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Evaluation of microbial surfactants for recovery of hydrophobic pollutants from soil

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SUMMARY

Several microbially produced biosurfactants were evaluated for their ability to remove hydrophobic compounds from soil. The biosurfactants produced by *Pseudomonas aeruginosa* UG2 and *Acinetobacter calcoaceticus* RAG-1 displayed the best results, with recovery of [^{14}C]hexachlorobiphenyl from soil slurries of 48.0 and 41.9%, respectively. *P. aeruginosa* UG2 produced higher levels of extracellular biosurfactants than four other *P. aeruginosa* strains. *P. aeruginosa* UG2 culture filtrate containing biosurfactants enhanced the recovery of several other individual hydrocarbons and polychlorinated biphenyl compounds, as well as several hydrocarbons in a mixture, from soil. The results suggest that biosurfactants produced by *P. aeruginosa* UG2 have the potential for remediation of hydrophobic pollutants in soil environments.

INTRODUCTION

Biosurfactants are compounds produced on microbial cell surfaces or excreted extracellularly that contain both hydrophillic and hydrophobic portions. Due to their amphipathic nature, surfactants aggregate at interfaces, causing a reduction in surface and interfacial tensions. Biosurfactants have many potential applications, including remediation of hydrophobic pollutants in soil [20,38]. Hydrocarbons and some halogenated compounds are serious environmental pollutants that cause public health concerns due to their environmental persistence and toxicity to biological systems.

Hydrophobic compounds bind to soil components and are difficult to remove or degrade. Efficient degradation requires that hydrocarbons be solubilized prior to microbial degradation [36,37]. Surfactants can emulsify hydrocarbons, thus enhancing their water solubility. Also, surfactants can decrease surface tension and increase displacement of oily substances from soil particles [5]. Many investigators have examined the use of chemical surfactants to enhance in situ aqueous washing of soil contaminated with hydrocarbons, pesticides, polychlorinated biphenyl (PCB) and pentachlorophenol (PCP) [1–5,13,25,33,39]. In each case, significant amounts of pollutants were removed from soils.

Biosurfactants may replace chemical surfactants in remediation of soil contaminated with hydrophobic compounds for several reasons. In contrast to some chemical surfactants, biosurfactants are degraded by microorganisms. Biosurfactants may be less toxic than synthetic surfactants, and novel compounds produced biologically may be different and more effective for specific purposes [38].

In this study, several bacterial and yeast strains known to produce surface-active substances were evaluated. *P. aeruginosa* UG2, isolated from oil-contaminated soil [27], produces high extracellular emulsifying activity when grown on several hydrocarbon and non-hydrocarbon substrates [7]. The UG2 bioemulsifier partitions high amounts of [^{14}C]hexachlorobiphenyl from soil slurries into the aqueous phase [7]. Numerous studies have been conducted on rhamnolipid production by *P. aeruginosa* PG201 [15,16]. In a continuous production process, 0.7 g rhamnolipid l^{-1} h^{-1} was obtained.

Other bacterial surfactants used in this study include emulsan, produced by *Acinetobacter calcoaceticus* RAG-1. Emulsan has been extensively studied and proposed for many applications [14,17]. *Bacillus licheniformis* JF-2, under both aerobic and anaerobic conditions, produces an anionic surfactant that lowers the surface tension of H_2O

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from 72 to 28 mN/m [21,29]. Surfactin is a lipopeptide produced by *B. subtilis* and is obtained in high amounts (0.8 g/l) in foam during fermentation [10]. *B. subtilis* Suf-1, a mutant derived from *B. subtilis* ATCC 21332, is able to produce over 3-times more surfactin than the parent strain [31]. Finally, *Rhodococcus erythropolis* forms trehalose mycolate biosurfactants which can lower the surface tension of H_2O to 28 mN/m. A maximum concentration of 2.1 g trehalose lipid/l was obtained in batch culture [32].

