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Benzo[*a*]pyrene removal by *Marasmiellus troyanus* in soil microcosms

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Benzo[*a*]pyrene (B[*a*]P) is a carcinogenic polyaromatic hydrocarbon that enters the environment as an incomplete combustion production of fossil fuels. Several species of filamentous fungi are capable of biotransforming and/or mineralizing B[*a*]P in liquid cultures, however there has been less success in soil habitats. In this study, the litter rot fungus *Marasmiellus troyanus* was encapsulated in alginate and delivered to B[*a*]P-spiked soil microcosms (100 μg B[*a*]P/g soil) for 1, 2 and 6 weeks, with and without a fertilizer solution. After 2 weeks, 32.5% of B[*a*]P was recovered from soil microcosms treated with *M. troyanus* compared to 55–70% for controls. After 6 weeks, controls demonstrated an average percent recovery of B[*a*]P of 54% while *M. troyanus*-inoculated samples gave an average percent recovery of 11%. Similar bioaugmentation of contaminated habitats with appropriately formulated fungi has potential for practical bioremediation in soil environments. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 116–119.

Keywords: benzo[*a*]pyrene; bioaugmentation; polyaromatic hydrocarbons; *Marasmiellus troyanus*

Introduction

Polycyclic aromatic hydrocarbons (PAHs) formed during the incomplete combustion of organic materials are potentially mutagenic and carcinogenic environmental pollutants. Many species of algae, bacteria and fungi degrade PAHs in liquid culture [5,29] and there is considerable interest in delivering such microbes (bioaugmentation) to polluted environmental sites for remediation [27,30]. Lower molecular weight PAHs such as naphthalene and anthracene are readily mineralized by bacteria, whereas higher molecular weight PAHs such as benzo[*a*]pyrene (B[*a*]P) have low water solubility and regularly bind to soil particles, resulting in low bioavailability for bacterial degradation [9,19]. Because of their nutritional strategy, fungi are uniquely suited to biodegradation of PAHs and many other environmental contaminants [2,28].

First isolated from coal tar in 1931, and frequently found in soils, B[*a*]P is both persistent and carcinogenic [25]. It has become the focus of many biodegradation studies using filamentous fungi [6,8,14,16] as well as a model for the study of PAH mobility in the environment [26]. The majority of early studies focused on *Cunninghamella elegans* [6–8] but many ligninolytic fungi are also effective degraders [3,15,16,19,31]. In particular, the white rot species *Phanerochaete chrysosporium* has become a model for studying fungal degradation of PAHs and other environmental pollutants [1,4,16]. Although most of the quantitative research on fungal biodegradation of B[*a*]P has been conducted in liquid culture, filamentous fungi are especially well suited to growth in terrestrial habitats. In fact, as bioaugmentation tools, fungi are uniquely suited to soil and soil-like habitats[2]. For example, *P. chrysosporium* can mineralize B[*a*]P in soils supplemented with carbon sources such as straw or wood chips [23].

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Recently, we isolated the litter rot mushroom *Marasmiellus troyanus* from a Superfund site north of Darrow, Louisiana, USA, and demonstrated that mycelia of this species can remove [32] and mineralize [33] B[*a*]P in liquid culture. Although *M. troyanus* grows well in the laboratory, it does not sporulate, and cultures must be maintained through transfer of mycelial plugs. Nevertheless, inoculation of soils can be accomplished by encapsulating hyphal fragments in sodium alginate [10,20]. We demonstrate here that alginate-encapsulated *M. troyanus* is capable of effective removal of spiked B[*a*]P from soil microcosms.

Materials and methods

Organism, culture media and culture conditions

M. troyanus TF 216-1867 was isolated from leaf litter on an abandoned oil refinery (Old Inger Refinery, Darrow, LA, USA). Stock cultures were maintained on malt extract agar plates (Difco, Detroit, MI, USA) and then grown on potato dextrose agar plates (Difco, Detroit, MI, USA) at 25°C for 7 days for use as inocula. Five plugs of mycelia (6 mm) were inoculated into 250-ml Erlenmeyer flasks containing 50 ml Sabouraud's dextrose broth (Difco, Detroit, MI, USA) and grown in shaker culture (250 rpm) at 25°C in the dark.

