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Pyrene degradation and detoxification in soil by a consortium of basidiomycetes isolated from compost: Role of laccases and peroxidases

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Abstract A consortium of three basidiomycetes isolated from compost was investigated for pyrene degradation in soil microcosms. Pyrene concentration, glucose and ammonium evolution, moisture content, ligninolytic enzyme activities and phytotoxicity (germination index) on Lepidium sativum L. seeds were monitored. The fungal consortium grown on straw was found able to efficiently colonize soil and remove about 56 out of 100 mg kg−1 of soil dry weight of pyrene in 28 days; in the meantime the germination index increased indicating a reduction of phytotoxicity. A glucose supply after 2 weeks was found useful to ensure fungal growth and activity; maintenance of moisture content below 70% allowed a good aeration of the system and improved degradation rates. Enzymatic assays showed that laccase and manganese independent peroxidase activity could have played a role in the degradation process.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants produced during incomplete combustion of fossil fuels, organic wastes and various industrial processes. Many of them have been found to exhibit cytotoxic, mutagenic and carcinogenic properties and therefore pose a serious risk to human health [1]. Pyrene is a toxic, recalcitrant, four fused ring PAH commonly found in soil [2]; its quinone-based metabolites are mutagenic and more toxic than the parent compound [3]. For these reasons, pyrene is listed among the 16 USEPA priority pollutants PAHs and considered as an indicator for monitoring PAH-contaminated wastes [2].

Composting or mature compost have been shown to be effective in the bioremediation of soil containing PAHs [4] and several works report a significant removal of pyrene from soil as sole contaminant or in mixture with other PAHs [5], [6] and [7]. The enormous potential of composts for PAHs bioremediation is mainly due to their support of microorganisms able to degrade a variety of aromatic pollutants [4].

Among microorganisms capable of degrading PAHs, extensive studies have been done on ligninolytic fungi, mainly basidiomycetes: their complement of enzymes comprises several peroxidases and laccases that are nonspecific, non-stereoselective and effective against a broad spectrum of aromatic compounds [3] and [8]. However, basidiomycetes are rarely isolated from compost and their role in composting (especially in humic acid degradation) and in compost applications has only recently been pointed out [8], [9] and [10].

Basidiomycetes in composting normally are not active during the thermophilic stage, when the temperatures can be higher than 50 °C [11], but they are recovered in the cooling and maturation phase during which they contribute to the cellulosolytic and ligninolytic process [12]. Loser et al. [6] found that the fastest and most extensive PAHs degradation during composting occurred at 30 °C and that higher temperatures inhibited the degradation of anthracene and pyrene. Martens [13] found that PAHs mineralization extent increased during the compost maturation phase, in which colonization by ligninolytic fungi increases too. Haderlein et al. [5] obtained the highest pyrene mineralization when this compound was in contact with composted soil from the curing stage, but they excluded the possible involment of fungi on account of the supplement of cycloheximide to suppress the fungal population. However, several fungal species are resistant to this antibiotic, as already demonstrated in a study on the mycoflora of different composts [14].

In a previous work we demonstrated the capacity of a consortium of three basidiomycetes isolated from compost to degrade naphthalene [8]. The aim of the present work is to study in soil microcosms the capacity of the same consortium to degrade a more recalcitrant PAH, pyrene, and to evaluate the toxicity due to pyrene and/or any of its metabolites by the calculation of the germination index (GI%) of Lepidium sativum L. Another objective of this work is to elucidate which of the main components of the lignin-degrading system of the consortium (different types of peroxidases and laccase) could play a role in the degradation process.

2. Material and methods

2.1. Chemicals

All chemicals used were reagent grade and were obtained from Fluka and Sigma Aldrich, except for mineral salts $MgSO4 \cdot 7H2O$, KCl and $FeSO4 \cdot 7H2O$ that were purchased from Merck.

