 1191). Ascomycetes belonged to genera *Alternaria* (HAMBI 3289), *Epicoccum* (HAMBI 3291), *Fusarium* (HAMBI 3292) or *Chaetomium* (HAMBI 3291). The growth rate of *P. rivulosus* belonged to three highest among the 14 tested basidiomycetes with five dyes, but not in the case of Remazol Brilliant Red F3B containing plates. The growth rate of *A. praecox* belonged to three lowest among the 14 tested basidiomycetes on Remazol Brilliant Blue R, Remazol Brilliant Yellow GL, Remazol Brilliant Red F3B, Reactive Black 5 and malt agar. The growth rate of *Chaetomium* sp. was the highest among the four ascomycetes on all tested plates. Decolorization was seen with 7 basidiomycetous strains on Remazol Brilliant Blue R, with 7 basidiomycetes on Remazol Brilliant Orange 3 R, with 8 basidiomycetes on Reactive Blue 4 and 11 basidiomycetes on Reactive Black 5 containing plates. *T. encephala* did not decolorize any of the tested six dyes. *Epicoccum* sp. and *Chaetomium* sp. decolorized Reactive Black 5 dye containing plates. None of the fourteen basidiomycetous or four ascomycetes were able to degrade all the tested six dyes.

Keywords: Synthetic Dye, Basidiomycetes, Ascomycetes, Biodegradation

Introduction

Synthetic dyes are widely used in mass production of textiles, food and paper (Pandey *et al.*, 2007). The synthetic dyes have complex chemical structures, which make them persistent against light, water and microbial attack (Saratale *et al.*, 2011). This persistence makes them harmful in the environment. Considerable amounts of synthetic dyes have been released from industrial production and discarded products to the environment.

Ligninolytic basidiomycetous fungi produce different combinations of extracellular oxidoreductive enzymes (laccases, manganese peroxidases, versatile peroxidases and lignin peroxidases), which can degrade natural biopolymers such as lignin and degrade recalcitrant xenobiotic synthetic compounds (Hatakka, 1994; Hatakka and Hammel, 2010). The growth of ascomycetes and basidiomycetes and their extracellular enzyme production are suitable indicators for the environmental health. Synthetic colour dye compounds can decrease the

growth of fungi and production of extra- and intracellular enzymes, which are needed in biodegradation of xenobiotic synthetic dyes. Some ascomycetes are able to produce extracellular oxidative enzymes (Palonen *et al.*, 2003; Sánchez, 2009). Ascomycetes produce mainly hydrolytic enzymes, which degrade carbohydrates (Hatakka and Hammel, 2010; Rodriguez *et al.*, 1996; Kluczek-Turpeinen *et al.*, 2003). Because the variety of different dyes is enormous, only little is known about capabilities of lignocellulose degrading ascomycetes (molds) while the capabilities of lignin degrading wood and litter decomposing basidiomycetes to degrade synthetic dyes have gained interest during last decades (Wesenberg *et al.*, 2003, Rodriguez Couto, 2009). Especially fungal laccases have been studied in larger screenings for bleaching of synthetic dyes.

The aim of the study was to assess the ability of taxonomically and functionally different fungi to grow in the presence of synthetic dyes and to degrade them as indicated by decolorization. We selected fourteen basidiomycetes and four ascomycetes. The selected six synthetic dyes were Remazol Brilliant Blue R, Remazol Brilliant Yellow GL, Remazol Brilliant Orange 3 R, Reactive Blue 4, Remazol Brilliant Red F3B and Reactive Black 5.

Materials and Methods

The four litter-decomposing saprotrophic ascomycetes from different genera were an *Alternaria* sp. (HAMBI 3289), an *Epicoccum* sp. (HAMBI 3291), a *Fusarium* sp. (HAMBI 3292) and a *Chaetomium* sp. (HAMBI 3291) deposited to the microbial culture collection HAMBI of the Department of Food and Environmental Sciences, Microbiology and Biotechnology Division, University of Helsinki, Finland. These fungi were isolated from straw, which was buried during winter time in arable soil in Southern Finland. The buried straw was surface-sterilized using 70% ethanol and 2% hypochlorite. The surface-sterilized straw was placed on agar plates, which contained 2% (w/v) Malt Extract (MEA). Isolated fungi were maintained as pure culture on 2% MEA. Pure cultures were identified morphologically under an optical microscope. Pure cultures selected for this study were stored on 2% MEA in glass tubes at 4°C.