Fifty-gram samples of a Commerce silt loam soil (fine-silty, mixed, nonacid, thermic Aeric Fluvaquent) from East Baton Rouge Parish, LA, USA [18] were placed in 250-ml Erlenmeyer flasks and spiked with 5 ml of a stock solution of B[*a*]P (Sigma Chemical Co., St Louis, MO, USA) in acetone (1000 ppm) to yield a soil B[*a*]P concentration of 100 μg/g. After thorough evaporation of the acetone, the samples were brought up to 30% water capacity by adding 15 ml of dH_2O or a fertilizer solution, and mixing thoroughly with a hand-held spatula. Samples of soil extracted immediately after spiking, evaporation, and mixing gave 77–91% recovery. The fertilizer

contained urea (Mallinckrodt Inc., St Louis, MO, USA) and disodium phosphate to result in a final soil concentration of 680 ppm N and 170 ppm P [12]. Each soil microcosm was inoculated with 5 g of alginate beads and incubated at 32°C in the dark for 1, 2 or 6 weeks. Moisture levels were maintained throughout the experiment with daily watering.

Preparation of fungi for alginate encapsulation

The method was adapted from Childress *et al.* [10], Daigle and Cotty [11], and Loomis *et al.* [22]. All water, amendments and glassware were kept sterile. Mycelial pellets from 1-week-old Sabouraud cultures were collected on cheesecloth and rinsed with 50 ml dH_2O. Washed mycelia from four cultures were pooled and blended on the high setting with a Waring blender for 1 min with 100 ml dH_2O.

Alginate used was Kelgin medium viscosity sodium alginate (Kelco, Division of Merck and Co., San Diego, CA, USA). Corncob grits (Anderson Cob, Maumee, OH, USA) were ground in a Fetsch Ultra centrifugal mill (Model 2M-1, Brinkman Co., Houston, Texas, USA) and sieved to pass through a no. 200-mesh screen (US Standard Mesh Designation) with 75 μm and sieved to pass through a no. 200-mesh screen (US Standard Mesh Designation) with 75 μm openings. To prepare alginate pellets, we mixed 10 g sodium alginate, 40 g corncob grits, 850 ml dH_2O and the fungal macerate from four pooled cultures. Control beads lacked the fungal macerate. The slurry was stirred for 1 h with a Dyna-mix overhead stirrer (Fisher Scientific, Pittsburgh, PA) at 250 rpm. The mixture was then transferred into a 2000-ml crystallizing dish and stirred with a magnetic stir bar while the slurry was drawn through silicon tubing (5-mm ID) using a peristaltic pump (Manostat Cassette, Barnant Company, Barrington, IL, USA) at a rate of approximately 1 ml/s. The solution was dropped into 4 l of a 0.25 M solution of calcium chloride, also being stirred by a magnetic stir bar. After the entire slurry had gelled, the resultant alginate-mycelial beads were kept for 15 additional minutes in the calcium chloride solution. The alginate beads were then collected on a screen and dried overnight in a fume hood on cheesecloth. Dry alginate pellets (approx. diam.=3 mm) were stored at 4°C.

For killed controls, alginate pellets containing *M. troyanus* were grown in unspiked soil under standard conditions for the appropriate time, killed with a 5-ml solution of 4% $HgCl_2$ (Sigma), spiked with the B[*a*]P solution (100 μg/g soil) and then incubated again for 1, 2 and 6 weeks.

