Journal of Hazardous Materials 260 (2013) 668–675

Contents lists available at SciVerse ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Mycoremediation of wood and soil from an old sawmill area contaminated for decades

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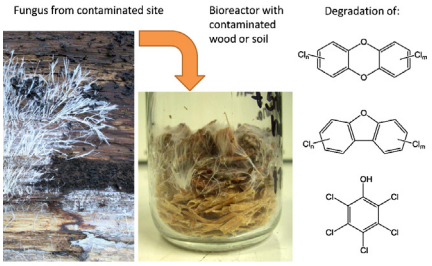
HIGHLIGHTS

- We performed experiments with non-sterile soil and wood with aged contamination.
- We isolated fungal strains from a saw mill site with chlorophenols contamination.
- Fungal strains were screened for tolerance to native microbes and contamination.
- The best fungi degraded chlorophenols and chlorinated dibenzo-*p*-dioxins and -furans.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 10 December 2012
Received in revised form 11 May 2013
Accepted 4 June 2013
Available online 11 June 2013

Keywords:
Fungi
Pentachlorophenol
Dioxin
Furan
Stropharia rugosoannulata

ABSTRACT

We investigated the potential of white-rot and litter-decomposing fungi for the treatment of soil and wood from a sawmill area contaminated with aged chlorinated phenols, dibenzo-*p*-dioxins and furans (PCDD/F). Eight screening assays with emphasis on application of non-sterile conditions were carried out in order to select the strains with capability to withstand indigenous microbes and contamination. Nine fungi were then selected for degrading pentachlorophenol (PCP), and 2,3,4,6-tetrachlorophenol (2,3,4,6-TeCP) and mineralizing radiolabelled pentachlorophenol (^{14}C-PCP) in non-sterile soil or wood during 15 weeks of incubation. Soil indigenous microbes and fungal inoculated soil (fungal inoculum + indigenous microbes) achieved similar degradation of PCP and 2,3,4,6-TeCP and mineralization of ^{14}C-PCP. However, the mineralization rate of ^{14}C-PCP by indigenous microbes was much slower than that boosted by fungal inoculum. The litter-decomposing fungus (LDF) *Stropharia rugosoannulata* proved to be a suitable fungus for soil treatment. This fungus mineralized 26% of ^{14}C-PCP and degraded 43% of 2,3,4,6-TeCP and 73% of PCP. Furthermore, *S. rugosoannulata* attained 13% degradation of PCDD/F (expressed as WHO-Toxic Equivalent). In wood, white-rot fungi grew and degraded chlorophenols better than LDF. No efficient indigenous degraders were present in wood. Interestingly, production of toxic chlorinated organic metabolites (anisoles and veratroles) by LDF in wood was negligible.

1. Introduction

Wood preservation plants and sawmills have used chlorinated phenols (CP) to treat timber against blue staining fungi. In Finland, nationally produced commercial preparation Ky-5 was widely used. In addition to CP, Ky-5 contained recalcitrant polychlorinated phenoxyphenols (PCPP), dibenzo-*p*-dioxins and furans (PCDD/F) as impurities [1,2]. Approximately 30,000 tonnes of CP products,

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http://dx.doi.org/10.1016/j.jhazmat.2013.06.014

including both Ky-5 and imported ones, were consumed for wood preservation in Finland from 1934 up to 1988, when they were banned due to their potential toxicity to humans and the environment [3,4]. The careless use of the preservatives together with the lack of proper waste disposal were common practices in sawmills. Thus, it is estimated that there are still 550 contaminated sawmill sites in Finland, and approximately 100 of them require urgent treatment [5].

At present, the common treatment for soil heavily contaminated with CP is either disposal to landfills or combustion. Combustion in high temperatures (1100 °C) destroys all the contaminants including PCDD/F. However, it is an expensive solution lacking sufficient capacity for all the sawmill soils. Furthermore, in landfilling the contamination remains to be a problem for further generations [5]. Bioremediation of soil is a sustainable way to eliminate the contamination entirely. Methods based on either bacterial or fungal degradation have been widely studied for sawmill soils as CP are rather easy to degrade by microorganisms [6]. Although PCDD/F compounds constitute a challenge to bioremediation, some bacteria are able to transform non-chlorinated and low chlorinated PCDD/F to catechols or chlorocatechols [7,8]. Wood-degrading fungi, such as *Phlebia* spp., have degradation capabilities for highly chlorinated dioxins and furans [9]. *Phlebia brevispora* was even able to degrade 1,3,6,8-tetrachlorodibenzo-*p*-dioxin in slurry-state conditions of a soil contaminated in the past [10].

Low specificity towards the organic contaminant type, hyphal growth mode and production of extracellular lignin modifying enzymes are the main advantages of white-rot (WRF) and litter-decomposing fungi (LDF), two main groups of fungi applied in bioremediation [11,12]. Especially, LDF possess potential for bioremediation, as they grow in close connection to the soil in their natural environment [11,13,14].

