IMPACTS OF HOLMIUM AND LITHIUM TO THE GROWTH OF SELECTED BASIDIOMYCETOUS FUNGI AND THEIR ABILITY TO DEGRADE TEXTILE DYES

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ABSTRACT

The impacts of Ho and Li (0, 10, 50, 200 mg/L) were tested towards the growth of four basidiomycetous fungal species, their ability to decolorise synthetic dyes (Reactive Green 19, Reactive Orange 16, Reactive Black 5), and produce oxidative enzymes. All species; *Agrocybe dura*, *Skeletocutis biguttulata*, *Exidia saccharina* and *Galerina paludosa;* grew with and without supplemented Ho or Li. The growth of *S. biguttulata* was the most tolerant species towards Ho or Li (200 mg/ L), whereas the growth of *G. paludosa* was the most sensitive of the studied species to both 200 mg Ho or Li/ L. All fungi oxidized ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) forming colour zone on a plate tests indicating production of lignin modifying laccase enzyme. *A. dura* and *G. paludosa*, formed black MnO_2 zone in Mn^{2+} plates, which indicates production of manganese peroxidase (MnP). *A. dura* and *G. paludosa* decolorised Reactive Black 5 indicating production of versatile peroxide (VP) enzyme. Our study presents two new candidate species able to produce VP. *A. dura* was capable of decolorising all tested synthetic dyes in the presence of Ho or Li (0 – 200 mg/ L) suggesting that this fungus is a promising species for bioremediation of multi dye containing wastes.

Key words: metals, fungi, enzyme, decolorisation

INTRODUCTION

Harmful metal waste is produced at all phases of products life cycle from production to consumption and after the product has been discarded to landfills or wastewaters. Despite recycling of metals, only proportion is completely separated from the original product and re-used, therefore metals are released to the environment. Metals, lithium (Li) and holmium (Ho), are used in various products, but only a little is known about their environmental fate and impacts on fungal communities. Li has been used in batteries (Bandhauer et al. 2011), lubricant greases, glass, polymers, nonlinear optics, various heat-transfer products and they have been added to strengthen alloys (Tarascon 2010). Ho is a rare element, which has been used in lasers (Mains et al. 2015) and improves corrosion resistance (Zhou et al. 2006). Ho and Li content in the soil have been reported 0.16 – 0.94 mg/ kg and 4 – 200 mg/ kg, respectively (Berger et al. 2014; Tyler Olsson 2001; Giannaccini et al. 2012).

Metals have been reported to cause deleterious impacts to the ability of fungi to degrade recalcitrant synthetic dyes by disturbing their physiology or inhibiting their enzyme activities (Gadd 2010; Hartikainen et al. 2013, Kähkönen et al. 2017). Synthetic dyes, which are used in large scale in production of textiles, paper products, end up in the environment at the different life stage (Hazrat 2010). Chemical structure of synthetic dyes has often aromatic structure (Hazrat 2010), which makes them recalsitrant compounds in the environment.

As the treatment of dye containing wastewater is still inefficient and expensive, decolorisation by microbes and their enzymes is a promising and eco-friendly alternative. Especially the use of fungal enzymes have been considered to be suitable for purification of waste waters and bioremediation of

contaminated soils (Kues 2015). Lignin degrading white-rot basidiomycetous fungi produce extracellular oxidoreductive enzymes, i.e. laccase and class II heme peroxidases, which are able to degrade recalcitrant aromatic structure containing compounds such as lignin (Hatakka and Hammel 2010). Decolorisation of aromatic textile dyes can been seen as indicator for the vitality of fungi. Fungi have ability to naturally attenuate impacts of harmful xenobiotic compounds and do intrinsic bioremediation in the soil. Wastewaters and contaminated soils can contain various xenobiotics including synthetic dyes and metals. The aim of the study was to test the ability of four basidiomycetous fungi to degrade and decolorise synthetic dyes; namely Reactive Green 19 (RG19), Reactive Orange 16 (RO16) and Reactive Black 5 (RB5); in the presence of Ho and Li.