Fourteen wood-rotting or litter-decomposing basidiomycetes were selected for this study from the Fungal Biotechnology Culture Collection (FBCC) at the Department of Food and Environmental Sciences, Microbiology and Biotechnology Division, University of Helsinki, Finland. The strains were *Phlebia radiata* (FBCC 43), *Tremella encephala* (FBCC 1145), *Dichomitus squalens* (FBCC 312), *Physisporinus rivulosus* (= *Obba rivulosa*, FBCC 939), *Cerrena unicolor* (FBCC 387), *Pleurotus abieticola* (FBCC 517), *Phanerochaete velutina* (FBCC 941), *Agrocybe*

praecox (FBCC 476), *Trametes pubescens* (FBCC 735), *Pleurotus ostreatus* (FBCC 498), *Fomitopsis pinicola* (FBCC 18), *Postia placenta* (FBCC 112), *Gloeophyllum trabeum* (FBCC 328) and *Piptoporus betulinus* (FBCC 1191). These fungi were maintained on 2% (w/v) malt extract agar.

Colour Dye Plate Tests

The ability of fungi to grow and biodegrade synthetic colour dyes were tested with six different dyes. These were Remazol Brilliant Blue R, Remazol Brilliant Yellow GL, Remazol Brilliant Orange 3 R, Reactive Blue 4, Remazol Brilliant Red F3B and Reactive Black 5. 0.5 g dm⁻³ of these colour dyes were added on malt agar plates. The control was malt agar plate without added colour dyes. ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid, Sigma-Aldrich, U.S.A.) was used as indicator dye in the malt agar plate to test ability of fungi to produce laccase and other oxidative enzymes. The formation of green colour in the ABTS plate indicates the production of oxidative enzyme(s). The decolorization of the other five dyes indicates degradation of the tested dye compound. A 4 mm diameter fully grown agar plug from each tested fungus was added to the center of the test plate. All was done as triplicate. Plates were incubated at 25°C. The diameter of the growth and colour change were measured on each sector of 90° and averaged over the four sectors of the plate.

Results

The growth rates of 14 basidiomycetes were tested with Remazol Brilliant Blue R, Remazol Brilliant Yellow GL, Remazol Brilliant Orange 3 R, Reactive Blue 4, Remazol Brilliant Red F3B, Reactive Black 5 and ABTS and without added synthetic dyes on malt agar plates (Fig. 1). All these fourteen fungi, namely *P. radiata*, *T. encephala*, *D. squalens*, *P. rivulosus*, *C. unicolor*, *P. abieticola*, *P. velutina*, *A. praecox*, *T. pubescens*, *P. ostreatus*, *F. pinicola*, *P. placenta*, *G. trabeum* and *P. betulinus* grew in the presence of all the six selected synthetic dyes and ABTS in the plates. The growth rate of *P. rivulosus* was the highest among the 14 tested basidiomycetes on five different dyes, ABTS plates and malt agar plates, but it was not the case on Remazol Brilliant Red F3B containing plates. The growth rate of *P. radiata* belonged to three highest among 14 tested basidiomycetes on five different colour dye and malt agar plates, but this was not the case on ABTS or Remazol Brilliant Yellow GL containing plates. The growth rate of *A. praecox* belonged to three weakest among the 14 tested basidiomycetes on Remazol Brilliant Blue R, Remazol Brilliant Yellow GL, Remazol Brilliant Red F3B, Reactive Black 5 and on malt extract agar without added dye.