The main themes of this study were to introduce new insights for further field-scale application of a fungal bioremediation technique by simulating natural conditions as precisely as possible and to compare the performance of LDF and WRF. Unfortunately, studies performed in soil so far with actual aged contamination obtained from the field and in non-sterile conditions are still exceptions and typical experimental arrangements have included spiked and autoclaved materials [12]. We used non-sterile soil and wood waste from a historically contaminated sawmill area as experimental materials. Fungal species were screened for the tolerance to these conditions, and selected species were studied for decontamination of hazardous soil and wood which contained CP and PCDD/F.

2. Experimental

2.1. Soil and wood samples

Soil and wood samples were taken in southern Finland from a sawmill area, which had been in use for almost 100 years from the beginning of the 20th century. From the 1930's timber was treated in the area with the commercial product Ky-5, which ended up to soil and wooden constructions. The process for Ky-5 production was unique in comparison with similar products made elsewhere, and thus also the composition of chlorophenols: 83% of 2,3,4,6-tetrachlorophenol (2,3,4,6-TeCP), 8% of pentachlorophenol (PCP) and 6% of 2,4,6-trichlorophenol (2,4,6-TCP). In addition, Ky-5 contained PCDD/F and PCPP as impurities [1]. Wood samples originated from buildings and constructions, which had been demolished and chopped. Soil was excavated from the most polluted hot spot according to local authorities who had earlier analyzed the soil. Litter was removed from the top and soil for experiments was taken from a depth of 0.8 m. The concentrations of the contaminants in soil and wood are shown in supplementary Table 1.

2.2. Fungi

For preliminary experiments, 16 fungal species were selected from the Fungal Biotechnology Culture Collection (FBCC) at the Department of Food and Environmental Sciences, University of Helsinki (Table 1). In addition, *Phlebiopsis gigantea* fungal culture, originated from commercial product Rotstop® (Verdera Oy, Espoo, Finland), was included in the screening together with two fungal strains isolated from the contaminated sawmill site. The isolated fungi were identified as *Gymnopilus penetrans* (FBCC1010) and *Trametes ochracea* (FBCC1011). The eco-physiological group and family of the fungi used in the screenings are shown in supplementary Table 2. As the isolation and screening with fungi from FBCC were performed in parallel, and the isolated fungi were screened later, only the most relevant screening tests were performed for these two fungi, namely growth on non-sterile contaminated wood chips and soil. All fungal strains were maintained on 2% malt extract agar (MEA) slants in 4 °C.

2.3. Screening assays of fungal strains

Fungal strains were screened in order to evaluate their tolerance to the contaminants and their competitiveness towards indigenous microbes. The suitability of wheat straw (*Triticum aestivum*) or Scots pine (*Pinus sylvestris*) bark as substrate of fungi during bioremediation was also tested. Tolerance to PCP was tested by cultivation of fungi in autoclaved PCP-containing liquid media and the competitiveness of fungi was tested by cultivation on non-sterile bark and wheat straw. Both characteristics were needed in cultivation on contaminated non-sterile wood chips or soil.

Eight screenings assays were performed:

(I) Fungi were cultivated in autoclaved liquid medium containing malt extract (ME) and 300 mg/l of PCP (Sigma Chemical Co., St. Louis, Mo, USA), which was added to the medium after autoclaving. Three plugs with fungal mycelium (diameter 10 mm) from ME agar (MEA) plates were placed in a flask containing 25 ml of ME medium (pH 5.0). The incubation was performed in triplicates at 25 °C for 20 days, after which fungal biomass was filtered (Whatman No. 4 paper filter, Whatman International Ltd., Maidstone, UK) and dried overnight at 105 °C to calculate dry biomass. The controls were cultivated in parallel without PCP.

(II–III) For cultivation experiments on non-sterile or autoclaved bark, bark was first soaked in water overnight, then drained and 30 g (wet weight, ww) placed in each incubation flask (dry matter content was 27%). Half of the flasks were autoclaved, i.e. surface sterilized (121 °C, 20 min.). Inoculum for each flask was obtained from a 19-day-old mycelium previously grown in 100 ml ME liquid medium. The incubation was performed in triplicates in the dark at 22 °C for 30 days.

(IV) For cultivation experiments on non-sterile or autoclaved wheat straw, straw was first cut to 3 cm pieces and soaked in water for one hour. Water was drained and 40 g (ww) of straw (dry matter content was 25%) was placed in each incubation flask. Half of the flasks were autoclaved, i.e. surface sterilized (121 °C, 20 min.). Straw was inoculated with four fungal plugs (diameter 10 mm) from MEA plate to each flask. The incubation was performed in triplicates in the dark at 22 °C for 30 days.

(V) Wood chip cultivation on MEA plate was performed by placing one previously grown fungal agar plug on one edge of a Petri dish. When fungal growth had reached the half way of the plate, a non-sterile wood chip from the sawmill site was placed on the other edge of the plate. Growth was followed visually to observe whether the fungus grew eagerly on wood or avoided it, and whether the fungus was able to compete with the native microbial population of the wood chip.