2.2. Microorganisms

The three basidiomycetes were previously patented and deposited in the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) collection (DSM 15214, DSM 15215, DSM 15216); they were isolated from compost as sterile mycelia and identified as basidiomycetes morphophysiologically. rDNA sequencing (18S, 28S, Internal Transcribed Spacer -ITS 1, 2) and comparison with sequences in gene databases showed that strains DSM 15214 and DSM 15215 had 100% similarity to Trametes versicolor (L.) Pilát, while strain DSM 15216 had 99% similarity to different species of basidiomycetes [Bjerkandera adusta (Willd.) P. Karst., Bjerkandera fumosa (Pers.) P. Karst., Lopharia spadicea (Pers.) Boidin] (Genbank, NCBI). All three strains had been shown to display laccase and/or peroxidase activity during their decolorization of Poly R-478 in single cultures [15]; when cultured together in consortium, they showed enhanced Lcc activity and efficient removal of naphthalene in soil [8] and [15].

2.3. Microcosms preparation

Microcosms were prepared by placing in 500 ml cotton-plugged Erlenmeyer flasks: 10 g of a sandy, uncontaminated soil (pH 6.63, total moisture 12%, sand 75%, silt 22%, clay 3%) sterilized with gamma rays; pyrene dissolved in acetone to a final concentration of 100 mg kg−1 (a value considered representative of polluted soils in Italy, Eurolab s.r.l. personal communication); 10 ml of an aqueous solution containing 40 g l−1 glucose; 0.5 ml of tween 80 (5%). The addition of surfactants as tween 80 in small concentrations can increase the accessibility of the PAHs to the white rot fungi degradation system without interfering with growth of fungi in the soil [16]. Our strains, in a previous study performed on agar medium containing tween 80, were found unable to use it as a carbon source [17]. The fungal inoculum consisted of 1 g of straw culture for each strain prepared by inoculating 3 disks of fungal mycelium (5 mm diameter) grown for 7 days on Malt Extract Agar in tubes containing 1 g wheat straw (autoclaved twice, 48 h apart, at 121 °C for 20 min) and 6 ml of a liquid medium (LM) composed (per litre of distilled water) of 20 g glucose, 2 g di-ammonium tartrate, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g $ZnSO_4 \cdot 7H_2O$, 0.005 g $CuSO_4 \cdot 5H_2O$ and 0.1 mg thiamine. Heat-killed controls (straw cultures autoclaved at 121 °C for 20 min) and abiotic (consortium-free) controls were set up. Microcosms were incubated statically at 24 °C in the dark and 5 ml of an aqueous solution containing 20 g l−1 glucose were added on day 14 and 5 ml of LM on day 28. Three replicates were performed per each microcosm set and each point of analysis; a total of 84 microcosms was set up.

2.4. Residual pyrene analysis

Pyrene residues were quantified weekly on three microcosms by drying them in toto at 40 °C for 8 h, followed by an initial extraction with 50 ml of a hexane:acetone mixture (80:20 v/v) in agitation for 8 h. Two further extractions were carried out by sonication for 10 min with 50 ml of the same mixture. The decanted extract was collected in a 100 ml balloon flask after each extraction. The entire extract was then purified in columns containing 5 g anhydrous Na_2SO_4 and concentrated (about 1 ml volume) on a rotating evaporator. The concentrated specimen was supplemented with 2 ml n-hexane and purified on a chromatography column (10 g silica gel and 2 g anhydrous Na_2SO_4) pre-eluted with 50 ml n-hexane according to the method EPA 3630 C [18]. Next, 25 ml n-hexane were flushed through to eliminate the aliphatic hydrocarbons [19]. Lastly, the purified extract was eluted with 50 ml of a 80:20 v/v n-hexane/dichloromethane mixture, concentrated to 10 ml on the rotating evaporator and analyzed by GC–MS (GC Agilent mod. 6890 with autosampler Agilent 7683—Detector MS Agilent, mod. 5973). The capillary column used was a DB-5 (30 m × 0.25 mm × 0.25 μm film thickness). The carrier gas was He at a constant flow rate of 1.0 ml/min. Residual pyrene was expressed as mg per kg of soil dry weight (mg kg−1). The nonparametric Mann–Whitney test for independent groups (Systat Version 10, Systat Software Inc., SPSS, 2000) was run to assess the significance ($p < 0.05$) of differences in its concentration between the biotic and control lines.