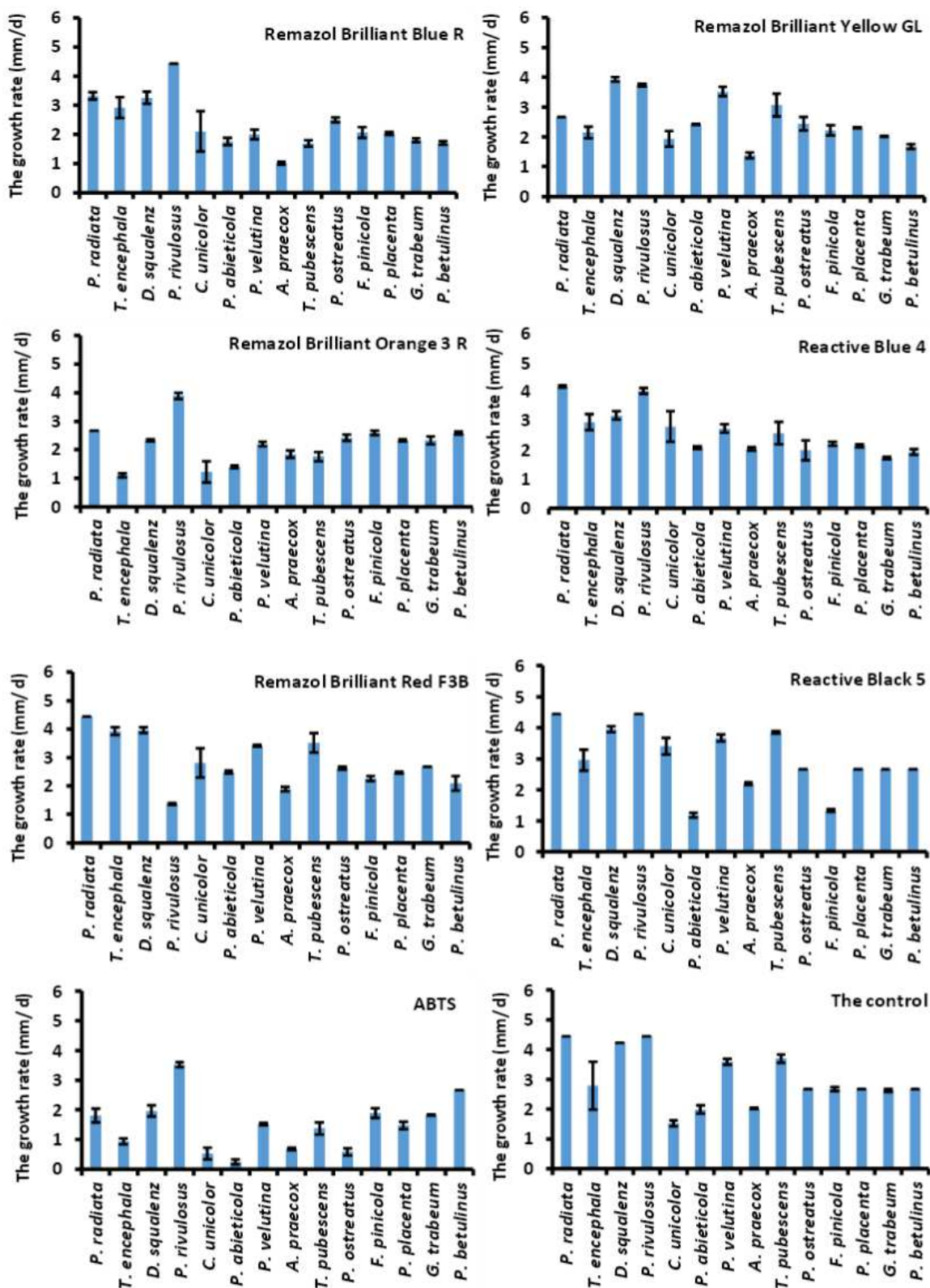


Fig. 1. The growth rate of 14 basidiomycetes on Remazol Brilliant Blue R, Remazol Brilliant Yellow GL, Remazol Brilliant Orange 3 R, Reactive Blue 4, Remazol Brilliant Red F3B, Reactive Black 5 or ABTS containing malt agar plates and the control without added dye