Of the yeasts tested, *Candida lipolytica* produces an extracellular emulsifier liposan when grown on water-immiscible carbon substrates [9]. *C. tropicalis* produces an emulsifier when grown on hydrocarbons [35]. *Torulopsis bombicola* produces a sophorolipid surfactant, with a maximum yield of 70 g/l. This was reported to be the most economical biosurfactant to produce [11].

In this paper, selected properties of these biosurfactants were compared, including the ability to remove hydrophobic compounds from soil. The objective was to identify the biosurfactant(s) that may be useful in remediation of hydrocarbon contaminated soils.

MATERIALS AND METHODS

Microorganisms

Bacterial strains and their sources are listed in Table 1. *P. aeruginosa* UG2 was isolated at the University of Guelph [27]. Three other *P. aeruginosa* strains PA01, PA02002 and ATCC 10145 were included in the later part of the survey for comparison with strains UG2 and PG201. Yeasts were maintained on yeast extract-peptone agar (Difco Laboratories, Detroit, MI, USA), and bacteria on either nutrient agar (Difco) or tryptic soy agar (Difco), at 4 °C and subcultured at monthly intervals.

Screening

Inoculum for each strain was grown by adding a loopful of cells to a 250-ml Erlenmeyer flask containing 50 ml of medium. The various media used were based on those reported in previous publications as capable of supporting maximal biosurfactant production by each strain (Table 1). All *P. aeruginosa* strains were grown in a minimal salts medium supplemented with 20 g glucose/l. The minimal

TABLE 1

Source and growth medium of microorganisms tested for biosurfactant production

Microorganism	Source	Growth medium	Carbon source
Acinetobacter calcoaceticus RAG-1	ATCC[a]	Goldman et al. [14]	hexadecane
Bacillus licheniformis JF-2	M.J. McInerney, University of Oklahoma, Norman, OK, USA	Javaheri et al. [21]	glucose and hexadecane
Bacillus subtilis ATCC 21331	ATCC	Cooper et al. [10]	glucose
Bacillus subtilis Suf-1	T. Chow, University of Sherbrooke, Sherbrooke, Que., Canada	Cooper et al. [10]	glucose
Candida lipolytica ATCC 34922	ATCC	Cirigliano and Carman [9]	hexadecane
Candida tropicalis ATCC 20326	ATCC	Kemp et al. [24]	glucose
Pseudomonas aeruginosa PG201	A. Koch, Institut für Biotechnologie, Zurich, Switzerland	[b]	glucose
Pseudomonas aeruginosa UG2	University of Guelph	[b]	glucose
Pseudomonas aeruginosa PAO1	ATCC	[b]	glucose
Pseudomonas aeruginosa ATCC 10145	ATCC	[b]	glucose
Pseudomonas aeruginosa PAO2002	J. Slater, University of Wales, Cardiff, Wales	[b]	glucose
Rhodococcus erythropolis ATCC 4277	ATCC	Margaritis et al. [28]	hexadecane
Torulopsis bombicola ATCC 22214	ATCC	Cooper and Paddock [11]	glucose and corn oil

[a] American Type Culture Collection, Rockland, MD, USA.

[b] See Materials and Methods.

salts medium contained (g/l): K_2HPO_4, 0.65; KH_2PO_4, 0.17; $MgSO_4 \cdot 7H_2O$, 0.1; $NaNO_3$, 0.5; NaCl, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; and $CaCl_2$, 0.02; pH 6.8. Cultures were incubated at 30 °C with shaking at 200 rpm.

After 48 h, a 0.5-ml sample of inoculum was added to each of ten flasks containing 50 ml of the same medium used to prepare the inoculum. Incubation conditions were those reported in the literature for each strain. Typically, maximum biosurfactant production was obtained during stationary phase of growth. *P. aeruginosa* strains were grown at 30 °C for 7 days with gyratory shaking at 200 rpm. After incubation, each culture was centrifuged at $5000 \times g$, 4 °C for 15 min to remove cells. The supernatants were pooled, and filtered through a Whatman GF/C glass fibre filter, followed by filtration through 0.8- and 0.45-μm cellulose acetate filters, respectively. The pH of *P. aeruginosa* culture filtrates was increased to 7 with 2 M NaOH before testing. Duplicate culture filtrates from each microorganism were obtained as above, using separate inoculum, and assayed using the following procedures.