MATERIALS AND METHODS

Fungal species

All fungal species used in this study are maintained in the fungal biotechnology culture collection (FBCC) at the University of Helsinki, Department of Microbiology, Finland. Three of isolates have not been studied previously. These selected fungi were following. Jelly fungus *Exidia saccharina* (FBCC 2434, *Auriculariales*, Basidiomycota) causes white-rot of conifer logs. It is one of the first wood-decay fungi to appear on recently fallen, still hard logs. *Skeletocutis biguttulata* (FBCC 2433, *Incrustoporiaceae*, *Polyporales*, Basidiomycota) is a white-rot fungus of conifers, though it grows and fruits on trunks that are more degraded, being evidently a strong competitor. *Galerina paludosa* (FBCC 2435, *Agaricales*, Basidiomycota) is a soil-inhabiting saprotroph found in *Sphagnum* swamps, where it evidently degrades moss remnants. Previously studied *Agrocybe dura* (FBCC 478, *Agaricales*, Basidiomycota) is soil-inhabiting decomposers forming mushroom-like fruiting bodies.

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103 Indicator color plate tests

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105 Indicator plates were used to test the impacts of metals (0, 10, 50 and 200 mg Ho/ L and 0, 10, 50 and
106 200 mg Li/ L) to the growth of fungi and decolorisation of synthetic dyes (250 mg/ kg of Reactive
107 Black 5 (RB5), Reactive Orange 16 (RO16), or Reactive Green 19 (RG19), Sigma-Aldrich, U.S.A.).
108 Metals were $HoCl_3$ * 6 H_2O (Sigma-Aldrich, U.S.A.) and LiCl (Sigma-Aldrich, U.S.A.). All plates
109 contained glucose 1.0 g/ L, KH_2PO_4 2.0 g/ L, $MgSO_4 \cdot 7\ H_2O$, 0.26 g/ L $CaCl_2$, 1.0 g/ L ammonium
110 tartrate, 3.56 g/ L succinic acid, 0.4 g/ L yeast extract and 25 g/ L agar. The pH was adjusted to 5.5
111 prior to autoclavation. Ability of fungi to produce oxidative enzymes were tested in indicator agar
112 plates containing 250 mg/ L 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS; Sigma-
113 Aldrich, USA) or 250 mg Mn/ L as $MnCl_2$.

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115 The fungi were precultured on 2% malt-extract plates at 25 °C for 8 days. Agar plug (4 mm
116 diameter) from the precultivation was aseptically inoculated to the center of test plates, which were
117 incubated at 25 °C. The diameters of the growth of fungi, formation of the colour zone around the
118 fungal mycelia in ABTS and Mn-plates, and decolorisation of synthetic dyes were measured
119 between 90° angels in each plates. The average was calculated from these four measurements for
120 each plate. The dark-green zone in ABTS plates indicates production of oxidative enzymes (such as
121 laccase, EC 1.10.3.2). Dark-brown flecks of MnO_2 in plates indicate manganese peroxidase (MnP
122 EC 1.11.1.14) activity. Decolorisation of Reactive Black 5 (RB5) has been used to detect versatile
123 peroxidase (VP, EC 1.11.1.16) activity, which has similar enzyme catalytic abilities as MnP and
124 lignin peroxidases (LiP, EC 1.11.1.14). All tests were performed in triplicates.

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127 Statistical tests

128 ANOVA test was performed to find out statistical difference ($p < 0.05$) between the growth, the
129 formed colour zone and decolorisation zone with certain fungus with added Ho or Li compared to
130 those without added metal. Tukey test was done as s post-hoc test. All statistical tests were performed
131 with SPSS Statistics software (IBMM).

