Biodegradation of polycyclic aromatic hydrocarbons by *Pichia anomala*

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Abstract

Pichia anomala 2.2540, isolated from soil contaminated by crude oil, degraded naphthalene, dibenzothiophene, phenanthrene and chrysene, both singly and in combination. The yeast degraded 4.5 mg naphthalene l⁻¹ within 24 h. Phenanthrene was degraded after a lag of 24 h. When a mixture of all four polycyclic aromatic hydrocarbons was treated at either 0.1–1.6 mg l⁻¹ or 3.1–5.3 mg l⁻¹, naphthalene was completely degraded first within 24 h, followed by phenanthrene and dibenzothiophene after 48 h. Chrysene, which remained in the mixture even after 96 h, could be degraded along with naphthalene. Chrysene at 0.7 and 1 mg l⁻¹, in the presence of 4.3 and 65 mg naphthalene l⁻¹, respectively, was removed within 96 h.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are formed mainly during the incomplete combustion of organic material. They are also present in petroleum oil. The release of PAHs into the soil and water environments may pose a threat to food safety, as PAHs are recalcitrant and are potentially hazardous.

Many authors have studied PAH-degrading microorganisms usually using either co-metabolism or low PAH concentrations (Cerniglia 1992, Chaineau *et al.* 1995). However, some bacteria and fungi have been isolated that utilize PAHs as sole carbon and energy sources (Mueller *et al.* 1990, Romero *et al.* 1998).

In this study, *Pichia anomala* 2.2540 was isolated from soil contaminated by weathered crude oil. This yeast uses naphthalene and phenanthrene as sole carbon sources but also degrades dibenzothiophene and chrysene through co-metabolism. Here we report the degradation of these four PAHs, either singly or in combination.

Materials and methods

Substrates

The polycyclic aromatic hydrocarbons (PAHs) used in this study are given in Table 1. All were of the highest purity available (Acros Organic Co.).

Isolation and identification of PAH-utilizing yeast

Samples of soil contaminated with weathered crude oil were taken from Jidong Oilfield in Hebei Province, China. About 1 g soil was suspended in 10 ml sterile water and added to a mineral basal salts (MBS) medium supplemented with 0.5% naphthalene at 27 °C at 150 rpm for 10 days. Yeast strains were isolated from MBS agar plates coated with naphthalene as the sole carbon source (Romero *et al.* 1998). The isolates were further transferred to phenanthrene-, dibenzothiophene- or chrysene-coated plates, respectively. The 26S rDNA D1/D2 domain sequence was determined to identify the yeast (Kurtzman & Robnett 1998).

Table 1. Properties of polycyclic aromatic hydrocarbons used in this study.

PAH	Structure	Aqueous solubility at 25 °C (mg l ⁻¹)
Naphthalene		31.7
Phenanthrene		1.29
Dibenzothiophene		Soluble
Chrysene		0.002

Degradation of PAHs in liquid cultures

For each PAH, 100 mg was dissolved in 10 ml acetone, added to 50 g Tween 80 in 50 ml distilled water, ultrasonicated, and the suspension diluted with water to make 200 ml (Andres *et al.* 1999).

In all experiments, 5 ml batch cultures grown in 40 ml sample tubes with screw caps and Teflon-lined septa. The isolate was pre-cultivated at 150 rpm at 27 °C in the following basal medium (BM): KH₂PO₄ 1 g, (NH₄)₂SO₄ 0.5 g, MgSO₄·7H₂O 0.5 g, yeast extract 0.5 g, distilled water 1000 ml, pH 5–6, supplemented with 10 g glucose (Yang *et al.* 2003). Cells were harvested by centrifugation, washed twice with sterile BM and inoculated into the modified BM supplemented with sole or mixtures of PAHs as the carbon source. The inoculum was controlled to approx. 2.4×10^8 cells ml⁻¹. Heat-killed cells (autoclaved) and non-inoculated flasks were used as negative and blank controls. Cultures were grown in the dark with tumble-shaking at 150 rpm and 27 °C (McNally *et al.* 1999).

All experiments were run in triplicate.

Analysis

Five ml hexane was added to each culture, shaken horizontally for 2 h at 150 rpm and approx. 2 ml of the hexane layer was placed in an autosampler vial, crimp-sealed with a Teflon-lined cap, then stored at -20 °C until analysis (Leblond *et al.* 2001). PAH concentrations were analyzed by gas chromatography interfaced

Fig. 1. Degradation of sole PAHs by *P. anomala*. The initial concentration of each PAH was: naphthalene, 4.5 mg l⁻¹; dibenzothiophene, 3.8 mg l⁻¹; phenanthrene, 5.2 mg l⁻¹; chrysene, 5.7 mg l⁻¹. Each point represents mean $\pm$ S.E. of triplication.

with mass spectrometer detector (GC/MSD) in single ion monitoring (SIM) mode.

