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Evaluation of a creosote-based medium for the growth and preparation of a PAH-degrading bacterial community for bioaugmentation

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Creosote was evaluated as an inexpensive carbon source for growing inocula of a polycyclic aromatic hydrocarbon (PAH)-degrading bacterial community (community five). Creosote was a poor growth substrate when provided as sole carbon source in a basal salts solution (BSM). Alternatively, peptone, yeast extract or glucose in BSM supported high growth rates, but community five could not subsequently degrade pyrene. A combination of creosote and yeast extract in BSM (CYEM) supported growth and maintained the pyrene-degrading capacity of community five. Optimum pyrene-degrading activity occurred when the inocula were grown in creosote and yeast extract concentrations of 2 ml L^{-1} and 1 g L^{-1} respectively: concentrations outside these values resulted in either low biomass yields or loss of PAH-degrading activity. CYEM-grown community five inocula degraded 250 mg L^{-1} of pyrene in BSM at a rate comparable to cultures inoculated with community five grown in BSM-pyrene. However, the CYEM-grown community showed a 40% lower rate of PAH degradation in a synthetic PAH mixture compared with pyrene-grown cells and there was an increase in the lag period before the onset of PAH degradation. This appears to reflect a weaker induction of PAH catabolism by CYEM compared to BSM-pyrene. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 277–284.

Keywords: polycyclic aromatic hydrocarbons; creosote; bioremediation; bioaugmentation; biodegradation; inoculum preparation

Introduction

Bioaugmentation is the addition of microorganisms, with known metabolic capabilities, to polluted sites to enhance the biodegradation rate of contaminants. Bioaugmentation may be considered when the indigenous microbial population is incapable of detoxifying the contaminated site or when the time required to remediate a site is of primary importance; the addition of a high population of pollutant-degraders can significantly reduce bioremediation times compared to indigenous biodegradation rates [14]. Pollutant-degrading microbial isolates can be used for *in situ* bioaugmentation of contaminated soils and for *ex situ* soil decontamination industries such as landfarming, biopiles and solid-phase bioreactors [20]. With regard to polycyclic aromatic hydrocarbon (PAH)-contaminated sites, a number of microorganisms have been isolated that have considerable potential for bioaugmentation of these sites. Most of these isolates are maintained and grown under laboratory conditions in either minimal or nutrient-rich media containing an appropriate PAH such as fluorene, pyrene, chrysene or fluoranthene [3,4,7,8,10,21] where the purpose of the PAH is to provide selective pressure on the isolates for retaining PAH-degrading capabilities.

The use of laboratory media on a commercial scale for the production of bioaugmentation inocula is expensive due to the high cost of the raw materials, especially pure forms of the PAHs, and the potentially large inoculum volumes required to treat contaminated sites. The development of less expensive media for growing inocula for bioaugmentation would make the process more economically viable. However, prospective media must meet a number of other criteria in addition to lower costs, such as maintaining PAH-degrading activity and supporting reasonable growth rates and biomass yields [19]. This particularly applies to the propagation of inocula for removal of high molecular weight PAHs, since such PAH-degraders are known frequently to lose their capacity to degrade these compounds if selective pressure is not maintained. Indeed, the use of inappropriate media for growing bioaugmentation inocula has been thought responsible in some studies for the subsequent failure of the seeded organisms to improve site remediation [13]. Despite the importance of developing culture media for commercial use that meets the above criteria, this issue has not been adequately addressed.

The objective of the work described in this report was to develop a growth medium for a high molecular weight PAH-degrading microbial community (designated community five) which had previously been isolated and maintained in BSM containing pyrene as the sole carbon and energy source [9,11]. Pyrene as a growth substrate for

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inoculum preparation is too costly for commercial operations. This paper reports the use of creosote as an inexpensive growth substrate for preparing inocula of community five that may be suitable for the bioaugmentation of PAH-contaminated sites.

Materials and methods

Chemicals

Phenanthrene, pyrene, benzo[*b*]fluorene, benz[*a*]anthracene, dibenz[*a*,*h*]anthracene and benzo[*a*]pyrene were purchased from Sigma Chemical Company (St Louis, MO, USA). Fluorene, fluoranthene and all solvents were purchased from Lab Chem (Ajax Chemicals, Sydney, Australia). Creosote was purchased from Sparko, Perth, Australia. Bacterial media and reagents were purchased from Oxoid (Unipath, Hampshire, UK). All solvents and chemicals were high purity grade reagents.

PAH-degrading bacterial community

Community five was isolated from soils obtained from an abandoned factory site located near Port Melbourne, Victoria, Australia. The site previously housed a gas manufacturing plant operation and was recently used as a defence facility site. Community five is comprised of only three microorganisms, all of which are strains of *Stenotrophomonas maltophilia*. All three strains are able to grow on and degrade pyrene as a sole carbon and energy source. Previous studies have shown that community five is capable of degrading fluorene, phenanthrene, fluoranthene, pyrene, benz[*a*]anthracene or dibenz[*a*,*h*]anthracene as sole carbon and energy sources [9,11].

