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To cite this version:

Rachel Rama-Mercier, Christian Mougin, Jean-Claude Sigoillot, Laurent Sohier, Véronique Chaplain, et al.. Wet sand cultures to screen filamentous fungi for the biotransformation of polycyclic aromatic hydrocarbons. Biotechnology Techniques, 1998, 12 (10), pp.725-728. 10.1023/A:1008819109005 . hal-02695466

HAL Id: hal-02695466

https://hal.inrae.fr/hal-02695466v1

Submitted on 12 Jan 2023

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Wet sand cultures to screen filamentous fungi for the biotransformation of polycyclic aromatic hydrocarbons

Rachel Rama-Mercier[1], Christian Mougin[2*], Jean-Claude Sigoillot[3], Laurent Sohier[1], Véronique Chaplain[2] and Marcel Asther[3]

[1]KREBS Réseau Eurisys, Division Réhabilitation de sites, 8 Rue Jean-Pierre Timbaud, BP 67, 78184 Saint Quentin en Yvelines, France

[2]Institut National de la Recherche Agronomique (INRA), Unité de Phytopharmacie et Médiateurs Chimiques, Route de Saint-Cyr, 78026 Versailles Cedex, France, e.mail address: mougin@versailles.inra.fr, Fax: +33-(0)1-30-83-31-19

[3]Institut National de la Recherche Agronomique (INRA), Laboratoire de Biotechnologie des Champignons Filamenteux, Faculté des Sciences de Luminy, Centre d'Enseignement Supérieur en Biotechnologies/ESIL, Parc Scientifique et Technologique, CP 925, 163 Avenue de Luminy, 13288 Marseille Cedex 09, France

Running title: Fungal biotransformation of PAHs

Key words: screening, triphasic cultures, filamentous fungi, biodegradation, organic pollutants

SUMMARY

The biodegradation of phenanthrene and benzo[a]pyrene was assayed in liquid and wet sand cultures in the presence of five filamentous fungi. In the controls, 85% volatilisation of phenanthrene occurred within 28 days in liquid cultures while it was only 50% in wet sand. In the later system, remaining phenanthrene and benzo[a]pyrene amounted to 6-51 and 53-92% of their initial levels, respectively, according to the strains. Then, wet sand used as a screening tool evidenced *Trametes versicolor* and *Cunninghamella elegans* as the most efficient polycyclic aromatic hydrocarbons degraders among ten strains.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants resulting from fossil fuel combustion and from by-products of industrial activities, and are widely dispersed in the environment (comprising air, soil and aquatic ecosystems). Many of them exhibit toxic, carcinogenic and mutagenic effects. Polluted sites represent a health risk to human populations because of the high persistence of mixtures of chemicals (estimated to reach several years at total concentrations of 200 to 20000 mg kg^{-1}, Wilson and Jones, 1993). Widespread occurrence and health implications are the motivating factors for site remediation.

Because of the high cost of classical remediation processes requiring, for example, soil excavation, incineration and/or secure landfilling, bioremediation is a promising cost-effective technology for cleaning up soils contaminated with PAHs (Wilson and Jones, 1993). There is a great interest in the use of filamentous fungi for site remediation. The degradation of aromatic hydrocarbons by microorganisms was reported in the past (Cerniglia, 1981) and more recently (Cerniglia, 1992; Launen *et al.*, 1995; Kiehlmann *et al.*, 1996; Vyas *et al.*, 1994), and evidenced the efficiency of fungi. Experiments were carried out with pure liquid cultures often optimised for oxidoreductase production and/or metabolite identification.

Inversely, there is little information concerning PAH degradation by fungi in soils from real polluted sites *in situ* or under laboratory conditions (Davis *et al*., 1993; Holroyd and Caunt, 1994). Additional experiments are required to confirm the efficiency of strains previously evidenced in liquid cultures, or to screen new PAH-degrading strains under intermediate conditions between liquid cultures and soil. For this reason, we chose wet sand as an adapted support allowing the fungi to develop in

a triphasic system including solid materials, and both liquid and gaseous phases. In that system, PAH metabolism was intended to be more related to the fungal metabolism occurring in soils than in liquid media, because it is well known that culture conditions interfere with the fungal metabolism of xenobiotics. The low interactions between sand and chemicals allow an easier and faster analysis of parent and transformation products compared to real soil degradation studies. In a first time, we compare the degradation of a mixture of phenanthrene and benzo[a]pyrene in liquid and wet sand cultures in the presence of strains selected among zygomycetes, deuteromycetes and basidiomycetes. Secondly, the screening system is extended to other strains for degradation studies.

MATERIAL AND METHODS

Chemicals

Unlabelled chemicals, [9-^{14}C]phenanthrene (2200 MBq mmol^{-1}) and [7-^{14}C]benzo[a]pyrene (980 MBq mmol^{-1}) were obtained from Sigma. Radiochemical purities were 98 and 99% for labelled phenanthrene and benzo[a]pyrene, respectively.

Culture support

The culture support was river sand, 3 to 6 mm diameter, heated to 65°C for 1 h in 10% (v/v) H_2O_2, rinsed with distilled water and heated overnight at 105°C before use.

