



## Effects of Gadolinium and Tin to the Production of Oxidative Enzymes and the Growth of Five Basidiomycetous Fungi

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### Abstract

Effects of gadolinium (Gd) and tin (Sn) on the growth and production of oxidative enzymes with five basidiomycetous fungi were tested. For this study we have selected well-known white-rot fungi *Obba rivulosa* and *Kuehneromyces mutabilis*, in addition to this we have tested three new isolates, the white-rot fungus *Phlebia subochracea*, the litter-degrading fungus *Gymnopus dryophilus* and the brown-rot fungus *Heliocybe sulcata*. This approach allowed us to find possible new sources for oxidative enzymes, such as laccases and versatile peroxidases (VPs). All five tested fungi grew in the presence of Gd (0-200 mg/l) or Sn (0-200 mg/l) on ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) containing plates. The growth rate of *H. sulcata* was tolerant to Gd and Sn (0-200 mg/l). The growth rates of *P. subochracea* and *G. dryophilus* were sensitive to Gd (5-200 mg/l) and Sn (5-200 mg/l). *O. rivulosa*, *K. mutabilis*, *P. subochracea* and *G. dryophilus* formed colour zones on the ABTS plates indicating that these fungi produced oxidative enzymes, most probably laccases. The brown-rot fungus *H. sulcata* did not form colour zone on the ABTS plate indicating that this fungus did not produce laccase. The production of laccase with *G. dryophilus* and *K. mutabilis* was tolerant to Gd (0-200 mg/l) and Sn (0-200 mg/l). The production of laccase with *P. subochracea* was sensitive to Gd (5-200 mg/l) and Sn (5-200 mg/l). *P. subochracea* decolorized the dye Reactive Black 5 without or with Gd and Sn (0-200 mg/l) indicating the production of VP. *O. rivulosa*, *K. mutabilis*, *G. dryophilus* and *H. sulcata* did not produce VP. The production of VP by *P. subochracea* was sensitive to 200 mg/l Gd and Sn.

### Keywords

Gadolinium; Tin; Basidiomycetous fungi; Oxidative enzyme; Laccase; Versatile peroxidase

### Introduction

Harmful xenobiotics are released from various sources to the soil and water. Contaminated soil and water need new technologies so that these are possible to remediate to clean and safe. All white-rot fungi are able to produce oxidative lignin modifying enzymes, such as laccases and versatile peroxidases (VPs) [1-3]. These two

oxidative enzymes are suitable for remediation of harmful xenobiotic compounds, particularly aromatic structure containing compounds in contaminated environment and recycling of carbon from natural compounds [3-6]. VP typically catalyzes oxidation reaction, where an aromatic structure containing dye compound, Reactive Black 5, in the presence of H<sub>2</sub>O<sub>2</sub> is transformed to the oxidized (bleached) form of Reactive Black 5 and water [7,8]. VP has properties of both manganese peroxidase and lignin peroxidases and has non-specific substrate specificity [9-11]. VPs are biotechnologically interesting since they do not need a mediator for the oxidation of substrate [2,10]. Laccases have broad substrate specificity and they are useful in many biotechnological applications [2]. Basidiomycetous fungi are able to decolorize several synthetic dyes [2]. Brown-rot fungi produce hydrolytic enzymes, which degrade cellulose and hemicellulose, but usually do not produce laccase or ligninolytic peroxidases [2]. Contaminated areas are often polluted with organic xenobiotic compounds and metals. Rare earth elements include the lanthanide gadolinium (Gd). The mean abundance of Gd is 5.4 (2.2-7.9) µg g<sup>-1</sup> dw at 30 sites in A-horizon in Swedish forest soil [13]. Gd containing compounds have been used as paramagnetic contrast agents for people who need magnetic resonance imaging [14,15]. Gd has been found in the waste water [14,15] and in contaminated soil [2]. Tin (Sn) is present in waste water effluents and contaminated soils [17] and sediments [18]. Sn is a post-transition metal, which belongs to group 14 in the periodic table. The white rot fungi *Obba rivulosa* (= *Physisporinus rivulosus*, [19]) and *Kuehneromyces mutabilis* [20,21] were selected for this study. New isolates the white-rot fungus *Phlebia subochracea*, the litter-degrading fungus *Gymnopus dryophilus* and the brown-rot fungus *Heliocybe sulcata* were selected for this study to find possible new sources of oxidative enzymes, namely laccases and versatile peroxidases. Only a little is known about impacts of gadolinium and tin on any basidiomycetous fungi. The aim this study of was to find out effects of gadolinium (Gd) and tin (Sn) to the growth rate and production of extracellular oxidative laccase enzyme of basidiomycetous fungi.