Stock solutions, PAH-containing media and growth conditions

Bacterial growth and degradation studies were performed in a basal salts medium (BSM) [12] containing added PAHs or growth substrates. Stock solutions of pyrene, a PAH mixture and creosote were prepared in dimethylformamide (DMF). Pyrene was prepared at a concentration of 25 mg ml^{-1} while the stock mixture of selected PAHs (fluorene, phenanthrene, fluoranthene, pyrene, benz[*a*]anthracene, dibenz[*a*,*h*]anthracene and benzo[*a*]pyrene) was prepared at a concentration of 5 mg ml^{-1} for each PAH. The creosote had a total hydrocarbon concentration of 710 mg L^{-1} and contained the following PAHs: fluorene (20 mg L^{-1}), phenanthrene (45 mg L^{-1}), fluoranthene (51 mg L^{-1}) and pyrene (60 mg L^{-1}). Creosote solutions were prepared by diluting the creosote stock solution with DMF at ratios of 1:1, 1:10, 1:100, 1:1000 and 1:10 000. Peptone, yeast extract, glucose and succinate stock solutions were prepared in Milli-Q water at a concentration of 50 mg ml^{-1}. Stock solutions were autoclaved at 15 psi for 20 min and added to BSM to achieve a final concentration in the range 0.1–2.0 g L^{-1}. BSM was supplemented with PAHs to achieve a final concentration of 250 mg L^{-1} for pyrene and 50 mg L^{-1} for each PAH in the PAH mixture. Creosote was added to BSM to achieve final concentrations of 2.0, 0.2, 0.02, 0.002 or 0.0002 ml L^{-1}. Unless otherwise stated, cultures were incubated at 30°C and 175 rpm in the dark.

Growth experiments

Inocula for growth studies were incubated for 7 days in BSM (400 ml) containing pyrene (250 mg L^{-1}) as the sole carbon and energy source. To evaluate the effectiveness of alternative carbon sources in sustaining rapid growth, for producing high biomass yields and for the induction of PAH catabolism, pyrene-enriched cultures (5 ml) were inoculated into BSM (45 ml) containing one of peptone, yeast extract, glucose, succinate (each at 1 g L^{-1}) or creosote (2 ml L^{-1}). Cultures were subcultured after 3 days incubation (30°C/175 rpm) for three successive subcultures. Microbial growth was monitored by measuring the increase in bacterial protein concentration; cells were collected from 10 ml of culture by centrifugation (3800 × *g* for 10 min) and washed twice in Ringers solution. Cell pellets were resuspended in 1.0 ml 4.6 M NaOH and boiled for 10 min to lyse cells, then protein concentrations were measured by the method of Lowry *et al* [15]. Growth of community five was also investigated using creosote (2.0, 0.2, 0.02, 0.002 and 0.0002 ml L^{-1}) plus one of peptone, yeast extract or glucose (0.1, 0.5, 1.0 and 2.0 g L^{-1}).

PAH degradation by bacterial inocula

Following growth on various substrates, community five was tested for its ability to grow on and degrade PAHs as sole carbon and energy sources in BSM. Inocula were used after incubating them for 30 h (peptone, yeast extract, glucose and succinate substrates in BSM), 3 days (creosote in BSM) or 7 days (pyrene in BSM). Serum bottles (30 ml) containing 9.0 ml of BSM were inoculated with 1.0 ml of unwashed community five inocula grown on the various substrates. Killed-cell control cultures were prepared by inoculating cells that had been killed with 0.2% $HgCl_2$ for 24 h into BSM containing PAHs [3]. Cultures in pyrene-BSM were incubated and samples were removed for analysis at 3, 7, 10, 14 and 21 days. Degradation of a synthetic PAH mixture (50 mg L^{-1} of each PAH) was evaluated using CYEM- or BSM-pyrene-grown inocula and sampled at 3, 7, 10, 14, 21, 28 and 42 days. Growth at the expense of PAHs was established by an increase in protein concentration and a decrease in PAH concentration. PAH degradation experiments were performed in triplicate for each set of culture conditions.

Analytical methods

PAHs were extracted from bacterial culture fluids with dichloromethane (DCM) as described previously [11]. Gas chromatographic analysis of DCM extracts and PAH standards was performed on a Varian Star 3400 gas chromatogram equipped with a flame ionisation detector (GC-FID), using a BPX-5 capillary column (25 m × 0.22 mm, SGE, Melbourne, Australia). The oven temperature was programmed at 50°C for 1 min, followed by a linear increase of 10°C min^{-1} to 320°C, holding at 320°C for 5 min. Injector and detector temperatures were maintained at 300°C.

Results

Effects of substrate on subsequent PAH degradation

Various carbon substrates were added to BSM and the media were compared for their ability to support com-

munity five growth to early stationary phase. Growth rates and biomass yields on peptone, yeast extract, glucose or succinate by community five were high compared to those observed on pyrene or creosote (Figure 1a). Microbial protein concentrations in media containing glucose, peptone, yeast extract or succinate were in the range 120–210 mg L^{-1} after 30 h, which was over 10 times greater than the biomass concentration in cultures grown on pyrene for the same time period. The biomass concentration of cultures grown on BSM-pyrene increased from 4.7 to 10.2 mg L^{-1} during the first 30 h and then to 53 mg L^{-1} after 168 h. The greatest biomass yield was obtained in cultures growing on glucose. Stationary phase was reached at around 24 h for cultures grown on peptone, yeast extract, glucose or succinate, but BSM-pyrene-grown cultures did not reach stationary phase until around 170 h incubation (data not shown). The growth of community five on creosote was limited with the biomass concentration increasing from 2.9 mg L^{-1} to 12.9 mg L^{-1} over a 30-day period (data not shown).