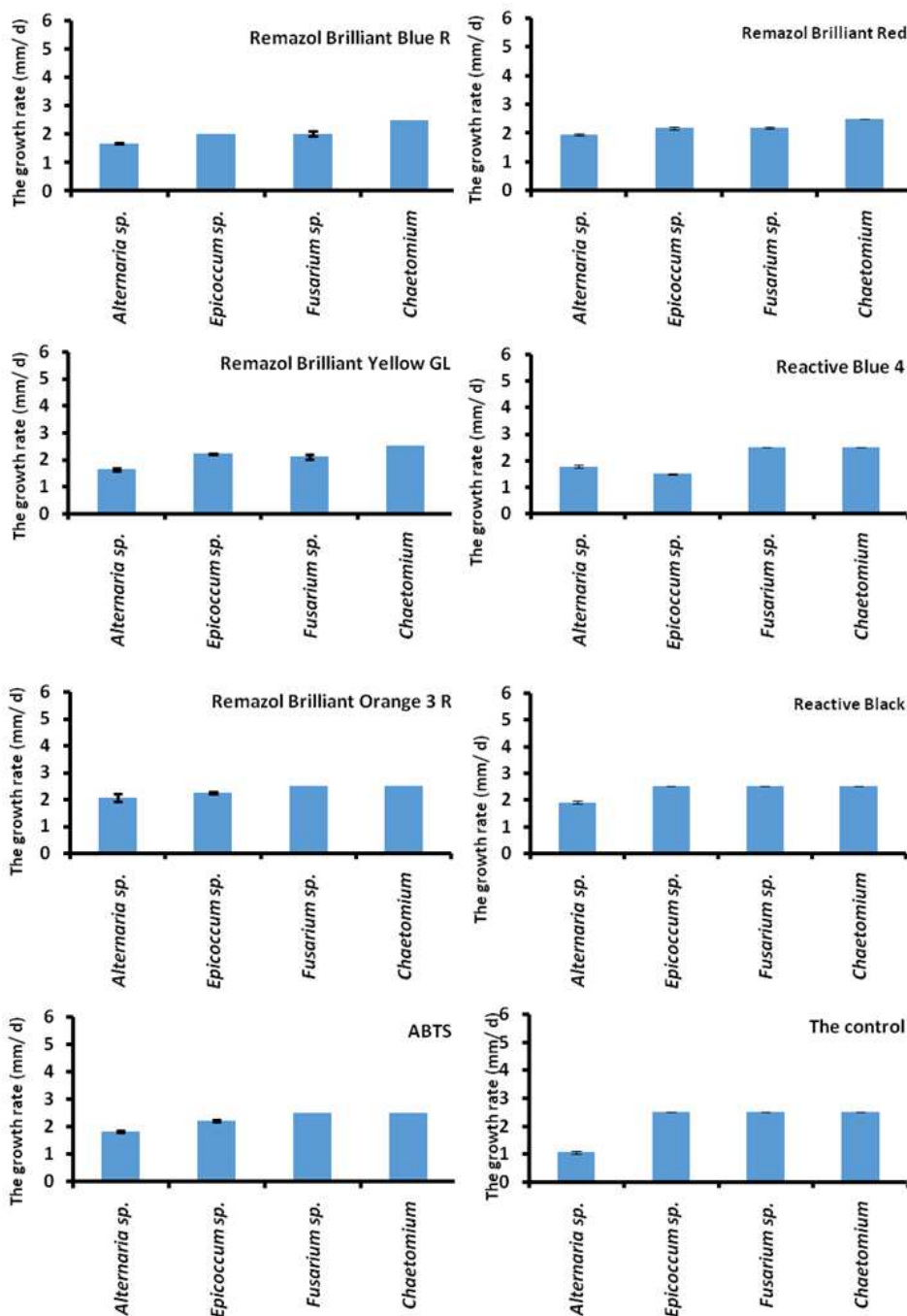


Fig. 2. The growth rate of four ascomycetes on Remazol Brilliant Blue R, Remazol Brilliant Yellow GL, Remazol Brilliant Orange 3 R, Reactive Blue 4, Remazol Brilliant Red F3B, Reactive Black 5 or ABTS containing malt agar plates and the control without added dye

The growth rates of four ascomycetes, namely *Alternaria sp.*, *Epicoccum sp.*, *Fusarium sp.* and *Chaetomium sp.*, were tested with Remazol Brilliant Blue R, Remazol Brilliant Yellow GL, Remazol Brilliant Orange 3 R, Reactive Blue 4, Remazol Brilliant Red F3B, Reactive Black 5 and ABTS and without added synthetic dyes on malt agar plates (Fig. 2). The growth

rate of *Chaetomium sp.* was the highest among the tested ascomycetes on all tested plates. The growth rate of *Alternaria sp.* was the lowest among the tested ascomycetes on Remazol Brilliant Blue R, Remazol Brilliant Yellow GL, Remazol Brilliant Red F3B, Reactive Black 5 and ABTS containing plates and malt extract agar plates without added dye.