Extraction and quantification of B[a]P

One hundred milliliters of ethyl acetate were added directly to the soil. The flasks were shaken overnight on a rotary shaker in the dark. The ethyl acetate–soil slurry was poured through a 100-mm top diameter funnel lined with Whatman (Fairfield, NJ, USA) no. 2 filter paper containing 5 g sodium sulfate and collected in a 250-ml beaker. The flask was washed twice with 50-ml aliquots of ethyl acetate. The pooled ethyl acetate extracts were dried in a chemical hood in the dark and resuspended in 10 ml acetone. Reverse phase thin layer chromatography (RPTLC) was performed immediately on reversed phase plates (10×20 cm, 150 μm thickness, Analtech Inc., Newark, DE, USA), according to the method of Nemergut *et al.* [24]. Extracts were spotted, in duplicate, using 1 μl microcapillary pipettes with a Nanomat III (Camag, Muttenz, Switzerland). Standards of 125, 250, and 500 ppm B[*a*]P were also spotted on each plate. The plates were developed in acetonitrile–methanol (1:1, vol/vol) and were scanned using a Shimadzu densitometer dual wavelength flying spot scanner CS-9000 (Shimadzu Scientific Instruments Inc., Kyoto, Japan) at 370 nm. Duplicated samples were averaged and standard curves were constructed using regression analysis of standard solutions. A *t*-test was performed to determine significant differences.

Results and discussion

Thick mycelia grew up to near the top of the flask in soil microcosms spiked with B[*a*]P. Mycelial growth was less abundant in unspiked control flasks (Figure 1).

The percent recovery of B[*a*]P from soil microcosms inoculated with and without alginate pellets, with and without fertilizer solution, and with living and dead *M. troyanus* is shown in Table 1. After 1 week, with the exception of microcosms containing dead *M. troyanus* and fertilizer, there were no significant differences in recovery between the controls and samples contain-

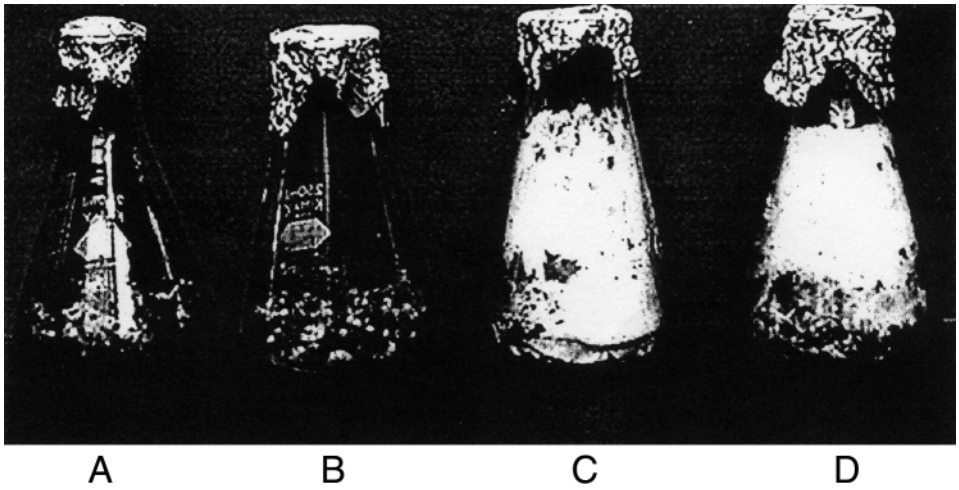

Figure 1 Alginate encapsulated *M. troyanus* growing in soil. (A) Unfertilized, (B) fertilized, (C) fertilizer and B[*a*]P, and (D) unfertilized with B[*a*]P.

Table 1 B[*a*]P recovery from fertilized and unfertilized soils with and without alginate-formulated *M. troyanus*

Treatment	B[*a*]P recovered (%)		
	1 week	2 weeks	6 weeks
Soil only	61.1 ± 2.8^{n}	68.1 ± 4.2^{p}	42.9 ± 2.3^{s}
Control beads	55.0 ± 2.9^{n}	62.1 ± 2.4^{p}	59.6 ± 4.2^{t}
Fertilizer solution	56.6 ± 3.1^{n}	55.3 ± 3.6^{q}	56.4 ± 5.3^{t}
Control beads and fertilizer	58.4 ± 8.9^{n}	59.9 ± 8.3^{q}	58.7 ± 3.9^{t}
Dead *M. troyanus*	52.0 ± 4.9^{n}	62.3 ± 8.1^{p}	47.8 ± 4.4^{s}
Dead *M. troyanus* and fertilizer	73.9 ± 3.8^{o}	69.7 ± 8.4^{p}	59.9 ± 2.4^{t}
Living *M. troyanus*	55.8 ± 4.1^{n}	32.5 ± 3.9^{r}	7.1 ± 3.9^{u}
Living *M. troyanus* and fertilizer	58.4 ± 8.9^{n}	54.5 ± 10.2^{q}	15.3 ± 3.5^{u}