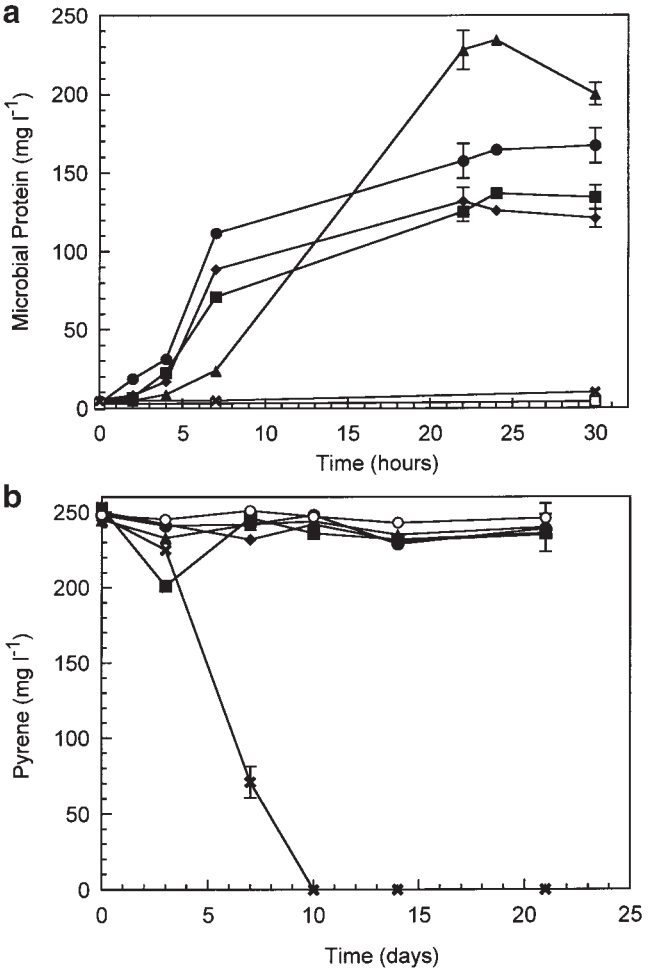

Figure 1 (a) Growth of community five in BSM containing either 1 g L^{-1} peptone (■) 1 g L^{-1} yeast extract (●), 1 g L^{-1} glucose (▲), 1 g L^{-1} succinate (◆), 2 ml L^{-1} creosote (□) or 250 mg L^{-1} pyrene (×). Media were inoculated with 1% unwashed pyrene-grown cells. (b) Pyrene degradation by community five inocula grown on the above substrates. BSM containing pyrene (250 mg L^{-1}) was inoculated with a 10% unwashed inoculum prepared from the cultures shown in panel (a); creosote-grown inocula were not used due to the low biomass concentration in the inoculum. The symbols for each culture reflect the source of the inoculum from panel (a). The pyrene concentration (○) in a killed control culture is also shown. All incubations were at 30°C and 175 rpm in the dark.

An unwashed inoculum was prepared from each of the cultures and added at 10% to BSM containing pyrene (250 mg L^{-1}) as the sole carbon source. Inocula from cultures grown on peptone, yeast extract, glucose or succinate in BSM were prepared after 30 h incubation and inocula from BSM-pyrene were prepared after 168 h incubation. The biomass levels in the inocula varied depending on the community five biomass yields in the different parent cultures, ie glucose-grown inocula had a biomass concentration which was approximately 4-fold greater than pyrene-grown inocula. Inocula from parent cultures containing peptone, yeast extract, glucose or succinate were unable to degrade pyrene (Figure 1b) in BSM during a 21-day period. Pyrene-grown inocula degraded all the pyrene in 10 days despite having the lowest initial biomass concentration. Pyrene degradation tests for creosote-grown inocula were not performed, as the cell population in these cultures was too small.

Evaluation of nutrient media supplemented with creosote

Although peptone, yeast extract, succinate and glucose promoted rapid growth and high biomass yields, inocula prepared from such cultures could not degrade pyrene as a sole carbon source. Conversely, creosote did not support a reasonable microbial growth rate, but its chemical composition would most likely provide the necessary selective pressure to propagate a community five inoculum with a primed PAH catabolism. In order to stimulate microbial growth and maintain active PAH catabolism in inoculum cultures, peptone, yeast extract or glucose was added to BSM containing creosote.

Community five growth rates on these substrate combinations were around 1.5 times greater than growth rates achieved in BSM cultures containing only pyrene (Figure 2a). After 96 h, nutrient-enriched creosote cultures attained protein concentrations of around 42 mg L^{-1} compared to 28 mg L^{-1} in BSM-pyrene cultures. A 10% unwashed inoculum from each of these cultures was transferred into BSM containing pyrene. All of these inocula were able to degrade pyrene as the sole carbon and energy source. The pyrene degradation rate by community five grown on yeast extract and creosote was comparable to that observed using pyrene-grown inocula (Figure 2b), with all the pyrene degraded in around 10 days. The pyrene degradation rate by inocula grown in creosote plus either glucose or peptone was slightly lower; however, pyrene was degraded to undetectable levels after 14 days.

Optimisation of creosote concentration

The combination of BSM, creosote and yeast extract was selected for further studies since inocula grown in this medium had a slightly higher pyrene degradation rate compared to inocula grown in BSM-creosote containing glucose or peptone.

The optimum creosote concentration in inoculum cultures required to achieve a high biomass yield and maintain active PAH catabolism was evaluated by adding different amounts of creosote, in the range 0.0002–2 ml L^{-1}, to BSM