### Materials and Methods

For this study five functionally different basidiomycetous fungi were selected from the Fungal Biotechnology Culture Collection (FBCC) at the University of Helsinki, Department of Food and Environmental Sciences, Microbiology and Biotechnology Division. The fungi were *Kuehneromyces mutabilis* (Agaricales, FBCC 508) and *Obba rivulosa* (= *Ceriporiopsis rivulosa*, *Physisporinus rivulosus*, Polyporales, FBCC 939). The new isolates of fungi, which were studied first time in the present study, were *Phlebia subochracea* (Polyporales), OM 19353, FBCC 2376, *Gymnopus dryophilus* (Agaricales), OM 19240, FBCC 2376 and *Heliocybe sulcata* (Gloeophyllales), OMC 1185, FBCC 2375. The draft genome of *H. sulcata* has been produced by Joint Genome Institute, CA, USA.

### Indicator plate tests

Effects of selected metals on the production of extracellular oxidative enzymes and the growth of six basidiomycetous fungi were tested with gadolinium (0, 5, 50, 200 mg Gd kg<sup>-1</sup>) and tin (0, 5, 50, 200 mg Sn kg<sup>-1</sup>) in the form of chlorides on the indicator plates. The test chemicals were GdCl<sub>3</sub> · 6 H<sub>2</sub>O (Sigma-Aldrich, U.S.A.) and

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$\text{SnCl}_4 \cdot 5 \text{H}_2\text{O}$  (Sigma-Aldrich, U.S.A.). The basic test medium had 10 g glucose, 2 g  $\text{KH}_2\text{PO}_4$ , 0,5 g  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ , 0,1 g  $\text{CaCl}_2$ , 0,5 g  $\text{NH}_4$ -tartrate, 2,2 dimethylsuccinate, 0,1 g yeast extract and 25 g agar per litre. The pH was adjusted to 5.0. The medium for indicator plates was supplemented with 250 mg  $\text{kg}^{-1}$  ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid, Sigma-Aldrich, U.S.A.) or 250 mg  $\text{kg}^{-1}$  RB5 (Reactive Black 5, Sigma-Aldrich, U.S.A.). All indicator plates were done as triplicate. For inoculum, a fungus-containing malt (2% w/v) agar plug, which had a 4 mm diameter, was added to the center of the indicator plate. The plates were incubated at 25.0 °C. The formed green colour in the ABTS containing plate indicated the production of oxidative enzyme, most probably laccase. The growth and the colour changes were measured at four positions across the plate at 90° angles, and averages of these four measurements per plate were calculated.

### Statistical tests

ANOVA was performed to test statistical difference between the growth rate and the formation of colour zone with certain fungi in the presence of added Sn or Gd compared to those without added metal. Tukey test was performed as a post-hoc test. Statistical tests were done with SPSS Statistics software (IBM).

### Results

Figure 1A displays the growth of five basidiomycetous fungi with and without tin (Sn). All five fungi grew in the presence of Sn. The growth rate of fungi without added metal decreased in the following order, which was *O. rivulosa*, *H. sulcata*, *P. subochracea*, *K. mutabilis* and *G. dryophilus*. The growth rate of *K. mutabilis* and *O. rivulosa* remained stable with 5-50 mg/l and decreased with 200 mg Sn/l compared to the control indicating that *K. mutabilis* and *O. rivulosa* were tolerant to Sn in the lower concentrations. The growth rate of *P. subochracea* and *G. dryophilus* decreased with 5-200 mg/l indicating that *P. subochracea* and *G. dryophilus* were sensitive to Sn. The growth rate of *H. sulcata* remained similar or even increased in the presence of Sn (5-200 mg/l) compared to the control indicating that *H. sulcata* also benefitted from the Sn.