All treatments were spiked with 100 μg B[*a*]P/g soil at time zero; moisture was maintained throughout the experiment. Data are means (±standard deviation) of three samples. For each time period, values followed by the same letter are not significantly different from one another as determined by a p value greater than 0.05.

ing *M. troyanus*. After 2 weeks of incubation, microcosms containing fertilizer alone, control beads and fertilizer, living *M. troyanus* alone or living *M. troyanus* with fertilizer, contained significantly less B[*a*]P than was removed from other samples. At 6 weeks, only 7–15% of the B[*a*]P was recovered from soil microcosms containing living *M. troyanus*, while control samples gave 43–60% recovery. At both 2 and 6 weeks, living *M. troyanus* microcosms without fertilizer had significantly less B[*a*]P than controls. After 6 weeks, *M. troyanus* inoculated samples, both with and without fertilizer, showed significant removal of B[*a*]P with an average percent recovery of 11%.

For killed controls, *M. troyanus* was grown for 1, 2 or 6 weeks in soil samples lacking B[*a*]P, at which point the entire soil microcosm was treated with $HgCl_2$ and mixed with B[*a*]P; these samples yielded similar recoveries to other controls indicating that nonspecific absorption of B[*a*]P to mycelia had not occurred. Soil microcosms in our study were not sterile and putative biodegradation by endogenous microbial populations could contribute to the lower B[*a*]P recovery observed in fertilized controls at 2 weeks. However, at 6 weeks, controls containing nutritional supplement (alginate–corn cob grits beads, fertilizer solution or combinations thereof) gave significantly less removal than unsupplemented controls. At both 2 and 6 weeks, microcosms inoculated with unfertilized living alginate-encapsulated *M. troyanus* showed significantly greater removal than controls lacking the alginate-encapsulated fungus. After 6 weeks there was no significant difference in B[*a*]P removal by *M. troyanus*-treated soils with or without the fertilizer solution. Although slower, the 85–93% removal of B[*a*]P from soil microcosms by *M. troyanus* at 6 weeks compares favorably with removal reported by Wunch *et al.* [32] in liquid culture where 8% of radio labeled B[*a*]P was mineralized and 61% was biotransformed into water soluble conjugates after three weeks of growth. Although B[*a*]P has not been reported to support microbial growth as a sole carbon source [9], visual examination of our soil microcosms indicated that cultures containing B[*a*]P had more profuse mycelial development than did controls (Figure 1).

The ability of filamentous fungi to degrade PAHs in liquid culture is not always reproducible in contaminated soils, nor is their ability to degrade pollutants in the laboratory readily translated into consistent field performance. The survival and activity of microorganisms introduced into soils is affected by many environmental parameters (e.g., pH, temperature, water, nutrient availability, soil composition, contaminant level, and biological competition) [30]. In addition, successful bioaugmentation requires that appropriate inocula be delivered in an expedient manner that preserves viability. Formulations consisting of pelleted mixtures of sawdust, starch, lignosulphonate and the like have been successfully used for the fungal degradation of pentachlorophenol in soils [21]. The coating of such pellets with fungal spores or hyphal fragments in sodium alginate improved effectiveness [20]. Our data indicate that alginate works well for encapsulation of hyphae of filamentous species such as *M. troyanus* that produce abundant mycelia without concomitant sporulation.

In summary, these data contribute to the growing literature that many filamentous fungal species such as *Bjerkandera* [19], *Pleurotus ostreatus* [3] and *Pleurotus* sp. Florida [30] are effective alternatives to the better-studied *C. elegans* and *P. chrysosporium* in the removal of higher PAHs such as B[*a*]P from contaminated soils. It remains to be seen whether the potential of these fungal biodegraders can be transferred successfully to field situations where pollutant "aging" limits the potential of many bioaugmentation strategies [13,17]. Experiments are underway in our laboratory to test the effectiveness of alginate-encapsulated *M. troyanus* in naturally aged PAH-contaminated soils.

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