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134 RESULTS AND DISCUSSION

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136 The fungal ability to grow in the presence of Ho or Li (10, 50 and 200 mg/ L) was tested in ABTS
137 containing indicator plates (Figure 1A, B). All tested fungi; *A. dura*, *E. saccharina*, *G. paludosa* and
138 *S. biguttulata*; grew in the presence of Ho and Li (10-200 mg/ L) and without added metals. The
139 growth of *G. paludosa* was reduced 27 % less with 200 mg Ho/ L compared to the control without
140 added Ho. The growth of three other tested fungi with 200 mg Ho/ L as well as *E. saccharina*, *G.*
141 *paludosa* with 10 – 50 mg Ho/ L remained stable or even increased compared to the control
142 cultivation. In the presence of Li (200 mg/ L), the growth of *G. paludosa* and *E. saccharina* was
143 reduced by 89 – 96 % less and *A. dura* grew 46 % less compared to the control cultivation. *S.*
144 *biguttulata* grew similarly or even more with 200 mg Li/ L compared to the control. *S. biguttulata*
145 grew similarly with 200 mg Li/ L compared to the control. Previously, it has been shown that
146 basidiomycetous litter decomposing fungus *Agrocybe praecox* and the wood rotting fungus *Pleurotus*
147 *pulmonarius* tolerate Li up to 100 mg/ L (Hartikainen et al. 2013). The growth of the related white
148 rot species *Pleurotus ostreatus* tolerated Li 1.65 g/ L (Nunes et al. 2014) Our results show that *S.*
149 *biguttulata* tolerates both tested metals indicating that fungus is therefore suitable for bioremediation
150 of xenobiotics in the Ho and Li contaminated soil.

In addition to the fungal growth, the ability to oxidize ABTS was determined by measuring the color forming zone around the fungal mycelium in ABTS indicator plates supplemented with Ho or Li (10, 50 and 200 mg/ L) and without added metals (Figure 1C, D). In all cultivation conditions, the tested fungal species were able to oxidized ABTS to green ABTS cation radical, which indicates extracellular oxidoreductase such as laccase activity. The formation of colour zone with *A. dura* decreased (65 – 70 %) with 10-200 mg Ho/ L. *A. dura* was the most sensitive to Ho among tested fungi. *S. biguttulata*, *E. saccharina* and *G. paludosa* formed colour zone in the ABTS plates similarly in the presence of 10-200 mg Ho/ L and without metal indicating that these fungi were tolerant to Ho. All tested fungi formed 11-96 % smaller colour zone in the presence of 200 mg Li/ L than without metal. However, the decrease was less than 10 % in the presence of 10 mg Li/ L with *A. dura*, *G. paludosa* and *S. biguttulata* indicating that Li is not toxic to these species at low concentrations. It is shown earlier that fungal tolerance towards Li varies. Based on the ABTS indicator plates *A. praecox* is tolerant to Li (up to 100 mg/ L), whereas *P. pulmonarius* and *P. radiata* are sensitive to Li (50 – 200 mg/ L and 200 mg/ L, respectively) (Hartikainen et al. 2013). Our results comparison indicates that the production of oxidative laccase enzyme by *S. biguttulata*, *E. saccharina* and *G. paludosa* was more vulnerable to Li than Ho.

Mn^{2+} oxidation, which indicates MnP activity, was followed by formation of brown zone of MnO_2. In the Mn containing plates supplemented with Ho or Li (10, 50 and 200 mg/ L) and without added metal (Figure 1 E, F) *A. dura* and *E. saccharina* formed 45 – 100 % smaller colour zone in the presence of 200 mg/ L Li or Ho than without added metals. Previously, it has been showed MnP activity decreased 50 % *in vitro* in the presence of 26 mg Cd l^{-1} and 13 mg Fe l^{-1} compared to those without added metal by a white-rot wood decaying fungus *Lentinula edodes* (Hatvani and Mees 2003). This is in line with our finding that MnP activity is sensitive to metals. The Mn^{2+} oxidation of