Analytical

Culture filtrates were assayed for emulsifying activity as described previously [7,19,42]. A 0.5-ml sample of test solution, 4.4 ml of 20 mM Tris buffer containing 10 mM $MgSO_4$ (pH 7), and 0.1 ml of a hexadecane/2-methylnaphthalene mixture (1:1, v/v) were added to 16 × 125-mm screw-capped glass tubes. The tubes were mixed by vortexing for 30 s, held stationary for 30 min and the turbidity of the liquid measured spectrophotometrically at 540 nm. Three replicates of each sample were performed, using H_2O as a control. One unit (U) of activity was defined as the amount of emulsifier giving an absorbance of 1.0. Surface tension of culture filtrates was measured using a surface Tensiomat as recommended by the manufacturer (Fisher Scientific, Toronto, Ont., Canada). Dilutions of each culture filtrate to reach the critical micelle dilution (CMD^{-1} or F_{CMC}) were determined as described by Jain et al. [19] and Margaritis et al. [28]. Estimates of the CMD^{-1} were only performed once.

The ability of each culture filtrate to partition hydrocarbons from soil into the aqueous phase was determined according to Berg et al. [7], with some modifications. The soil used was a Conestoga silt loam which contains 54% silt, 30% sand and 16% clay [34]. Water holding capacity of the soil was found to be 0.47 ml/g. Soil was air dried for 2 days, passed through a 2-mm sieve and dried at 80 °C for two additional days prior to use.

In the standard partition assay, 1 g of dried soil was added to a 50-ml screw-capped teflon centrifuge tube. Water holding capacity of soil was increased to 40% using ultrapure H_2O, and 0.04 ml (10000 dpm) of a 2.5×10^5 dpm/ml 3,3′,4,4′,5,5′-hexachlorobiphenyl solution in hexane (3.0 mCi/mM, Sigma Chemical Co., St. Louis, MO, USA) was added to each tube and mixed well. Five ml of biosurfactant solution were then added to each tube. Three replicates of each sample were prepared, and control tubes contained 5 ml ultrapure H_2O. After gyratory shaking at 250 rpm for 2 h at 22 °C, the tubes were centrifuged at $3000 \times g$ for 15 min to sediment the soil. Two ml of supernatant were added to 10 ml of Scintiverse II scintillation cocktail (Fisher Scientific) and the radioactivity counted using a Beckman model LS6000 Liquid Scintillation System (Beckman, Fullerton, CA, USA). In some experiments, a Biological Oxidizer OX300 (R.J. Harvey Instrument Corp., NJ, USA) was used to determine the amount of [^{14}C]hexachlorobiphenyl remaining in soil after biosurfactant washing. The combined recovery of radioactivity from the aqueous and soil phases was consistently greater than 80% of the [^{14}C]hexachlorobiphenyl used (data not shown).

The ability of *P. aeruginosa* UG2 culture filtrate to solubilize various ^{14}C-labelled compounds in soil slurries was also tested. In these experiments, a 4-day culture filtrate was used and dilutions of this culture medium were made in ultrapure H_2O. Therefore, the 0% UG2 culture filtrate data were H_2O controls. In addition to [^{14}C]3,3′,4,4′,5,5′-hexachlorobiphenyl, other labelled compounds tested included pyridine (15.0 mCi/mM), naphthalene (10.3 mCi/mM), anthracene (13.3 mCi/mM), fluorene (14.2 mCi/mM), phenanthrene (13.1 mCi/mM), and 2,2′,5,5′-tetrachlorobiphenyl (14.2 mCi/mM). All radiolabelled compounds were obtained from Sigma Chemical Co. and dissolved in methanol, except tetrachlorobiphenyl and hexachlorobiphenyl which were dissolved in hexane. As pyridine is water soluble, it served as a control. Partitioning experiments were carried out as described above.