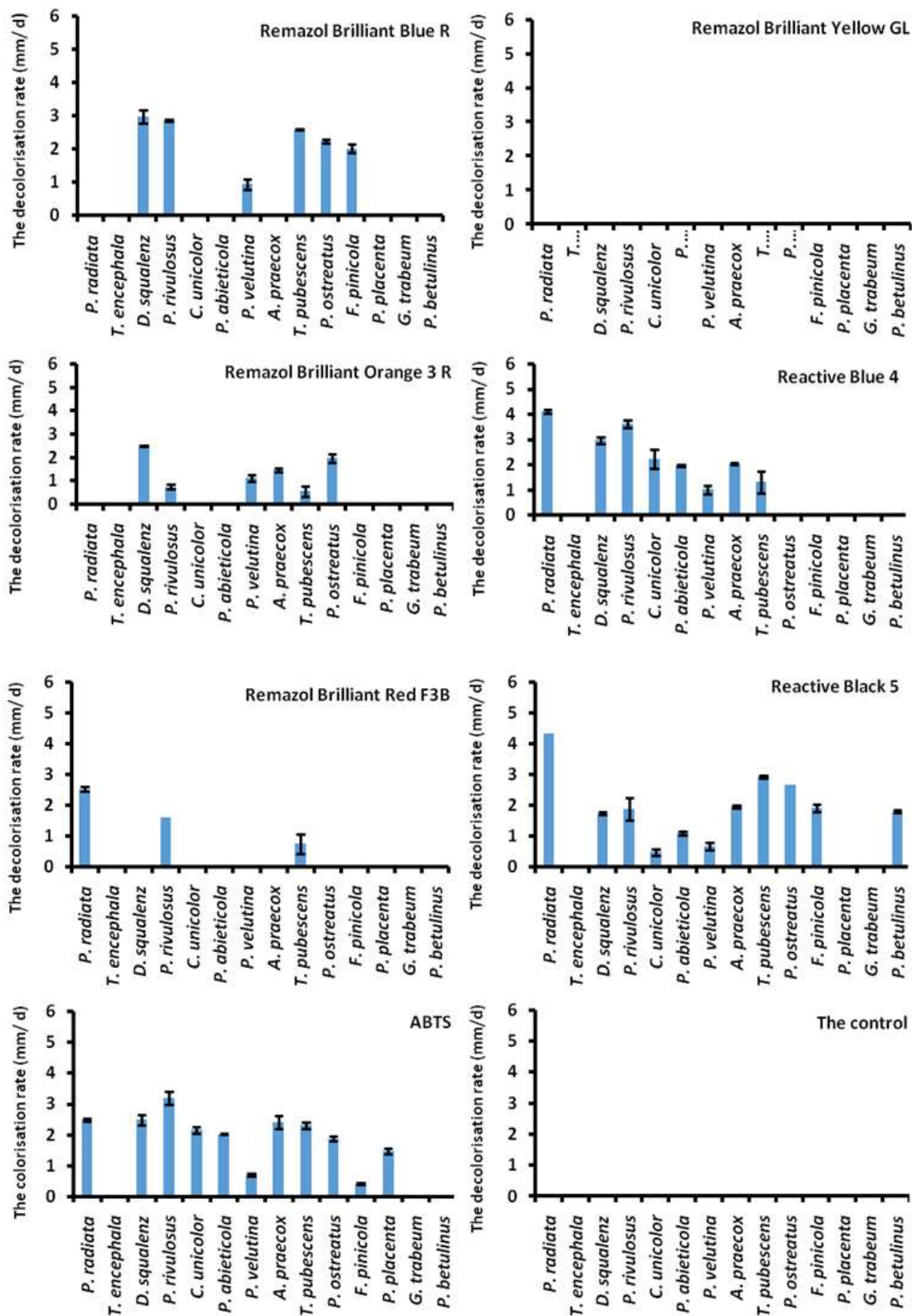


Fig. 3. The decolorization rate with 14 basidiomycetes on Remazol Brilliant Blue R, Remazol Brilliant Yellow GL, Remazol Brilliant Orange 3 R, Reactive Blue 4, Remazol Brilliant Red F3B, Reactive Black 5 and the formation of colour zone with ABTS containing malt agar plates and the control without added dye

Decolorization was seen with 7 basidiomycetes on Remazol Brilliant Blue R, with 7 basidiomycetes on Remazol Brilliant Orange 3 R, with 8 basidiomycetes on Reactive Blue 4 and 11 basidiomycetes Reactive Black 5 containing plates (Fig. 3). *D. squalens*, *P. rivulosus*, *P. velutina* and *T. pubescens* decolorized Remazol Brilliant Blue R, Remazol Brilliant Orange 3 R, Reactive Blue 4 and Reactive Black 5. Decolorization was seen with *P. radiata*, *P. rivulosus* and *T. pubescens* on Remazol Brilliant Red F3B containing plates. It is noteworthy that none of the tested fourteen basidiomycetes was able to decolorize Remazol Brilliant yellow GL and as to the other five dyes (Remazol Brilliant Blue R, Remazol Brilliant Orange 3 R, Reactive Blue 4, Remazol Brilliant Red F3B and Reactive Black 5), none of the basidiomycetes was able to decolorize them all. *Tremella encephala* did not decolorize any of the tested six colour dyes.