Figure 1B displays the growth of five basidiomycetous fungi in the presence of gadolinium (Gd). All five fungi grew in the presence of Gd. The growth rate of *K. mutabilis* and *O. rivulosa* remained stable with 5-50 mg Gd/l and decreased in the presence of 200 mg Gd/l indicating that these fungi were sensitive to higher Gd concentrations. The growth of *H. sulcata* remained similar or even increased in the presence of 5-200 mg Gd/l indicating that *H. sulcata* was tolerant to Gd. The growth of *P. subochracea* and *G. dryophilus* decreased in the presence of 5-200 mg Gd/l indicating that *P. subochracea* and *G. dryophilus* were sensitive to Gd.

Figure 2A displays the formation of colour zone in ABTS containing plates in the presence of tin (Sn). *K. mutabilis*, *O. rivulosa*, *P. subochracea* and *G. dryophilus* formed colour zone in the ABTS containing plates in the presence of Sn indicating production of oxidative enzyme, most probably laccase. *H. sulcata* did not form colour zone indicating that *H. sulcata* does not produce any oxidative enzymes. The formation rate of colour zone with *G. dryophilus* increased with 5-200 mg Sn/l indicating that the production of laccase enzyme with *G. dryophilus* benefitted from Sn. The formation rate of colour zone with *P. subochracea* decreased in the presence of 5-200 mg Sn/l indicating that Sn was harmful to *P. subochracea*. The formation rate of colour zone remained stable or even increased

with *K. mutabilis* in the presence of 5-200 mg Sn/l indicating that *K. mutabilis* was tolerant to Sn. The formation rate of colour zone with *O. rivulosa* in the presence of 200 mg Sn/l decreased indicating that *O. rivulosa* was sensitive to Sn.

Figure 2B displays the formation of colour zone in ABTS containing plates in the presence of gadolinium (Gd). The formation of colour zone in ABTS plate indicates the production of oxidative laccase enzyme. *K. mutabilis*, *O. rivulosa*, *P. subochracea*, and *G. dryophilus* formed colour zone in the ABTS containing plates indicating production of laccase type activity. The production rate of colour zone decreased with *P. subochracea* in the presence of 5-200 mg Gd/l indicating that *P. subochracea* is sensitive to Gd. The production rate of colour zone even increased with *G. dryophilus* in the presence of 5-200 mg Gd/l indicating that *G. dryophilus* even benefitted from Gd. The production rate of colour zone with 200 mg Gd/l remained stable with *K. mutabilis* and *O. rivulosa* indicating that these fungi are tolerant to Gd.

The decolorization rate of Reactive Black 5 (RB5) in the presence of tin (Sn) or gadolinium (Gd) was studied. *P. subochracea* decolorized RB5 in the presence of Sn or Gd or without added metal indicating that this fungus produced versatile peroxidase (VP) activity. The other four fungi *K. mutabilis*, *O. rivulosa*, *G. dryophilus* and *H. sulcata*, did not decolorize RB5 indicating that these fungi did not produce VP. The production rate of VP with *P. subochracea* remained similar with 5-50 mg/l Sn and Gd and decreased 27% with 200 mg Sn/l and 9 % with 200 mg Gd/l indicating that the production rate by *P. subochracea* was sensitive to higher Sn and Gd concentrations.