The ability of the UG2 biosurfactants to partition a hydrocarbon mixture from soil was also studied. The hydrocarbon mixture contained (% w/w): undecane, 20; pentadecane, 20; hexadecane, 20; octadecane, 20; pristane, 8; naphthalene, 6; phenanthrene, 4 and pyrene, 2. All hydrocarbons were obtained from Sigma Chemical Co. Partitioning experiments were performed as described above. The hydrocarbon mixture was added at 10, 25 and 50% hydrocarbon holding capacity (0.025; 0.063 and 0.098 g hydrocarbon mixture/g soil) and soil samples were held stationary for 3 h at 22 °C. Five ml of a 7-day UG2 culture filtrate were added to each soil sample. After shaking for 2 h as described above, the soil was centrifuged and the aqueous phase removed. The aqueous phase was extracted three times with 2 vols. of hexane, and the volume reduced by drying under N_2. The internal standard tetradecane was added to a final concentration of 0.5% (w/v). Hydrocarbons were analyzed using a Varian 2100 gas chromatograph equipped with a 2 m × 2 mm, 3% OV-17

on WHP Chromosorb column (Chromatographic Specialties, Brockville, Ont., Canada) as described [20].

RESULTS AND DISCUSSION

Initially, some properties of eight different extracellular biosurfactants produced under optimal batch culture conditions were compared. Surfactants are able to lower surface tensions and have emulsifying properties. However, emulsifiers only display emulsification activity and may not lower surface tension [38]. The emulsifying activity, minimal surface tension and CMD^{-1} value for biosurfactants produced by each strain were compared to partitioning of 3,3′,4,4′,5,5′-hexachlorobiphenyl to determine whether any correlation existed between these four parameters (Table 2).

Emulsifying activities were based on the ability of surfactants to stabilize a dispersion of hexadecane/2-methylnaphthalene in water. Culture filtrates of *A. calcoaceticus* RAG-1 and *B. subtilis* Suf-1 exhibited the highest emulsifying activities of 22.0 and 21.4 U/ml filtrate, respectively, followed by *T. bombicola* ATCC 22214 (15.8 U/ml), *P. aeruginosa* UG2 (15.5 U/ml) and *B. subtilis* ATCC 21331 (12.7 U/ml) (Table 2).

Low minimal surface tensions (less than 40 mN/m) were achieved by culture filtrates of all strains except *A. calcoaceticus* RAG-1, *C. lipolytica* ATCC 34922 and *C. tropicalis* ATCC 20326 (Table 2). The lowest minimal surface tension of 27.6 mN/m was achieved with culture filtrates of *B. subtilis* Suf-1. Critical micelle dilution (CMD^{-1}) estimates the amount of surface-active material in the culture filtrate. Increasing CMD^{-1} values indicate increasing concentrations of biosurfactants [15,19]. CMD^{-1} values were highest for filtrates of *B. subtilis* Suf-1 (40.0), *B. subtilis* ATCC 21331 (15.9) and *P. aeruginosa* UG2 (15.4) (Table 2). Moderately high CMD^{-1} values were exhibited by culture filtrates of *P. aeruginosa* PG201 (9.5), *T. bombicola* ATCC 22214 (9.0) and *B. licheniformis* JF-2 (4.8). Filtrate of *A. calcoaceticus* RAG-1 displayed a low CMD^{-1} value, although it had an emulsifying activity of 22.0 U/ml. *A. calcoaceticus* RAG-1 produces an extracellular bioemulsifier, which stabilizes emulsions by forming a strong polymeric film at the interface, but does not form micelles [17]. Hence, the surface tension of water was not appreciably reduced, and the CMD^{-1} value was the same as the control.

It has been reported that with *C. tropicalis* ATCC 20326 and *R. erythropolis* ATCC 4277, significant amounts of biosurfactants remain attached to the cell wall [35,40]. Since we are measuring the activity of cell-free culture medium, this may account for the low values recorded with these strains.