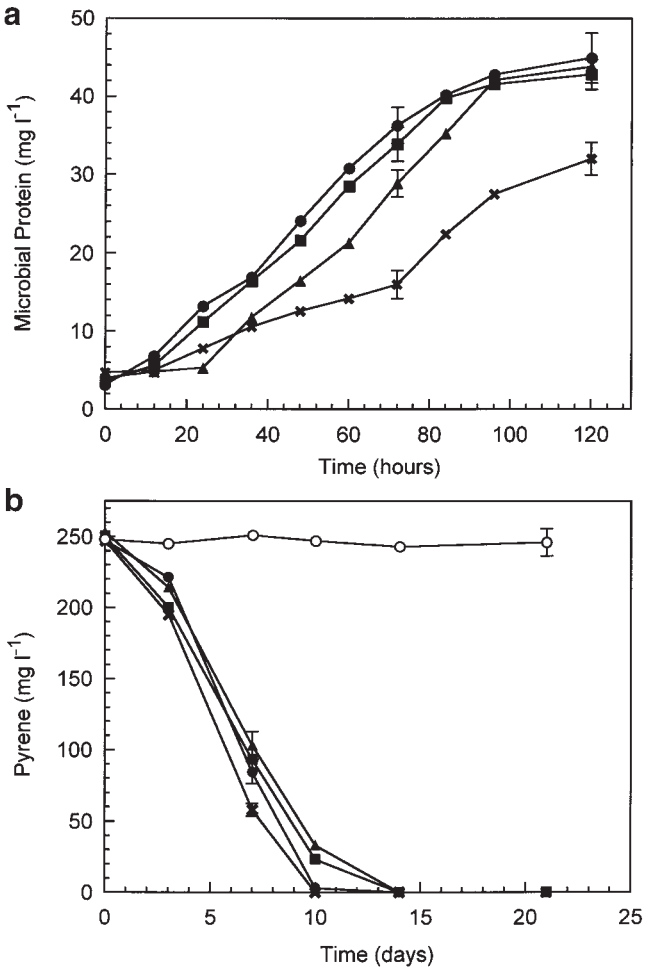

Figure 2 (a) Growth of community five in BSM containing 250 mg L^{-1} pyrene (×) or 2 ml L^{-1} creosote combined with 1 g L^{-1} peptone (■), 1 g L^{-1} yeast extract (●) or 1 g L^{-1} glucose (▲). Media were inoculated with 1% unwashed pyrene-grown cells. (b) Pyrene degradation by community five inocula grown on the above substrates. BSM containing pyrene (250 mg L^{-1}) was inoculated with a 10% unwashed inoculum prepared from the cultures shown in panel (a). The symbols for each culture reflect the source of the inoculum from panel (a). The pyrene concentration (○) in a killed-cell control culture is also shown. All incubations were at 30°C and 175 rpm in the dark.

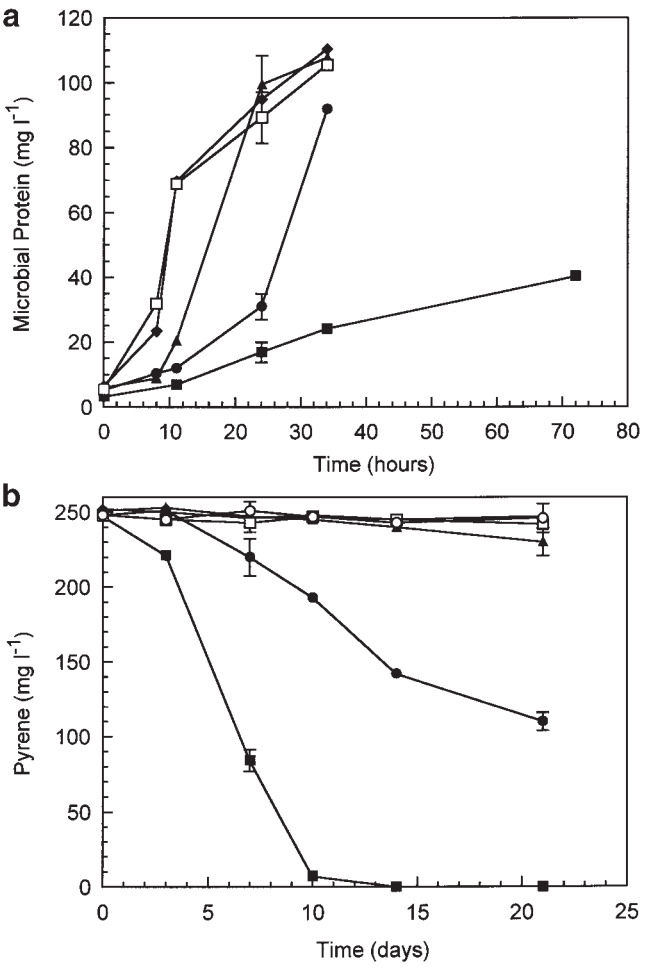

Figure 3 (a) Growth of community five in BSM containing 1 g L^{-1} yeast extract and creosote at: 2 ml L^{-1} (■), 0.2 ml L^{-1} (●), 0.02 ml L^{-1} (▲), 0.002 ml L^{-1} (◆) or 0.0002 ml L^{-1} (□). Media were inoculated with 1% unwashed pyrene-grown cells. (b) Pyrene degradation by community five inocula grown on the above substrates. BSM containing pyrene (250 mg L^{-1}) was inoculated with a 10% unwashed inoculum prepared from the cultures shown in panel (a). The symbols for each culture reflect the source of the inoculum from panel (a). The pyrene concentration (○) in a killed-cell control culture is also shown. All incubations were at 30°C and 175 rpm in the dark.

containing 1 g L^{-1} of yeast extract. The highest growth rates for community five occurred in media containing the lower creosote concentrations (0.02, 0.002 and 0.0002 ml L^{-1}) with biomass concentrations reaching 90–110 mg L^{-1} after 36 h (Figure 3a), but subsequent cultures in BSM-pyrene did not significantly degrade pyrene after 21 days. Creosote concentrations above 0.02 ml L^{-1} were more inhibitory to microbial growth and at the highest concentration of creosote (2.0 ml L^{-1}) the microbial protein concentration reached only 40 mg L^{-1} after 72 h. However, a 56% decrease in pyrene concentration after 21 days was observed using community five inocula grown in BSM containing yeast extract and 0.2 ml L^{-1} creosote. The highest pyrene degradation rate (97% pyrene degraded in 10 days) occurred when a community five inoculum grown in yeast extract and the highest creosote concentration (2 ml L^{-1}) was used. These results indicate that the lower creosote concentrations, in the presence of yeast extract, failed to support pyrene-degrading activity in community five. It is also apparent that high biomass yields did not necessarily equate with increased pyrene degradation rates.