176 *A. dura* has been reported previously, but it was not in the presence of added metals (Steffen et al.
177 2000). Ligninolytic enzyme activities of three other tested species have not studied before. *S.*
178 *biguttulata* did not form the colour zone in Mn plates with or without added metals suggesting that
179 the species did not produce MnP even though it causes white rot type decay. Other tested white rot
180 causing fungi; *A. dura*, *G. paludosa* and *E. saccharina*; oxidized Mn^{2+}, which indicates MnP activity.
181
182 The ability of four selected basidiomycetous fungi to decolorise RB5 were tested in the plates
183 supplemented with Ho or Li (10, 50 and 200 mg/ L) and without added metal (Figure 2 A, B). Azo
184 dye RB5 is used to measure VP activity, which is able to directly oxidize RB5, when LiP needs redox
185 mediator for example veratyl alcohol (Hatakka and Hammel 2010). VP is unspecific enzyme, which
186 catalyses degradation of various xenobiotic compounds. The decolorisation zone of RB5 by *G.*
187 *paludosa* decreased 17 – 31 % and by *A. dura* decreased 55 – 86 % in the presence of 10 – 200 mg
188 Ho/ L indicating that the enzymatic decolorisation of RB5 was sensitive to Ho. *E. saccharina* and *S.*
189 *biguttulata* did not decolorise RB5 plates at any tested conditions (0 – 200 mg Ho or Li/ L) indicating
190 that these fungi did not produce VP. RB5 decolorisation has been detected in white-rot fungal species
191 *Bjerkandera adusta* (Mohorčič et al. 2004) and *Funalia trogii* (Chulhwan et al. 2007). The present
192 study is first to show that fungi, *A. dura* and *G. paludosa*, decolorise RB5 indicating production of
193 VP enzyme. Since VPs have been described only in some white-rot species, mainly in the genera
194 *Pleurotus* and *Bjerkandera* (Hatakka and Hammel 2010), our study presents new candidate species,
195 *G. paludosa* and *A. dura*, able to produce VP.
196
197 Decolorisation of other tested synthetic dyes, namely RG19 and RO16, were tested with four
198 basidiomycetous fungi in the plates supplemented with Ho or Li (10, 50 and 200 mg/ L) and without
199 added metal (Figure 2 C-F). *A. dura* and *G. paludosa* decolorized RG19 in the presence of Ho and Li
200 even at the highest concentrations (10 – 200 mg/ L). The decolorisation of RG19 decreased 41 – 82

% with *A. dura* in the presence of 10 – 200 mg Ho/ L and 43 % with *G. paludosa* in the presence of 200 mg Ho/ L. The decolorisation of RG19 decreased 12 – 60 % with *A. dura* and 52 – 100 % with *G. paludosa* in the presence of 10 – 200 mg Li/ L. Our results show that *A. dura* and *G. paludosa* were sensitive to both Ho and Li. *E. saccharina* and *S. biguttulata* did not decolorise RG19, RO16 or RB5 with or without Ho and Li indicating that these compounds were recalcitrant to these fungi. Previously, it has reported that RG19, which is the same as in our study, was decolorized by white-rot species *Cordyceps militaris* MTCC3936 (Kaur et al. 2015), white rot fungal species *Trametes versicolor* (Sari et al. 2016) and saline-pH tolerant fungus *Pestalotiopsis* sp. strain NG007 (Yanto et al. 2014). The white-rot fungi *Irpex lacteus* (Svobodova et al. 2007) and *Ganoderma* sp in simulated textile wastewater (Ma et al. 2014) decolorized RO16, which is the same dye as in our study.

Based on our indicator plate tests, litter decomposing species *A. dura* decolorised all three tested synthetic dyes i.e. R19, RO16 and RB5 and formed colour zone in ABTS and MnP plates indicating production of oxidative enzymes (laccase, MnP and VP). However, the growth of *A. dura* was inhibited by Li. In addition, Ho and Li inhibited laccase, MnP and VP activity of *A. dura*. The results indicate that the *A. dura* was sensitive to Ho and Li. In spite of sensitivity to added metals, *A. dura* had wide ability to degrade synthetic recalcitrant dyes and therefore fungi is suitable for bioremediation of multi dye contaminated wastes.

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226 On behalf of all authors, the corresponding author states that there is no conflict of interest.