The amount of [^{14}C]hexachlorobiphenyl removed from soil into the aqueous phase was determined by partition-

TABLE 2

Comparison of biosurfactants produced by selected microorganisms

Strain	Average emulsifying activity (U/ml filtrate)	Average minimum surface tension (mN/m)	CMD^{-1} [a]	Average % HCB[b] recovered in aqueous phase
Water (control)	0	72.5	1	1.0
A. calcoaceticus RAG-1	22.0 (20.3–23.7)	54.4 (53.7–55.3)	1	41.9 (30.3–53.5)
B. licheniformis JF-2	0.2 (0–0.4)	40.0 (35.6–44.4)	4.8	2.7 (2.6–2.8)
B. subtilis ATCC 21331	12.7 (9.0–16.4)	28.6 (28.5–28.7)	15.9	13.1 (8.4–17.8)
B. subtilis Suf-1	21.4 (14.6–28.2)	27.6 (27.5–27.7)	40.0	22.2 (20.2–24.2)
C. lipolytica ATCC 34922	0	56.7 (55.2–58.2)	1	1.2 (1.0–1.4)
C. tropicalis ATCC 20326	0	65.0 (63.6–66.4)	1	1.1 (0.6–1.6)
P. aeruginosa PG201	10.4 (8.7–12.1)	30.5 (28.9–32.1)	9.5	38.9 (30.0–47.8)
P. aeruginosa UG2	15.5 (13.8–17.2)	31.4 (31.3–31.5)	15.4	48.0 (45.8–50.2)
R. erythropolis ATCC 4277	0.6 (0.2–1.0)	35.8 (35.0–36.6)	1	1.1 (0.6–1.6)
T. bombicola ATCC 22214	15.8 (8.3–23.3)	34.0 (33.9–34.1)	9.0	8.2 (3.2–13.2)
P. aeruginosa PAO1	5.6 (5.2–6.0)	32.5 (32.3–32.7)	7.4	16.7 (14.6–18.8)
P. aeruginosa ATCC 10145	4.1 (2.8–5.4)	30.6 (29.8–31.4)	3.3	15.5 (5.9–25.1)
P. aeruginosa PAO2002	0.2 (0.1–0.3)	31.3 (31.1–31.5)	1	0.6 (0.5–0.7)

[a] CMD^{-1} values were based on one determination.

[b] HCB = [^{14}C]hexachlorobiphenyl.

Range values are expressed in parentheses.

ing experiments (Table 2). Culture filtrates of *P. aeruginosa* UG2 and *A. calcoaceticus* RAG-1 exhibited the highest partitioning ability, with recoveries of 48 and 41.9% of the radiolabelled compound, respectively. Culture filtrates from both strains also displayed high emulsifying activities. However, culture filtrate of *B. subtilis* Suf-1, with an emulsifying activity of 21.4 U/ml, resulted in only 22.2% recovery of [^{14}C]hexachlorobiphenyl from soil slurries. Therefore, a direct correlation between the ability to remove hydrophobic contaminants from soil and emulsifying activity was not apparent. Our results show that only the partitioning test can accurately measure the ability of a biosurfactant solution to remove hydrophobic pollutants from soil.

It has been shown that hydrophobic compounds bind to organic matter in soil, and can be measured using the distribution coefficient $K_{oc} = K/OC$, where K is the adsorption constant of the compound and OC is the mass fraction of organic carbon in soil [22]. The largest fraction of organic matter is humic material, which has been modelled as membrane-like aggregates with a hydrophillic exterior and hydrophobic interior [41]. Chiou et al. [8] have shown that the mechanism of sorption of hydrophobic organic compounds from water to soil is a partition process from aqueous soil solution to the interior of soil humic material. Non-hydrophobic interactions, such as particle size distribution, clay mineral composition, pH, and cation-exchange capacity, can also contribute to sorption under conditions of high polarity or low organic content in soil [30].