Optimisation of yeast extract concentration

Community five was grown in BSM containing creosote (2 ml L^{-1}) and yeast extract at concentrations of 0.1, 0.5, 1.0 or 2.0 g L^{-1}. Microbial growth at the lower yeast extract concentrations (0.1 and 0.5 g L^{-1}) was typified by low growth rates and biomass yields; biomass concentrations reached 6 mg L^{-1} and 18 mg L^{-1} respectively after 96 h from initial biomass concentrations of 1.75–4.7 mg L^{-1} (Figure 4a). A higher growth rate occurred in community five cultures containing 1.0 and 2.0 g L^{-1} yeast extract with biomass concentrations reaching 65 mg L^{-1} and 110 mg L^{-1} respectively after 30 h; microbial growth slowed considerably in these cultures after 30 h incubation.

A 10% unwashed inoculum was prepared from these cul-

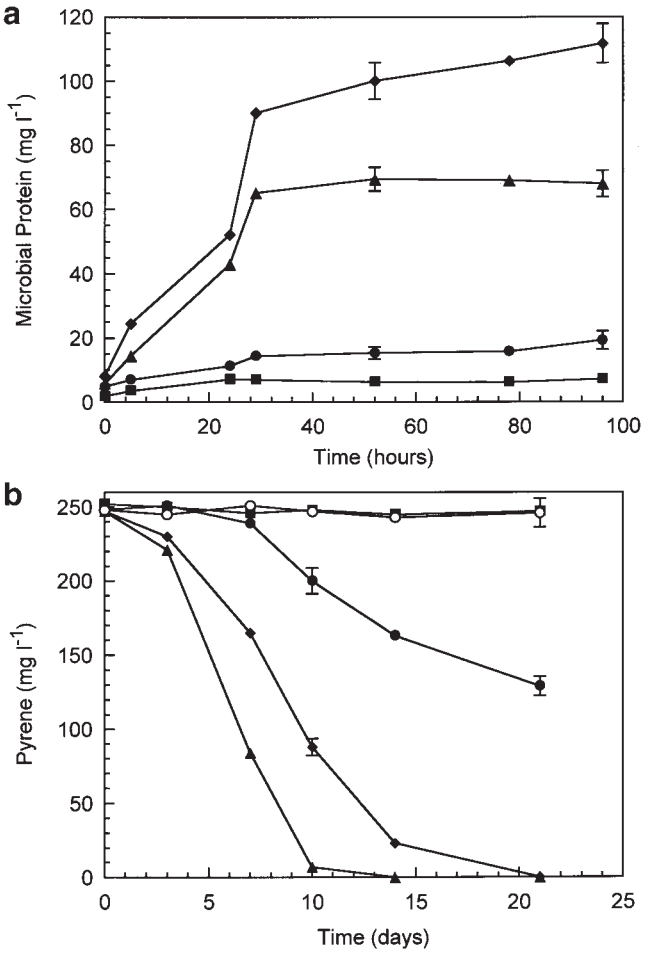

Figure 4 (a) Growth of community five in BSM containing 2 ml L^{-1} creosote and yeast extract at: 0.1 g L^{-1} (■), 0.5 g L^{-1} (●), 1.0 g L^{-1} (▲) and 2.0 g L^{-1} (◆). Media were inoculated with 1% unwashed pyrene-grown cells. (b) Pyrene degradation by community five inocula grown on the above substrates. BSM containing pyrene (250 mg L^{-1}) was inoculated with a 10% unwashed inoculum prepared from the cultures shown in panel (a). The symbols for each culture reflect the source of the inoculum from panel (a). The pyrene concentration (○) in a killed-cell control culture is also shown. All incubations were at 30°C and 175 rpm in the dark.

tures and added to BSM containing only pyrene. There was no significant pyrene degradation by community five inocula grown in BSM containing yeast extract (0.1 g L^{-1}) and creosote (Figure 4b). The pyrene degradation rate by community five inocula grown in BSM containing creosote (2.0 ml L^{-1}) and yeast extract at concentrations of 0.5 or 1.0 g L^{-1} increased with increasing yeast extract concentration. The rate of pyrene degradation by community five inocula grown in BSM containing creosote and 1.0 g L^{-1} yeast extract was comparable to the pyrene degradation rate of inocula grown in BSM-pyrene (Figure 1b). Inocula grown in BSM containing creosote and 2.0 g L^{-1} yeast extract had a lower pyrene degradation rate despite having a higher biomass level. The higher nutrient concentration in this case negatively affected the activity of the pyrene catabolism of community five inocula.

Degradation of a synthetic PAH mixture by community five grown on yeast extract and creosote

Community five was grown in BSM containing 2 ml L^{-1} creosote and 1 g L^{-1} yeast extract (CYEM) and a 10% unwashed inoculum was then added to a synthetic PAH mixture in BSM. A 10% unwashed community five inoculum was also prepared from BSM-pyrene and added to BSM containing a synthetic PAH mixture. The synthetic PAH mixture comprised PAHs identified in the creosote (fluorene, phenanthrene, fluoranthene and pyrene) as well as some higher molecular weight compounds (benz[*a*]anthracene, dibenz[*a,h*]anthracene and benzo[*a*]pyrene). The concentration of each component (50 mg L^{-1}) approximately represented the concentrations of phenanthrene, fluoranthene and pyrene measured in the creosote.