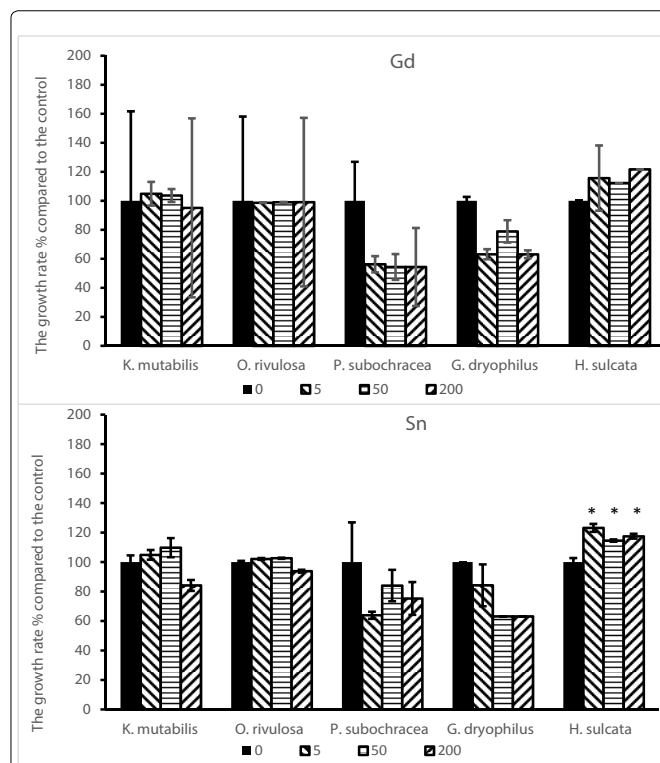
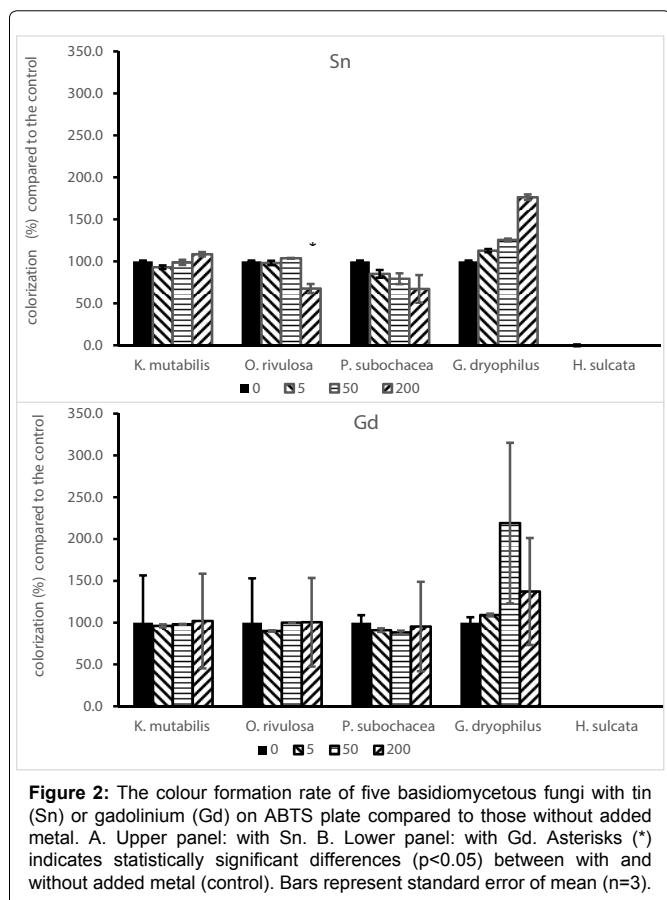


Figure 1: The growth rate of five basidiomycetous fungi with tin (Sn) or gadolinium (Gd) compared to those without added metal. A. Lower panel: with Sn. B. Upper panel: with Gd. Asterisks (\*) indicates statistically significant differences ( $p < 0.05$ ) between with and without added metal (control). Bars represent standard error of mean ( $n=3$ ).