2.5. Moisture, ammonium and glucose monitoring

Moisture and glucose and ammonium content were monitored fortnightly on three microcosms. Moisture was calculated as the difference between the mass of the specimen before and after drying to a constant mass at 105 °C. Mineral N was extracted by agitating the microcosms for 1 h with KCl 2 M; 60 ml of the suspension thus obtained were then centrifuged for 10 min at 3000 rpm. The centrifugate was distilled for spectrophotometric determination of its ammonium content [20]. Glucose was monitored by agitating the microcosms for 1 h with 150 ml deionised H2O; 2 ml of the suspension were then filtered and their glucose content was determined spectrophotometrically with the Starch Assay Kit (Sigma, Code SA-20).

2.6. Detoxification assay

Toxicity was evaluated by means of a phytotoxicity test on Lepidium sativum L. seeds, applied directly in microcosms with and without pyrene, at the start and at the end of the experiment according to the method of Maila and Cloete [21]. The test was performed in triplicate and L. sativum was used since it proved to be the most sensitive over 6 plant species (L. sativum, Raphanus sativus, Cucumis sativa, Lycopersicon esculentum, Allium cepa, Lolium perenne) to different PAHs (naphthalene, phenanthrene, fluoranthene, anthracene, pyrene) [22]. The germination index (GI%) was calculated from the number of germinated seeds and the root length values in the control and in the samples according to the formula: GI% = (Gs × Ls)/Gc × Lc) × 100, where Gs is the mean number of germinated seeds in the sample, Ls is the mean root length of the sample, Gc is the mean number of germinated seeds in the control, Lc is the mean root length of the control.

2.7. Enzymatic assays

Extracellular ligninolytic enzymes activities were assessed fortnightly on three microcosms. Extracellular ligninolytic enzymes were extracted by adding 50 ml acetate buffer (50 mM, pH 5.0) to each flask, agitating at 4 °C for 30 min and centrifuging at 10,000 rpm, 4 °C for 20 min [23]. Supernatants were collected and stored at −18 °C. The activities of manganese peroxidase (MnP), laccase (Lcc) and manganese independent peroxidase (MiP) were assayed spectrophotometrically according to Tanaka et al. [24] monitoring the oxidation of 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS). Activities were expressed as international units per litre of enzyme extract, where one unit is defined as the amount of enzyme oxidising 1 μmol of substrate per minute (U l−1). Lignin peroxidase (LiP) activity was assayed spectrophotometrically according to Arora and Gill [25] monitoring the oxidation of the dye azure B; one unit of enzyme activity was expressed as an O.D. decrease of 0.1 U/(min l) of the enzyme extract (U l−1).

3. Results

The time course of pyrene concentration is reported in Fig. 1. A distinction between killed, abiotic and biotic lines was evident from the start. The substantially constant pyrene values in the killed and abiotic controls throughout the experiment indicated a good extraction yield. In the biotic line, the consortium colonized the polluted soil with a dense mycelial growth and reduced pyrene concentration. A degradation of about 56% was achieved after 28 days. The sharpest fall in pyrene concentration (about 30 mg kg–1) was already evident in the first incubation week and coupled with enhanced glucose and ammonium consumption. Addition of the glucose solution on the 14th day prolonged the metabolic and degradative activity of the fungi to day 28. No significant decrease in the pollutant, however, occurred after this time despite the addition of a complete nutrient solution (LM) and persistently intensive metabolic activity of fungi. Moisture within the microcosms remained between 60 and 71%. This range ensures sufficient aeration of the soil and favours degradation. Our preliminary results (not shown), in fact, demonstrated that degradation was markedly reduced when incubation began with more water (20 ml of a 2% glucose solution instead of 10 ml at the double concentration) and 80% humidity.