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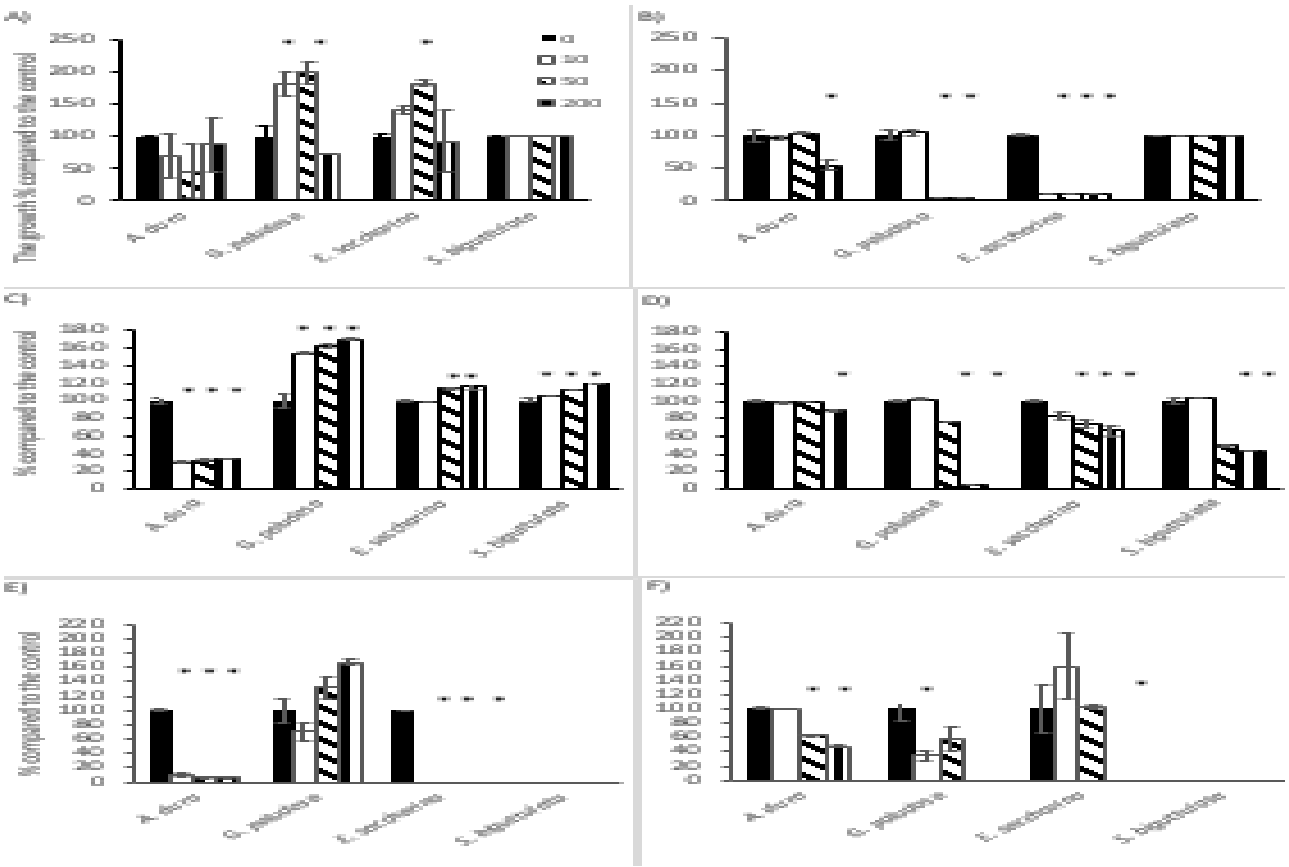

Figure 1. The growth of four selected basidiomycetous fungi in the presence of Ho (A.) or Li (B.) on ABTS indicator plates, formation of colour zone on ABTS plates in the presence of Ho (C.) or Li (D.) and formation of colour zone on Mn plate in the presence of Ho (E.) or Li (F.). Fungi were *A. dura*, *S. biguttulata*, *E. saccharina* and *G. paludosa*. Tested Ho or Li concentrations were 0, 10, 50 and 200 mg/ L.