## Discussion

All five tested fungi grew in the presence of Sn and Gd. Our study is the first to test impacts of Sn and Gd on the growth rate of the brown-rot fungus *H. sulcata*, and it was found to be tolerant to Sn and Gd. The growth rates of the white-rot fungi *K. mutabilis* and *P. rivulosus* were tolerant to Sn and Gd with 5-50 mg/l and sensitive in the higher Sn and Gd concentrations (200 mg/l) indicating that these fungi are suitable for bioremediation of the low level Sn or Gd contaminated soil. It was earlier reported that *K. mutabilis* grows less than 30% in the presence of ZnO or Cu<sub>2</sub>O at concentration of 1 g/l in the basic agar medium compared to those without added metal [21,22]. Thus high ZnO or Cu<sub>2</sub>O concentrations seem to inhibit the growth of *K. mutabilis*, as was seen in the present study with 200 mg/l Sn and Gd concentrations. Impacts of different metals greatly vary on the growth rate of *P. rivulosus*. The growth of *P. rivulosus* was vulnerable in the presence of Cd (5-10 mg kg<sup>-1</sup>), Co (20 mg kg<sup>-1</sup>), Cr (20-100 mg kg<sup>-1</sup>), Li (20-100 mg kg<sup>-1</sup>) [10] and of Al (50-200 mg kg<sup>-1</sup>), Mo (10-50 mg kg<sup>-1</sup>) and V (20 mg kg<sup>-1</sup>) [23]. The growth of *P. rivulosus* was tolerant to Zr (0-50 mg kg<sup>-1</sup>) and Ga (20-100 mg kg<sup>-1</sup>) [23]. Our study is the first test on the growth rates of the white-rot fungus *P. subochracea* and the litter-degrading fungus *G. dryophilus* along with their sensitivity to Sn and Gd. The growth rates of these two fungi, *P. subochracea* and *G. dryophilus*, were most sensitive to Sn and Gd, indicating that these fungi are not suitable for bioremediation in the contaminated soil.

Four of the five tested fungi, *K. mutabilis*, *O. rivulosa*, *P. subochracea* and *G. dryophilus*, showed formation of the colour zone

in the ABTS containing plates indicating the production of oxidative enzymes, namely laccases and peroxidases. The production of laccase has been reported by *P. rivulosus* [24], *K. mutabilis* [25] and other isolate of *G. dryophilus* (= *Collybia dryophila*, K209) and tested in the present study [26]. The white-rot fungus *P. rivulosus* showed colour zone on ABTS plates [12] with Al, W, Ga, Zr, Mo and V and without metals [23] as was observed also in the present study. *P. rivulosus* was sensitive to Al, Mo, V, W and Zr and tolerant to Ga [23]. Ten of eighteen tested *Phlebia* species including *Phlebia subochracea* HHB8494 were able to degrade over 50% organochloride pesticide heptachlor [27]. Our study shows formation of the colour zone in the ABTS containing plates indicating production of laccase type activity by *P. subochracea* OM19353 and *G. dryophilus* OM19240. *H. sulcata* did not form colour zone in ABTS plates indicating that it does not produce oxidative enzymes such as laccases and peroxidases, which indicates that *H. sulcata* is a typical brown-rot fungus. The brown-rot fungi do not usually produce laccases or lignin modifying peroxidases [2], although there are some exceptions. Along with this gene encoding laccases have been found in the whole genomes of some brown-rot fungi [28].

Reactive Black 5 (RB5) is a specific substrate to test the activity of versatile peroxidase (VP), which oxidizes directly RB5 without mediators, and it can be seen as decolorization of RB5. This dye has been used to screen VP activity in a number of lignin degrading fungi [29]. Lignin peroxidase needs a redox mediator to oxidize this compound [2]. Our study is the first study which shows that *P. subochracea* decolorized RB5 in the presence of Sn or Gd or without added metal indicating that this fungus produces VP activity. The other two white-rot fungi *K. mutabilis* and *O. rivulosus* did not produce VP activity in the present study. We also showed that *G. dryophilus* and *H. sulcata* did not decolorize RB5 indicating that these fungi did not produce VP activity.

## Conclusions

The three new isolates, the white-rot fungus *P. subochracea*, the litter-degrading fungus *G. dryophilus* and the brown-rot fungus *H. sulcata* as well as the earlier isolated and much studied white-rot fungi *P. rivulosus* and *K. mutabilis* grew in the presence of Sn (0-200 mg/l) and Gd (0-200 mg/l). The formation rate of colour zones with *P. rivulosus*, *K. mutabilis*, *P. subochracea* and *G. dryophilus* on the ABTS plates indicated that these four fungi produce laccase type oxidative enzyme. No formation of colour zone with the brown-rot fungus *H. sulcata* was seen on the ABTS plates indicating that *H. sulcata* did not produce any oxidative enzymes, which was also expected as it is a brown-rot fungus.

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