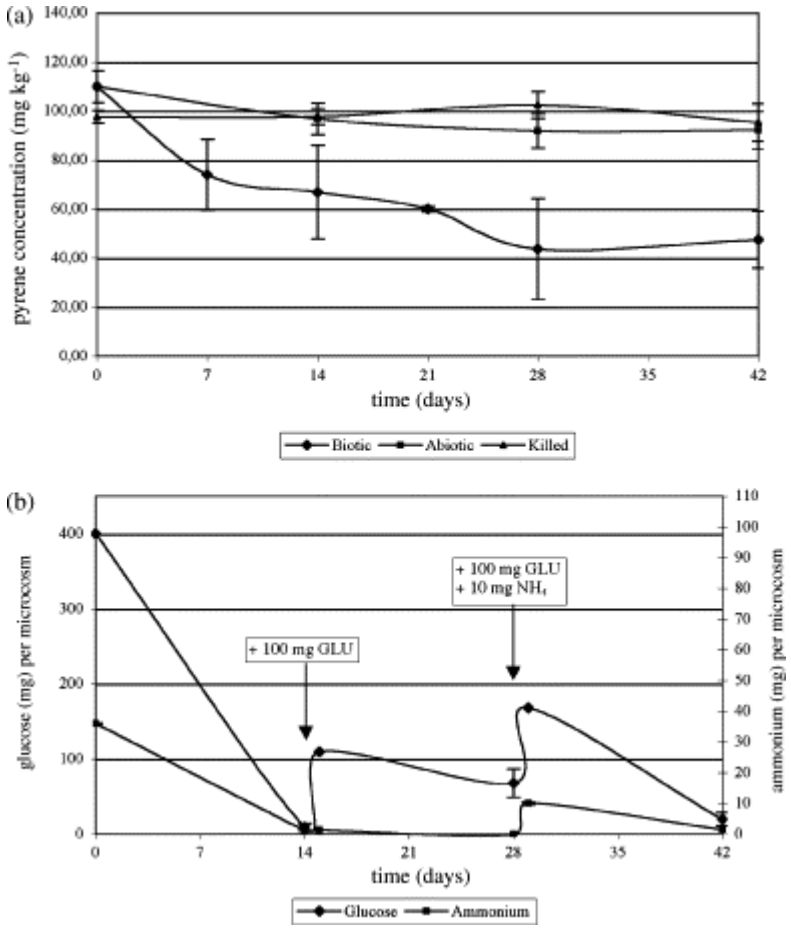

Fig. 1 (a) Time course of pyrene and (b) glucose and ammonium concentration (average of 3 replicates ± S.E.) in microcosms containing the fungal consortium. In boxes are shown the amounts of glucose and ammonium added at days 14 and 28.

The GI% at the start of the incubation was 74.1% and rose to 154.3% at the end of the experiment. This result indicates that phytotoxicity was reduced and growth was actually stimulated compared to the controls without pyrene.

Extracellular ligninolytic enzyme activities were detected throughout the experiment, but varied greatly from one enzyme to another and in function of the sampling time, as shown in Fig. 2. Lcc activity was the highest (maximum 397 U l−1 on day 14), especially during the first 20 days when pyrene was degraded. MiP activity was low in the first 2 weeks, then increased to a peak on day 28 (124 U l−1). MnP and LiP activities were low throughout the experiment; their highest values (48 and 19 U l−1) were reached on day 14.