Eleven of the tested basidiomycetes, namely the white-rot fungi *P. radiata*, *D. squalens*, *P. rivulosus*, *C. unicolor*, *P. abieticola*, *P. velutina*, *T. pubescens* and *P. ostreatus*, the litter-decomposing fungus *A. praecox* and the brown-rot fungi *F. pinicola* and *P. placenta* formed colour zone in ABTS plates (Fig. 3) indicating production of extracellular oxidative enzymes, either peroxidases or laccases. The colour zone formation was not seen on ABTS containing plates with *T. encephala*, *G. trabeum* and *P. betulinus* indicating that these fungi did not produce extracellular oxidative enzymes. *Tremella encephala* is a fungal parasite and *G. trabeum* and *P. betulinus* are brown-rot fungi.

The ability of four ascomycetes, *Alternaria* sp., *Chaetomium* sp., *Epicoccum* sp. and *Fusarium* sp., was tested to decolorize Remazol Brilliant Blue R, Remazol Brilliant Yellow GL, Remazol Brilliant Orange 3 R, Reactive Blue 4, Remazol Brilliant Red F3B, Reactive Black 5 or the formation of colour zone with ABTS containing malt agar plates. The formation of colour zone was seen with *Alternaria* sp., *Epicoccum* sp. and *Fusarium* sp. on ABTS plates indicating production of extracellular oxidative enzyme(s). The formation of colour zone was not seen with *Chaetomium* sp. on ABTS plates. Decolorization was not seen with any of tested six colour dyes with *Alternaria* sp. Decolorization was seen with *Epicoccum* sp. and *Chaetomium* sp. on Reactive Black 5 dye plates. Decolorization was seen with *Fusarium* sp. on Reactive Black 5 dye plates.

Discussion

Our results show that anthraquinone dye Remazol Brilliant Blue R, azo dye Remazol Brilliant Orange 3 R, anthraquinone dye Reactive Blue 4, azo dye Remazol Brilliant Red F3B and azo dye Reactive Black 5 were decolorized by some of the 14 tested basidiomycetes.

None of the tested fungi was able to decolorize all the synthetic dyes in our experiments. Azo dye Remazol Brilliant Yellow GL was not decolorized by any of the 14 tested basidiomycetes. *P. ostreatus* decolorized well Drimarene Orange K-GL, Procion BluePX-5R and Cibacron Blue P-3RGR in liquid culture during 10 days except Remazol Brilliant Yellow (Asgher *et al.*, 2006). Our results and the results of Asgher *et al.* (2006) indicate that Remazol Brilliant Yellow GL is persistent in the environment.

Remazol Brilliant Orange 3 R, Reactive Blue 4 and Reactive Black 5 dye were decolorized by *D. squalens*, *P. rivulosus*, *P. velutina*, *A. praecox* and *T. pubescens* indicating that these fungi are potentially useful for biodegradation and bioremediation in multi contaminated soils. Our results showed that Remazol Brilliant Red F3B was decolorized by *P. radiata*, *P. rivulosus* and *T. pubescens* indicating that they could be useful in the treatment of soil or water contaminated by this dye. Earlier it was found that the white-rot fungi *Bjerkandera* sp., *Phanerochaete chrysosporium*, *T. hirsuta* and *T. versicolor* are able to decolorize Remazol Black B, Reactive Blue 15 and Remazol Brilliant Orange in solid plate during 10 days (Swamy and Ramzay, 1999).