Long degradation lag periods were observed for the CYEM-grown cells when inoculated into BSM containing the synthetic PAH mixture (Table 1). Degradation lag periods were approximately 10–14 days for the three-ring compounds, 14–21 days for the four-ring compounds and 28 days for the five-ring compounds. However, the pyrene-grown inocula generally had shorter degradation lag periods of 3–7 days for the three- and four-ring PAHs; benz[*a*]anthracene was the exception with a degradation lag period of 7–14 days. Degradation of the five-ring PAHs by the pyrene-grown inocula commenced after 14–28 days.

In addition to the longer degradation lag periods, CYEM-grown inocula degraded the PAHs at a slower rate than the pyrene-grown inocula. The combination of longer lag periods and slower degradation rates by CYEM-grown inocula resulted in lower amounts of each PAH (27–37% less phenanthrene, fluoranthene pyrene and benz[*a*]anthracene, and 47–54% less fluorene, dibenz[*a,h*]anthracene and benzo[*a*]pyrene) degraded by this inocula after 42 days compared to the pyrene-grown cells. The CYEM-grown cells were able to grow in the PAH mixture albeit slowly (Figure 5). Pyrene-grown inocula grew in the PAH mixture at a faster rate and with a shorter lag period than CYEM-grown inocula (Figure 5).

Discussion

The success of a bioaugmentation program for a PAH-contaminated site depends on, among other factors, the characteristics of the microbial inocula. In this regard it is important that the inoculum has a high concentration of cells in good physiological condition and which have PAH catabolic capabilities [19]. Such features of the inoculum are largely influenced by the medium used to grow the biomass. Previous laboratory experiments with community five had demonstrated that these inoculum characteristics are satisfied if the cells were grown in BSM-pyrene [11]; however, for economic reasons this medium is not practical for commercial use. In developing a less expensive commercial-scale inoculum medium, this study demonstrated that PAHs are an essential medium component if community five is to have an active PAH catabolism upon inoculation. Media comprising only glucose, yeast extract, peptone or succinate in BSM stimulated high growth rates and biomass yields, but this was at the expense of PAH-degrading activity. On the other hand, BSM-creosote did

Table 1 Degradation of a PAH mixture by community five inocula grown in either BSM-pyrene or BSM-creosote-yeast extract

PAH[a]	Inoculum[b]	PAH degraded (%)[c]				Killed-cell control (42 days)[d]
		7 days	14 days	28 days	42 days	
Fluorene	BSM-pyrene	23.2 ± 0.0	53.4 ± 0.0	72.3 ± 0.0	76.2 ± 0.0	4.9 ± 0.0
	CYEM	2.7 ± 0.0	11.9 ± 0.0	22.8 ± 0.0	35.5 ± 0.0	
Phenanthrene	BSM-pyrene	11.0 ± 0.0	25.1 ± 0.0	61.3 ± 0.0	65.8 ± 0.0	1.4 ± 0.0
	CYEM	2.5 ± 0.0	20.0 ± 0.0	37.1 ± 0.0	45.4 ± 0.0	
Fluoranthene	BSM-pyrene	5.2 ± 0.2	16.0 ± 0.2	37.4 ± 0.2	44.7 ± 1.2	1.6 ± 0.9
	CYEM	4.6 ± 0.0	11.5 ± 0.0	22.6 ± 0.0	32.4 ± 0.0	
Pyrene	BSM-pyrene	3.4 ± 0.0	17.2 ± 1.8	49.5 ± 0.0	59.1 ± 0.0	0.8 ± 1.6
	CYEM	0.0 ± 0.0	7.6 ± 0.0	26.0 ± 0.0	36.8 ± 0.0	
Benz[*a*]anthracene	BSM-pyrene	0.4 ± 0.1	1.2 ± 0.2	10.3 ± 0.8	21.0 ± 1.3	1.2 ± 0.8
	CYEM	0.0 ± 0.0	3.9 ± 0.0	8.8 ± 0.0	14.0 ± 0.0	
Dibenz[*a*,*h*]anthracene	BSM-pyrene	4.0 ± 0.3	3.9 ± 0.3	6.1 ± 1.3	15.8 ± 0.4	1.8 ± 1.2
	CYEM	0.0 ± 0.0	0.0 ± 0.0	2.7 ± 0.0	7.4 ± 0.0	
Benzo[*a*]pyrene	BSM-pyrene	0.0 ± 1.0	0.0 ± 0.4	7.4 ± 0.8	17.3 ± 0.7	2.8 ± 0.1
	CYEM	2.4 ± 0.0	1.0 ± 0.0	4.3 ± 0.0	9.4 ± 0.0	

[a]Each PAH comprised a component of a PAH mixture in BSM; the concentration of each PAH was 50 mg ml^{-1}.
[b]Inocula were grown in either BSM-pyrene (BSM containing 250 mg L^{-1} pyrene) or CYEM (BSM containing 2 ml L^{-1} creosote and 1 g L^{-1} yeast extract).
[c]%PAH removal was calculated as the amount of PAH that disappeared from each culture compared to the initial PAH concentration; averages are from triplicate experiments. Inocula comprised unwashed cells which were transferred directly into BSM containing a PAH mixture. All incubations were performed at 30°C and 175 rpm in the dark.
[d]Killed-cell cultures were prepared by inoculating pyrene-grown cells that had been killed with $HgCl_2$ immediately prior to inoculation. The amount of PAH that disappeared in these cultures after 42 days is shown.