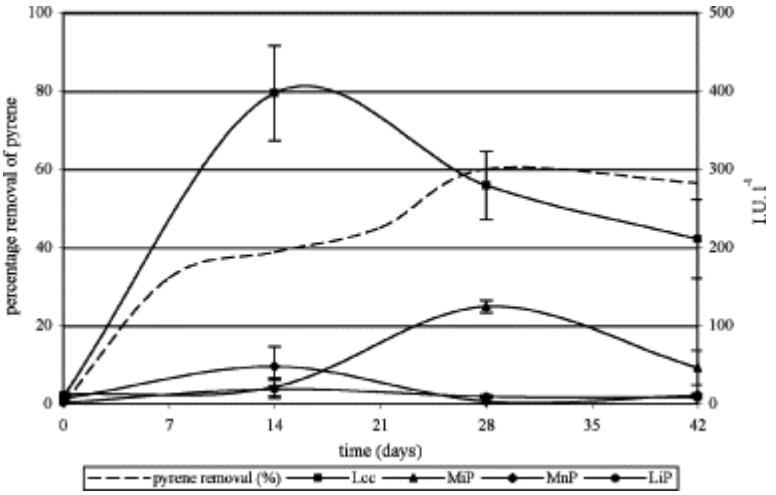

Fig. 2 Laccase (Lcc), manganese peroxidase (MnP), manganese independent peroxidase (MiP) and lignin peroxidase (LiP) activity (U l−1) (average of 3 replicates ± S.E.) and pyrene degradation percentage (%) in microcosms containing the fungal consortium.

4. Discussion

The setting up of microcosms closely simulates PAHs biodegradation in soil without prejudice to the maintenance of controlled culture conditions (artificial pollution, constant incubation temperature, absence of competitors, periodic addition of nutrients and water, and monitoring of metabolic activity) [8]. The ability to grow in and efficiently colonise soil is obviously a critical factor in PAH degradation. As shown by Novotny et al. [26], some lignicolous fungi with a good degradative capacity in liquid conditions are poor colonisers of soil, a feature that weakens their chances of application. Our fungal consortium efficiently colonised the soil, maintained a good metabolic activity throughout the experiment and brought about the removal of 56 mg kg−1 of pyrene in 28 days. This removal is considerable compared to the 1.5–23 mg kg−1 in 60 days (50 mg kg−1 initially) and 35 mg kg−1 in 90 days (67 mg kg−1 initially) reported for white-rot fungi in conditions similar to ours [26] and [27], and 30 mg kg−1 in 9 days (50 mg kg−1 initially) for Aspergillus terreus in liquid culture [28].

Monitoring of the removal of PAHs, however, is not enough to indicate bioremediation of the soil. Incomplete degradation and formation of toxic intermediary metabolites may enhance soil toxicity during bioremediation [3]. To investigate this crucial aspect we used in our study

the germination test on L. sativum seeds. The use of bioindicators to evaluate hazardous chemical waste sites provides a direct, inexpensive and integrated estimate of bioavailability and contaminant toxicity. However, these methods can have some disadvantages, as reviewed by Maila and Cloete [29]: (i) the inability to distinguish toxicity resulting from parent compound and metabolites; (ii) bioindicators response does not always correspond to contaminant concentration; (iii) different tests respond differently to individual toxicants; (iv) sensitivity depends on the toxicant and soil (i.e. the test can be sensitive to other factors of the soil).

At the start of the experiment, a GI% of 74.1% was observed indicating that L. sativum has a moderate sensitivity to the initial concentration of 100 mg kg−1. This starting value, however, made it possible to track both a decrease or an increase of toxicity that could be due to the degradation products (i.e. the pyrene quinone-based metabolites) [3]. Following the action of the fungal consortium an increase of the GI% up to 154.3% was observed, indicating a reduction of toxicity and a promotion of the growth (the value over 100% is the result of an equal number of seeds germinated in the samples compared to the controls without pyrene but with higher root length values), as already demonstrated with the same consortium in the presence of naphthalene [8] and in other PAHs bioremediation systems [30].