Some of these dyes are commonly used as substrate in enzyme activity assays for different peroxidases. The azo dye Reactive Black 5 (RB5) is used to assay versatile peroxidase activity since this enzyme is able directly oxidize RB5 whereas lignin peroxidase requires the presence of redox mediator such as veratryl alcohol. Typical versatile peroxidases are produced by *Pleurotus eryngii* (Ruiz-Dueñas *et al.*, 2009). Another strain of *P. eryngii* F032 decolorizes Reactive Black 5 in liquid medium, while producing lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Hadibarata *et al.*, 2013), but versatile peroxidase activity was not determined. *D. squalens* is able to decolorize Orange G, Amaranth, Orange I, Remazol Brilliant Blue R (RBBR), Cu-phthalocyanin, Poly R-478, Malachite Green and Crystal Violet on agar plates (Eichlerova *et al.*, 2006). It was reported by Máximo *et al.* (2003) that *Geotrichum* sp. is able to decolorize Reactive Black 5, Reactive Red 158 and Reactive Yellow 27. However, the previously isolated *Geotrichum* sp. Dec-1, which was found to be an efficient dye decolorizing fungus, has been after several re-identifications confirmed to be a strain of the white-rot fungus *Bjerkandera adusta* (Gomi *et al.*, 2011). *B. adusta*, *Ganoderma* sp., *Irpex lacteus*, *Phanerochaete magnoliae*, *Rigidoporus* sp. and *Trametes versicolor* do not decolorize Reactive Red 158 and Reactive Yellow 27, but these fungi decolorize Remazol Brilliant Blue R (Máximo *et al.*, 2003), which in our experiments was decolorized by *P. pulmonarius*, *P. rivulosus*, *P. velutina* and *P. ostreatus*. *Pleurotus ostreatus* (Palmieri *et al.*,

2005) and *Trametes trogii* (Mechichi *et al.*, 2006) decolorize Remazol Brilliant Blue R with their laccases. *Trametes pubescens* decolorizes Reactive Red 243 (azo dye) and Remazol Brilliant Blue R (anthraquinone dye) (Casieri *et al.*, 2008). Our results and the results in literature (Swamy and Ramzay, 1999; Hadibarata *et al.*, 2013; Eichlerova *et al.*, 2006; Máximo *et al.*, 2003; Palmieri *et al.*, 2005; Mechichi *et al.*, 2006; Casieri *et al.*, 2008) show that Remazol Brilliant Blue R, Remazol Brilliant Orange 3 R, Reactive Blue 4, Remazol Brilliant Red F3B and Reactive Black 5 are all decolorized by at least some lignin degrading white-rot basidiomycetes. We conclude that Remazol Brilliant Yellow GL is persistent and can therefore cause harmful impacts in the environment.

Our results from ABTS plate tests indicate that the white-rot fungi *P. pulmonarius*, *P. radiata*, *D. squalens*, *P. rivulosus*, *C. unicolor*, *P. abieticola*, *P. velutina*, *T. pubescens*, *P. ostreatus*, the litter-decomposing fungus *A. praecox* and the brown-rot fungi *F. pinicola* and *P. placenta* produced laccase or other oxidative enzymes. *T. encephala*, *G. trabeum* and *P. betulinus* did not produce any extracellular oxidative enzyme (laccase) under our conditions. Recent comparisons of basidiomycete genomes show that the borderline between white-rot and brown-rot fungi is not as strict as previously thought and many brown-rotters such as *F. pinicola*, *P. placenta* and *G. trabeum* do have laccase genes in their genomes (Riley *et al.*, 2014). Laccase is produced by *D. squalensis* (Susla *et al.*, 2007), *P. rivulosus* (Hakala *et al.*, 2005), *T. pubescens* (Palmieri *et al.*, 2000), *F. pinicola* (Gramss *et al.*, 1999), *P. placenta* (Wei *et al.*, 2010), *P. pulmonarius* (De Souza *et al.*, 2004), *P. radiata* (Arora and Gill, 2001), *P. abieticola* (Koutrotsios and Zervakis, 2014), *P. velutina* (Boddy, 2000), *A. praecox* and *P. ostreatus* (Homolka *et al.*, 2006). Although the studied basidiomycetes *P. pulmonarius*, *P. radiata*, *D. squalens*, *P. rivulosus*, *C. unicolor*, *P. abieticola*, *P. velutina*, *A. praecox*, *T. pubescens*, *P. ostreatus*, *F. pinicola* and *P. placenta* produced laccase, they did not decolorize all studied dyes indicating that other enzymes than laccases were involved in decolorization of the tested dyes. Lignin degrading peroxidases (PODs, Class II peroxidases) produced the white-rot fungi have certainly an important role in the degradation of dyes, but it does not explain the decolorization caused by brown-rot fungi because the most common brown-rot fungi do not produce Class II peroxidases, since there are no POD encoding genes in their genomes (Riley *et al.*, 2014).