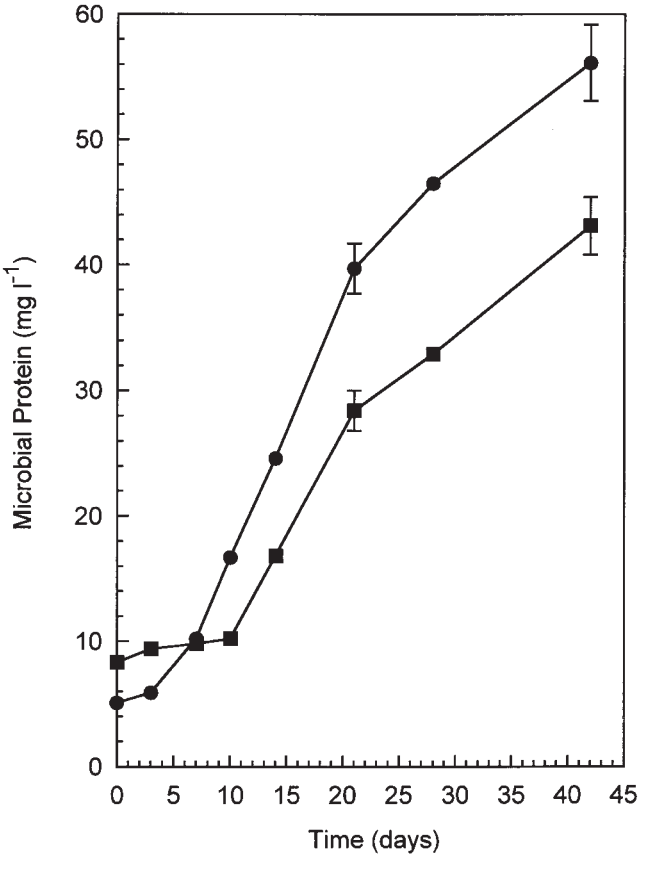

Figure 5 Growth of community five in BSM containing 50 mg L^{-1} each of fluorene, phenanthrene, fluoranthene, pyrene, benz[*a*]anthracene, benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene. BSM containing the PAH mixture was inoculated with a 10% unwashed inoculum prepared from either pyrene-grown cells (●) or CYEM-grown cells (■). All incubations were at 30°C and 175 rpm in the dark.

not support the growth of community five, resulting in very low final biomass concentrations. A compromise was found by combining yeast extract with creosote in BSM; this medium stimulated the community five PAH catabolism and supported reasonable microbial growth rates and biomass yields.

Although addition of yeast extract to BSM-creosote stimulated microbial growth, the results demonstrated the importance of optimising the concentration of each medium component to achieve maximal rates of inoculum growth and subsequent PAH degradation. Achieving high biomass growth rates and yields in BSM-creosote-yeast extract medium did not necessarily result in the highest PAH degradation activity in the bioaugmentation inocula. Two opposing effects take place with the use of these two medium components. If the yeast extract concentration is too high, there is a loss of PAH-degrading activity. Although this may be counteracted by the stimulatory effect of creosote on PAH catabolism, a creosote concentration threshold existed at which point community five growth and PAH catabolism was inhibited by creosote components.

An important aspect of these results was the need for nutrient addition to BSM-creosote in order to support microbial growth. This was not expected, given that BSM-pyrene supported community five growth and in comparison there was around 50 mg L^{-1} each of pyrene, fluoranthene and phenanthrene in BSM-creosote, suggesting that the creosote should have provided suitable substrates for bacterial growth. The most likely reason is the inhibitory effects of other compounds in the creosote. Creosote is a complex mixture of more than 200 constituents encompassing diverse chemical structures. PAHs comprise approximately 85% of creosote, while phenolic and heterocyclic aromatic compounds containing nitrogen, sulphur or oxygen (NSO-compounds) comprise the remaining con-

stituents [16]. The inhibitory effects of NSO-compounds in creosote on the microbial degradation of benzene, toluene and other creosote compounds have been documented [1,5,6]. The explanation for this result was that NSO-compounds inhibit the catabolic pathways of other aromatic compounds, presumably due to similarities in molecular structure. This hypothesis could be extended to account for the lack of community five growth on BSM-creosote, assuming that creosote NSO-compounds inhibited PAH catabolism. The addition of alternative carbon substrates to BSM-creosote enabled non-aromatic catabolic pathways that presumably are not inhibited by the compounds to be used for growth.

The optimum combination of these medium components in BSM for growing community five inocula was 1 g L^{-1} yeast extract and 2 ml L^{-1} creosote. Compared to BSM-pyrene, the CYEM medium is approximately 11 times less expensive so that its use potentially represents a substantial cost saving for large-scale bioaugmentation operations. The sacrifice for this cost saving appears to be a decline in PAH-degrading activity, where changes seen were due to an extended lag period and lower PAH degradation rates; however, the range of PAHs degraded in the mixture did not appear to be affected. The reason for the decrease in PAH degradation activity could be due either to the transfer of inhibitory creosote compounds along with the inoculum or to poor stimulation of PAH catabolism in the inoculum. The former explanation is the least likely due to dilution of the creosote components when inoculated into the PAH mixture. The dilution effect on creosote inhibitory compounds is demonstrated by the absence of inhibition of PAH degradation in BSM-pyrene inoculated with CYEM-grown cells; in this case both BSM-pyrene and CYEM-grown inocula degraded pyrene at equivalent rates. The latter effect has been reported in other studies [2,17,18] and repression of PAH degradation by community five due to the yeast extract components in CYEM medium might be expected. This is evident in our results that show a proportional loss in subsequent pyrene degradation activity as the yeast extract concentration in the inoculum medium increases past the optimum concentration. Although community five inocula grown in optimum creosote and yeast extract concentrations were able to degrade pyrene at equivalent rates to that of pyrene-grown inocula, the PAH catabolism of CYEM-grown inocula appears to have not been sufficiently primed, compared to the pyrene-grown inocula, to cope with the PAH range that was present in the PAH mixture. This is consistent with the longer lag periods observed before the onset of PAH degradation by BSM-creosote-yeast extract-grown inocula in the PAH mixture compared to pyrene-grown inocula, representing a longer adaptation or induction period of the community five PAH catabolism.