This disappearance of toxicity, even in presence of a remaining 40 mg kg−1 of pyrene, could have different explanations. Sorption of PAHs onto soil organic matter [31] or onto the fungal biomass [32], owing to the presence of the exopolymeric substances produced by the fungus and accumulated around the fungal hyphae, could reduce bioavailability as well as biotoxicity. Another reason could be that at low concentrations of PAHs a stimulation rather than an inhibition of the growth could be observed, an effect called hormesis, as also observed on plants exposed to creosote [33] and to a mixture of PAHs [34]. However, taking also into account the moderate sensitivity of the test to these concentrations of pyrene, these results on the detoxification capacity of the consortium, although encouraging, could be strengthened by further investigations with a battery of bioassays involving various organisms that, as stated by Hubalek et al. [35], should encompass different trophic levels.

The high pyrene degradation and the detoxification caused by the consortium could be due to its synergic production of ligninolytic enzymes, as in the case of its Lcc production during degradation of the anthraquinone dye Poly R-478 [8]. Several experiments have shown that degradation of hydrocarbons in nature may require the combined efforts of several microorganisms, since few are able to degrade high-molecular-weight PAH on their own [36] and [37]. Recently, Li et al. [38] showed that inoculation of microbial consortia (bacteria, fungi and bacteria–fungi complex) was a promising method to degrade PAHs in oil-contaminated soil. Chi et al. [39] have demonstrated that co-cultures of white rot fungi increase lignin degradation and lignin-degrading enzyme production. Hence, co-cultures of selected fungus strains boost enzyme production through synergic interactions [39] and [40].

The literature indicates that several enzymes, such as peroxidases and Lcc, may be involved in PAHs degradation by fungi, though very little is known about their combined role and interactions [41]. This is mainly due to the high complexity of the biodegradation mechanisms

involved, where in addition to the ligninolytic enzymes, other biochemical systems may influence the rate of the bioremediation process, namely cytochrome P450 mono-oxygenase system, hydroxyl radicals and the level of H2O2 produced by the fungi [42]. However, the measure of high levels of activity of a specific enzyme, together with the removal of a pollutant, is considered indicative of a relation between the detected enzyme and the degradation [42].

In our case, Lcc seemed the main class of enzymes involved in pyrene degradation: Lcc activity was the highest throughout the experiment, particularly in the first 3 weeks when we also recorded the largest removal of pollutant. The key role of Lcc in pyrene degradation had already been noted by other authors [37] and [43] and it underscores the importance of the synergic interactions of our consortium in the production of this class of enzymes [8].

Enhancement of MiP activity from the 14th day in our experiment suggests that versatile peroxidase may be involved in pyrene degradation, mainly in the later stages by catalyzing oxidative reactions of metabolites resulting from pyrene destruction by the other enzymes. Versatile peroxidases are a new family of ligninolytic peroxidases independent of manganese and with characteristics intermediate between MnP and LiP. They are generally found in Pleurotus, Bjerkandera, Lepista, Trametes and are known to degrade dyes [44], polychlorinated dibenzo-p-dioxins [45] and the PAH benzo(a)pyrene [46].

MnP and LiP activities were low and no apparent relationship can be observed between them and the degradation of pyrene. However, even with weak activities, we cannot exclude that these enzymes may have been indirectly involved in pyrene removal, both at the start of the reaction and in the following oxidative steps involving intermediates resulting from the first attack. As observed by Gavril et al. [41] during an azo naphthalenic dye degradation by T. versicolor low amounts of LiP initiate the reaction, which is then completed by Lcc. Each enzyme activity during the degradation depends on the level of the other enzymes present, and on the characteristics of the environment [41]. Ligninolytic enzymes, in fact, compete for the same substrates and the radicals formed interact with each other or with the fungal metabolites and generate other radicals that continue the process.

5. Conclusions

The data presented in this study show that composts comprise basidiomycetes capable of removing recalcitrant PAHs such as pyrene from soil, producing a broad spectrum of ligninolytic enzymes. Investigation of the potential of these microorganisms throws light on an important and often overlooked part of compost microflora, and serves as the foundation for the devising of ways and means of enhancing the activity of ligninolytic fungi by bioaugmentation or optimisation of parameters favouring their growth and ability to degrade. This could lead to the elaboration of techniques based on composting or the addition of ripe composts for the bioremediation of contaminated soils.

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