All fourteen tested basidiomycetes were able to grow in the presence of all tested six dyes and the redox indicator ABTS. The growth rate of *P. rivulosus* and *P. radiata* belonged to three highest and the growth rate of *A. praecox* to three lowest among the 14 tested

basidiomycetes on Remazol Brilliant Blue R, Remazol Brilliant Yellow GL, Remazol Brilliant Orange 3 R, Remazol Brilliant Red F3B, Reactive Black 5 and on the control malt extract plates without any added dyes. We have previously studied the effect of different metals on the growth of many fungi studied also in the present work. The growth of *A. praecox* was tolerant to Mn, Cr and Li (Hartikainen *et al.*, 2013). The growth of *P. radiata* was almost completely inhibited with 2 mg Co/kg (63%) and with 100 mg Li/kg (88%) (Hartikainen *et al.*, 2013). The growth of *P. rivulosus* was sensitive to Mn (50-400 mg kg⁻¹), Cr (20-100 mg kg⁻¹), Cd (5-10 mg kg⁻¹), Li (20-100 mg kg⁻¹) and Co (20 mg kg⁻¹) (Hartikainen *et al.*, 2013). The growth of *P. radiata* and *P. rivulosus* was sensitive to Al, Mo and W and tolerant to Zr. The growth was sensitive to Ga in the case of *P. radiata* and sensitive to V in the case of *P. rivulosus* (Kluczek-Turpeinen *et al.*, 2014). The growth of the tested ascomycete *Chaetomium* sp. was sensitive to Cr, Co and Li and *Alternaria* sp. to Cd (Hartikainen *et al.*, 2013). The growth of the ascomycetes *Alternaria* sp. and *Fusarium* sp., was sensitive to six metals (Al, Mo, V, Zr, W, Ga) (Kluczek-Turpeinen *et al.*, 2014). Our results showed that *P. radiata* and *P. rivulosus* were fast growing, but the growth of these two fungi is sensitive to several metals (Hartikainen *et al.*, 2013; Kluczek-Turpeinen *et al.*, 2014) indicating that their ability to grow is vulnerable in the metal contaminated waste or soil. Our results indicate that *P. rivulosus* and *P. radiata* are tolerant and suitable for the fast bioremediation of the dyes; namely Remazol Brilliant Blue R, Remazol Brilliant Yellow GL, Remazol Brilliant Orange 3 R, Remazol Brilliant Red F3B, Reactive Black 5 and malt agar plates. The slow growth rate of *A. praecox* was not useful for efficient decolorization of the dyes. The growth rate of *Chaetomium* sp. was the highest among tested ascomycetes on all tested plates indicating that it was tolerant to all tested dyes. Basidiomycetes were generally more efficient to decolorize dyes than ascomycetes.

Conclusion

All fourteen selected basidiomycetous fungi *P. radiata*, *T. encephala*, *D. squalens*, *P. rivulosus*, *C. unicolor*, *P. abieticola*, *P. velutina*, *A. praecox*, *T. pubescens*, *P. ostreatus*, *F. pinicola*, *P. placenta*, *G. trabeum* and *P. betulinus* grew in the presence of all six selected synthetic dyes and ABTS in the plates. The growth rate of *P. rivulosus* belonged to three highest among the tested basidiomycetes with five dyes, Remazol Brilliant Blue R, Remazol Brilliant Orange 3 R, Reactive Blue 4, Remazol Brilliant Red F3B and Reactive Black 5. The growth rate of *Chaetomium* sp. was the highest among four ascomycetes on all tested plates. The fast growth rate is beneficial in

bioremediation of synthetic dye contaminated environment. Remazol Brilliant Blue R, Remazol Brilliant Orange 3 R, Reactive Blue 4, Remazol Brilliant Red F3B and Reactive Black 5 were decolorized with 14 tested basidiomycetes. None of tested fungi was able to decolorize all tested synthetic dyes indicating that abilities of several fungi are needed in the bioremediation of multi contaminated sites. Remazol Brilliant Yellow GL was not decolorized by any of 14 tested basidiomycetes indicating that it is a very persistent dye in the environment.

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Author's Contributions

E. Samuel Hartikainen: Designed and organized the study, collected the data, performed analysis of the data and wrote the manuscript.

Otto Miettinen: Gave expert opinions concerning fungi.

Annele Hatakka: Gave expert opinions and provided facilities to perform this study.

Mika A. Kähkönen: Designed and organized the study, performed analysis of the data and wrote the manuscript and supervisor in the study.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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