Microorganisms and culture conditions

Bjerkandera sp. strain BOS 55 was a kind gift from Dr J.A. Field (Dept. of Industrial Microbiology, Wageningen Agricultural University, The Nederlands). *Cunnighamella elegans* (ATCC 26269), *Lentinus lepideus* (ATCC 12653) and *Postia placenta* (ATCC 11538) were obtained from the American Type Culture Collections. *Bjerkandera adusta* CBS 595-78 and *Aspergillus niger* 28816 MUCL were purchased from the Centraal Bureau voor Schimmelculture (The Nederlands) and the Mycotech of the Université Catholique de Louvin la Neuve (Belgium), respectively. *Pycnoporus cinnabarinus* SS 16 and SS 39, as well as *Trametes versicolor* (ATCC 32745) and *Phanerochaete chrysosporium* BKM-F-1767 (ATCC 24725) originated from the culture collection of INRA Marseille-Luminy. Culture medium was from Gross *et al.* (1990) and Lesage-Meessen *et al.* (1996). Carbon sources were 5g glucose for biomass production and 20g maltose for PAH degradation. A modified medium was used for *P. chrysosporium* cultures (Mougin *et al.*, 1994). Carbon source was 10g glycerol for biomass production and PAH degradation. Biomass production was achieved in Roux flasks developed from 5 agar discs (5 mm) of mycelium.

Biodegradation assays

Two mats were harvested and ground with an Ultra Turrax (10 000 rpm) in 100 ml distilled water. The mycelium homogenate was used to inoculate 150 ml Erlenmeyer flasks containing 10 ml medium (liquid cultures) and 10 ml medium in 35g sand (wet sand cultures). The Erlenmeyer flasks were sealed with cotton stoppers and incubated in one liter sealed flasks in the presence of 10 ml vials containing NaOH M and water (Mougin *et al.*, 1997). Cultures were maintained without shaking at

25°C in the dark. The flasks were flushed with ambient air at the beginning of the incubations, and then aerated twice a week. Each Erlenmeyer was supplied with 35 mg phenanthrene l^{-1} and 35 mg benzo[a]pyrene l^{-1} consisting in a mixture of labelled (3.3 kBq of each compound) and unlabelled chemicals dissolved in 100 µl acetone. Three prepared flasks were used for each timepoint. Results are expressed as means. The standard deviation was less than 10% of the mean.

Analytical procedures for PAH compounds

The extent of PAH transformation was determined by extracting the cultures at the beginning of the assays and after 28 days of incubation, and HPLC analysis. Dichloromethane (20 ml) was added to each flask sealed with a rubber stopper, and the extraction was carried out for 1 h at room temperature with shaking in the dark. After filtration of the cultures on a stainless steel sieve (0.5 mm) to retain solid materials, aqueous and organic phases were separated with a separatory funnel. A second extraction of sand and mycelium was then performed. Organic phases were pooled, concentrated and dissolved in 500 µl acetonitrile. Aliquots were filtered through a 0.2µm nylon membrane and injected in HPLC. Elution of chemicals was then achieved onto the column Supelcosil LC-PAH (15 cm × 4.6mm *id*) with a pump delivering a solvent system composed of acetonitrile and water, each acidified with 0.5g phosphoric acid l^{-1}. Elution began with 45 % acetonitrile for 1 min, followed by a linear increase to 100 % of acetonitrile over 13 min, and a stationary phase of 2 min. The flow rate was 1.5 ml min^{-1}. Radioactivity and UV absorbance (254 nm) of the column eluate were monitored. PAH mineralisation was measured by liquid scintillation counting of the NaOH solution.

RESULTS

Evaluation of wet sand as a screening system

The amounts of phenanthrene and benzo[a]pyrene remaining in the organic extracts from liquid and wet sand cultures after 28 days of incubation are shown in Table 1. In the sterile controls from liquid cultures, phenanthrene amounted to only 15% of its initial value while its amount was 50% in wet sand cultures. Benzo[a]pyrene levels corresponded to 100 and 90% of their initial amounts in liquid and wet sand cultures, respectively, after 28 days incubations.

Phenanthrene and benzo[a]pyrene amounts were decreased in the presence of the fungal strains when compared to initial values. Except in the presence of *P. chrysosporium*, phenanthrene levels in liquid cultures were very low (in the range of the levels related to volatilisation), and the extent of the degradation was difficult to quantify. In wet sand cultures, phenanthrene levels were 3 to 6 fold higher and were clearly lower than the control levels, indicating biodegradation. No phenanthrene was detected in *C. elegans* liquid cultures, whilst an appreciable amount of chemical (12%) was quantified in wet sand cultures. In general, benzo[a]pyrene amounts were not decreased when compared with the control in the liquid cultures. At the opposite, no or little chemical was found in the presence of *C. elegans* and *B. BOS 55*. In wet sand cultures, amounts of benzo[a]pyrene were in general lower when compared to these of the liquid cultures, and were also lower to the corresponding control. In *C. elegans* and *B. BOS 55* cultures, 53 and 67% of the initial chemical amount were still noticed after 28 days incubations, respectively.