This study has demonstrated the important role of bioaugmentation inoculum medium in determining PAH degradation activity in the receiving polluted medium. This issue was addressed by a recent study that demonstrated the failure of *Sphingomonas paucimobilis* strains to degrade PAHs in contaminated soil due to the high salinity of the inoculum medium [13]. In this case the medium in the inoculum affected the salinity of the contaminated soil such that it inhibited PAH degradation activity. The findings of this study emphasise the importance of optimising media used to grow bioaugmentation inocula according to the subsequent biodegradation activity of the inocula rather than inoculum growth rates and biomass yields. We have also shown that creosote can be used as a substrate for growing bioaugmentation inocula for the treatment of PAH-contaminated sites provided it is supplemented with a nutrient source at an appropriate concentration. Although creosote is traditionally recognised as being inhibitory to microbial activity, the advantages of using this compound for growing microbial inocula reside in its ability to maintain microbial PAH catabolism, its low unit cost and its ready availability.

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References

1 Arvin E, BK Jensen and AT Gundersen. 1989. Substrate interactions during aerobic biodegradation of benzene. Appl Environ Microbiol 55: 3221–3225.
2 Barnsley EA. 1975. The induction of the enzymes of naphthalene metabolism in pseudomonads by salicylate and 2-aminobenzoate. J Gen Microbiol 88: 193–196.
3 Boonchan S, ML Britz and GA Stanley. 1998. Surfactant-enhanced biodegradation of high molecular weight polycyclic aromatic hydrocarbons by *Stenotrophomonas maltophilia*. Biotechnol Bioeng 59: 482–494.
4 Caldini G, G Cenci, R Manenti and G Morozzi. 1995. The ability of an environmental isolate of *Pseudomonas fluorescens* to utilize chrysene and other four-ring polynuclear aromatic hydrocarbons. Appl Microbiol Biotechnol 44: 225–229.
5 Dyreborg S, E Arvin and K Broholm. 1996. Effects of creosote compounds on the aerobic bio-degradation of benzene. Biodegradation 7: 191–201.
6 Dyreborg S, E Arvin and K Broholm. 1996. The influence of creosote compounds on the aerobic degradation of toluene. Biodegradation 7: 97–107.
7 Grifoll M, SA Selifonov, CV Gatlin and PJ Chapman. 1995. Actions of a versatile fluorene-degrading bacterial isolate on polycyclic aromatic compounds. Appl Environ Microbiol 61: 3711–3723.
8 Heitkamp and CE Cerniglia. 1989. Polycyclic aromatic hydrocarbon degradation by a *Mycobacterium* sp in microcosms containing sediment and water from a pristine ecosystem. Appl Environ Microbiol 55: 1968–1973.
9 Juhasz AL, GA Stanley, B Davey and ML Britz. 1997. Evaluation of high molecular weight PAH degradation by a pyrene-enriched microbial community in inoculated soils. In: Global Environmental Biotechnology (Wise DL, ed), pp 475–488, Kluwer Academic Publishers, Dordrecht, The Netherlands.
10 Juhasz AL, ML Britz and GA Stanley. 1996. Degradation of high molecular weight polycyclic aromatic hydrocarbons by *Pseudomonas cepacia*. Biotechnol Lett 18: 577–582.
11 Juhasz AL, ML Britz and GA Stanley. 1997. Degradation of fluoranthene, pyrene, benz[*a*]anthracene and dibenz[*a*,*h*]anthracene by *Burkholderia cepacia*. J Appl Microbiol 83: 189–198.
12 Juhasz AL. 1998. Microbial degradation of high molecular weight polycyclic aromatic hydrocarbons. PhD thesis, Victoria University of Technology, Melbourne, Australia.
13 Kastner M, M Breuer-Jammali and B Mahro. 1998. Impact of inoculation protocols, salinity, and pH on the degradation of polycyclic aromatic hydrocarbons (PAHs) and survival of PAH-degrading bacteria introduced into soil. Appl Environ Microbiol 64: 359–362.

14 Liu S and JM Suflita. 1993. Ecology and evolution of microbial populations for bioremediation. Tibtech 11: 344–352.
15 Lowry OH, NJ Rosebrough, AL Farr and RJ Randall. 1951. Protein measurement with the folin phenol reagent. J Biol Chem 193: 265–275.
16 Mueller JG, DP Middaugh, SE Lantz and PJ Chapman. 1991. Biodegradation of creosote and pentachlorophenol in contaminated groundwater: chemical and biological assessment. Appl Environ Microbiol 57: 1277–1285.
17 Rohm M and D Werner. 1985. Regulation of the β-ketoadipate pathway in *Rhizobium japonicum* and bacteroids by succinate. Archiv Microbiol 140: 375–379.
18 Rotert KH, LA Cronkhite and PJJ Alvarez. 1995. Enhancement of BTX biodegradation by benzoate. In: Microbial Processes for Bioremediation (Hinchee R E, C M Vogel and F J Brockman, eds), pp 161–168, Battelle Press, Ohio.
19 van Veen JA, LS van Overbeek and JD van Elsas. 1997. Fate and activity of microorganisms introduced into soil. Microbiol Mol Biol Rev 61: 121–135.
20 Wilson SC and KC Jones. 1993. Bioremediation of soils contaminated with polynuclear aromatic hydrocarbons (PAHs): a review. Environ Pollut 81: 229–249.
21 Ye D, A Siddiqi, AE Maccubbin, S Kumar and HC Sikka. 1996. Degradation of polynuclear aromatic hydrocarbons by *Sphingomonas paucimobilis*. Environ Sci Technol 30: 136–142.