Results and discussion

Isolation and identification of PAHs utilizing yeast

Ten yeast strains were isolated from naphthalene-coated agar plates. One isolate, which rapidly degraded in liquid medium, was selected for further study. It could grow on agar plates coated with phenanthrene or dibenzothiophene but not on those coated with chrysene. The isolate has been lodged in the China General Microbiological Culture Collection Center (CGMCC) as CGMCC 2.2540. The BLAST search result through GenBank indicated that the D1/D2 sequence of this strain was identical to that of the type strain of *Pichia anomala*.

Degradation of PAHs in liquid cultures

Degradation of sole PAHs by *P. anomala* 2.2540 in liquid cultures is shown in Figure 1. A total of 3.1 mg phenanthrene l⁻¹ was removed within 96 h following a lag time of 24 h. No obvious degradation of dibenzothiophene and chrysene was detected over the cultivation period although the isolate could grow on agar plates coated with dibenzothiophene.

Figure 2 shows the results when *P. anomala* was grown in a mixture of four PAHs at two concentrations. The degradation of dibenzothiophene was observed at each concentration after 48 h. The co-existence of naphthalene may be helpful for the degradation of dibenzothiophene. Two *Pichia anomala* strains have previously been reported to degrade the

Fig. 2. Degradation of PAHs by *P. anomala* in mixture solutions at different concentrations. Initial concentrations of PAHs: (A) 0.1–1.6 mg l⁻¹; (B) 3.1–5.3 mg l⁻¹. Each point represents mean $\pm$ S.E. of triplication.

biaryllic compound, dibenzofuran, which has a structure similar to that of dibenzothiophene (Romero *et al.* 2002).

Figure 2A shows that 0.8 mg phenanthrene l⁻¹ and 0.7 mg dibenzothiophene l⁻¹ were completely degraded within 96 h. Figure 2B shows that similar amounts of phenanthrene and dibenzothiophene were degraded within 96 h at higher substrate concentrations, indicating that the concentrations in this experiment were not a factor inhibiting PAH degradation. The degradation rate of phenanthrene in the combined PAH solutions, however, was lower than that in sole PAH solutions (Figure 1), indicating the possible occurrence of competitive inhibition between dibenzothiophene and phenanthrene. Chrysene could not be degraded over 96 h at either concentration.

Although chrysene was not degraded in the mixtures containing the four PAHs, it was significantly degraded when mixed with naphthalene only. With naphthalene at 4.3 mg l⁻¹, 0.7 mg chrysene l⁻¹ could be removed within 96 h after a lag time of 24 h (Figure 3A). However, with naphthalene at 64.7 mg l⁻¹,

Fig. 3. Degradation of binary mixtures of naphthalene and chrysene by *P. anomala* at different naphthalene concentrations. (A) 4.3 mg l⁻¹; (B) 64.7 mg l⁻¹. Each point represents mean $\pm$ S.E. of triplication.

no lag period was observed before the chrysene was degraded (Figure 3B) and 38 mg naphthalene l⁻¹ and 1 mg chrysene l⁻¹ were removed within 96 h. Chrysene was possibly being removed through a co-metabolism mechanism.

A bacterium, *Pseudomonas paucimobilis* EPA505, which could utilize fluoranthene as the sole source of carbon and energy for growth, failed to grow in the saturated liquid culture of naphthalene (Mueller *et al.* 1990). Six PAH-degrading bacteria strains were isolated using six different PAHs as sole carbon and energy sources and naphthalene showed limited co-metabolism capacities for other PAHs (fluorine, phenanthrene, anthracene, fluoranthene and pyrene) but was toxic to bacterial strains isolated on other compounds (Bouchez *et al.* 1995). In both cases, the high aqueous solubility of naphthalene (see Table 1) had apparently inhibited the growth of bacteria. In this present study, however, naphthalene as high as 64.7 mg l⁻¹ did not inhibit *P. anomala* but promoted the degradation of chrysene. It is possible that naphthalene was inducing the enzymes required for the degradation of chrysene, and a higher enzyme concentration was produced under higher naphthalene concentration. The competitive inhibition between

dibenzothiophene and chrysene may also explain why chrysene was not degraded in the mixtures of the four PAHs.

Conclusion

Pichia anomala degraded four PAHs, either through direct metabolism (for naphthalene and phenanthrene) or co-metabolism (for dibenzothiophene and chrysene). The isolate tolerated 65 mg naphthalene l⁻¹ and effectively degrade chrysene in the presence of naphthalene. Chrysene degradation, however, was not observed in solutions containing the above four PAHs over 96 h.

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