Exocellular enzymatic activities have been measured after 14 days incubations in the culture media (data not shown). They represented 3, 10 and 4 nkat/ml in liquid

cultures for lignin peroxidase (*P. chrysosporium*), laccase (*P. cinnabarinus* SS16) and manganese-dependent peroxidase (*B. BOS* 55), respectively. The three enzymes were detected in wet sand cultures, but with lower activities.

Use of wet sand as a screening system

Table 2 reports the mass-balance analysis of the cultures for the control and ten fungal strains in 28 days incubations treated with the two PAHs. ^{14}C recovery in the sterile controls was 100% at the beginning of the experiments, showing the efficiency of our simple protocol. It was 74% after 28 day incubations. The loss of radioactivity is mainly attributed to phenanthrene volatilisation (quantified with traps, unpublished results). In most cases in the presence of the fungi, ^{14}C recovery showed values comprised between 61 and 71% of initial radioactivity. The highest recoveries were obtained with *L. lepideus* and *P. placenta* (80% and 86%).

No or little PAH mineralisation was oberved.

No metabolites were detected following extraction by dichloromethane in both organic and aqueous phases from control cultures, also confirming the hypothesis of volatilisation. For all strains, most of the radioactivity was associated with organic extractable compounds, mainly as residual benzo[a]pyrene. These results strengthened the data shown Table 1 after analysis of the organic extracts. Small amounts of organic soluble metabolites were also noticed during the incubations, with a maximal value of 6% obtained with *T. versicolor*. By contrast, water-soluble compounds ranged from 1 to 22% of the initial radioactivity, the highest value being obtained with *C. elegans.* Radioactivity associated with sand and mycelial fragments following solvent extraction represented 4% in the controls, and 10 to 17% in the presence of fungi.

DISCUSSION

The data presented above indicated that the filamentous fungi known as able to transform individual PAHs in liquid cultures were also able to degrade a mixture of PAHs during incubations on wet sand cultures.

However, the results obtained with liquid and wet sand cultures are different due to culture conditions modulating chemical bioavailability and degradation catalysis. Sufficient PAH bioavailability was ensured by the low interactions of the chemicals with the sand. Yet, it is likely that the sand decreased the liquid medium/air interface and then limited phenanthrene volatilisation. Consequently, wet sand offered much more reliable and useful controls than liquid cultures, especially in the presence of volatile chemicals. To our opinion, this last system gave also a more realistic image of the biodegradation occurring in soils especially in allowing the fungi to growth in a triphasic system, acting on enzyme production and fungal metabolism.

Wet sand screening showed that the most efficient strains to degrade PAHs were *T. versicolor* and *C. elegans*. The results also confirmed the relative recalcitrance of benzo[a]pyrene towards biodegradation as compared to phenanthrene. It is also noteworthy than the levels of metabolites remained lower than these obtained in classical liquid cultures.

Our screening system with wet sand appears as an efficient tool to evaluate fungal abilities to degrade xenobiotics in mimicing natural systems, but with easier analysis procedures. The technique (or some variant of it) could be used with other chemicals and/or fungi.

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Table 1 Recovery of phenanthrene and benzo[a]pyrene in liquid and wet sand cultures after 28 days incubations.

	Remaining polycyclic aromatic hydrocarbons (% of initial amounts)			
	Phenanthrene		Benzo[a]pyrene	
Fungal strains	liquid	wet sand	liquid	wet sand
Sterile controls	15	50	100	90
Zygomycete				
Cunninghamella elegans	0	12	0	53
Deuteromycete				
Aspergillus niger	1	6	100	92
Basidiomycetes				
Bjerkandera BOS 55	0	14	16	67
Phanerochaete chrysosporium	22	51	90	75
Pycnoporus cinnabarinus SS16	10	34	100	72

Table 2 Percentage distribution of initial radioactivity in fractions of 28 days cultures treated with a mixture of phenanthrene and benzo[a]pyrene.

Fungal strains	Organic soluble compounds (%)			Water soluble compounds (%)	Compounds associated to solid phase (%)	CO_2 (%)	^{14}C recovery (%)
	Phenanthrene	Benzo[a]pyrene	Metabolites				
Initial amounts	50	50	0	0	0	0	100
Sterile controls	25	45	0	0	4	0	74
Zygomycete							
Cunninghamella elegans	6	26	2	22	12	0.0	69
Deuteromycete							
Aspergillus niger	3	46	1	7	12	0	69
White-rot fungi							
Bjerkandera adusta	10	39	2	9	14	0.2	75
Bjerkandera BOS 55	7	34	1	9	17	2.1	70
Phanerochaete chrysosporium	26	37	2	3	10	0	78
Pycnoporus cinnabarinus SS16	17	36	1	4	13	0	71
Pycnoporus cinnabarinus SS39	14	29	1	4	14	0	61
Trametes versicolor	7	28	6	11	12	0.5	64
Brown-rot fungi							
Lentinus lepideus	28	40	0	1	10	0.0	80
Postia placenta	29	45	0	1	10	0	86