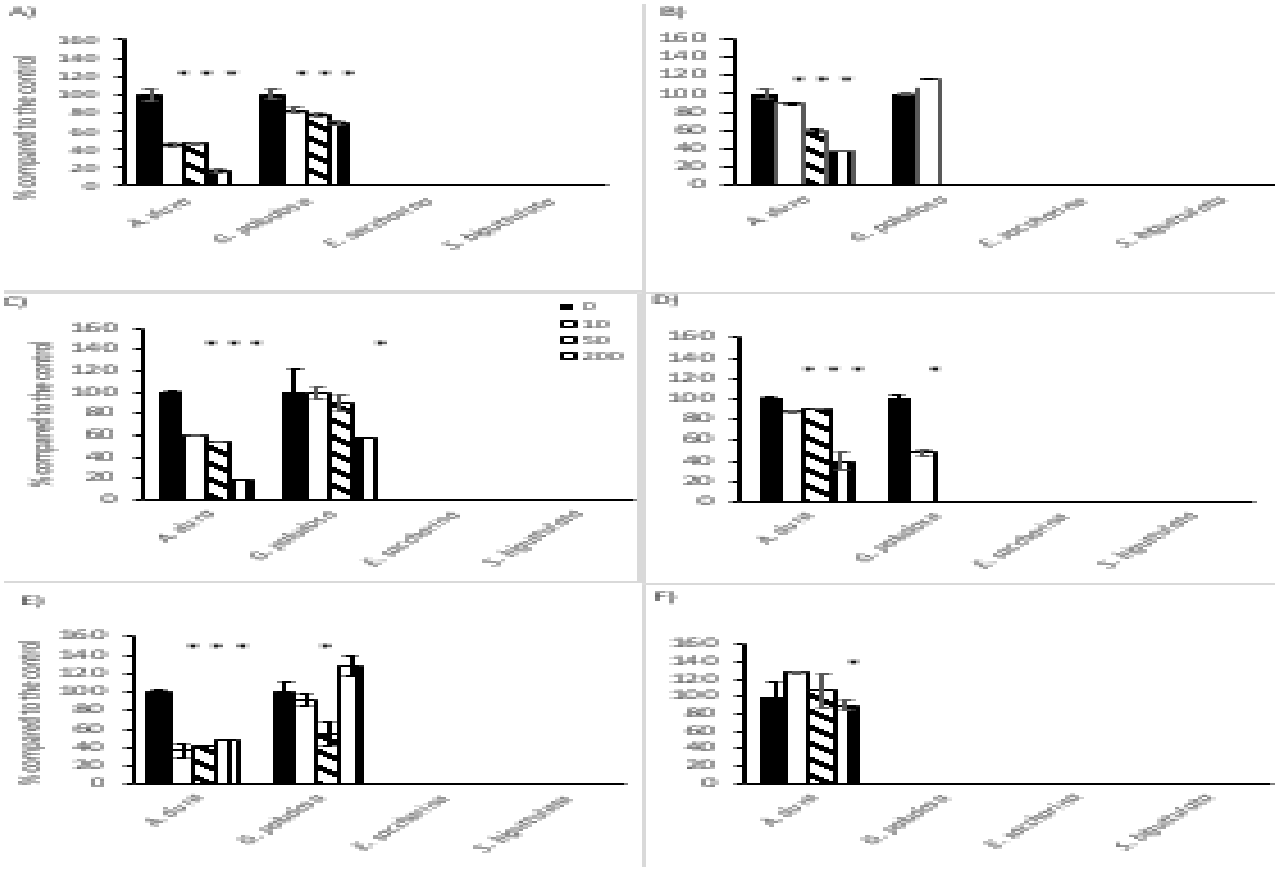

Figure 2. The ability of four selected basidiomycetous fungi to decolorise Reactive Black 5 (RB5) in the precence of Ho (A.) or Li (B.), Reactive Green 19 (RG19) in the presence of Ho (C.) or Li (D.) and Reactive Orange 16 (RO16) in the presence of Ho (E.) or Li (F.). Fungi were *A. dura*, *S. biguttulata*, *E. saccharina* and *G. paludosa*. Tested Ho or Li concentrations were 0, 10, 50 and 200 mg/ L.