All hydrophobic chemicals have relatively similar affinities for soil, with their affinities for water determining the difference in sorption behaviour [23]. Therefore, desorption of hydrophobic compounds from soil particles can be enhanced by the use of surfactants. Surfactants may increase the water solubility of hydrophobic compounds by partitioning into the hydrophobic interior of micelles [6]. Kile and Chiou [25] have also shown that significant water solubility enhancements below the critical micelle concentration (CMC) of surfactant are possible for extremely water-insoluble compounds. However, removal of contaminants is hindered due to a slower rate of desorption than sorption of hydrophobic compounds to soil [12], and the increased adsorption to soil particles over time [22].

Removal of hydrophobic contaminants from soil using chemical surfactants have been tested. With the anionic surfactant dodecylsulphate, surfactant micelles and the soil solid phase had about the same affinity or sorption potential for non-polar PAH compounds [18]. Ellis et al. [13] reported a 90% recovery of crude oil and 70% recovery of PCB using a 2% blend of Adsee 799 and Hyonic NP-90 surfactants in batch washing of contaminated soil. Vigon and Rubin [39] were able to desorb up to 70% biphenyl compound and 58% anthracene from soil using 1% solutions of alkylphenolethoxylate surfactants. Laha and Luthy [26] removed approx. 80% phenanthrene from soil with 1.5% Triton X-100 and Brij 30 surfactants. Abdul et al. [1] compared a number of surfactants in the washing of automatic transmission fuel (ATF) from sandy soil. Using 0.5% surfactant concentration, recoveries ranged from 83.8% for Witconol 1206 to 33.1% for Adsee 799. The effectiveness of surfactants in removing ATF from soil was not dependent on whether the surfactants were nonionic or anionic.

The screening experiments revealed that extracellular biosurfactants produced by *P. aeruginosa* UG2 and *A. calcoaceticus* RAG-1 exhibited the best ability to partition a hydrophobic compound such as hexachlorobiphenyl into the aqueous phase of soil slurries. *P. aeruginosa* PG201 also displayed excellent partitioning results. Screening results suggest the UG2 biosurfactants possess excellent potential for use in washing hydrocarbons from soil. A study was done to compare the hydrocarbon partitioning ability of surfactants produced by several other *P. aeruginosa* strains using the same criteria as those in the screening experiment. Culture filtrates from all strains lowered the surface tension to approx. 31 mN/m (Table 2). UG2 culture filtrate exhibited the highest CMD^{-1} value (15.4), emulsifying activity (15.5 U/ml), and partitioning recovery (48.0%) of the five strains tested.

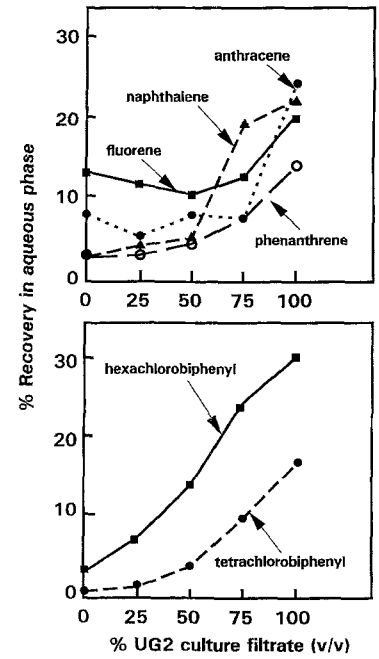

Fig. 1. Recovery of selected hydrocarbons from soil slurries into the aqueous phase containing varying dilutions of UG2 culture filtrate. Culture filtrate was diluted with ultrapure H_2O.

The ability of *P. aeruginosa* UG2 culture filtrate to partition various hydrophobic compounds, such as polyaromatic hydrocarbons and chlorinated biphenyls, was further tested using a 4-day culture filtrate. Aqueous phase recovery by undiluted culture filtrate was about 20% for anthracene, naphthalene and fluorene, and 15% for phenanthrene (Fig. 1, top panel). Recovery of hexachlorobiphenyl was slightly higher at 30%, while that for tetrachlorobiphenyl was 18% (Fig. 1, bottom panel). Recovery of the water-soluble pyridine was 80 to 90% at all culture filtrate dilutions (data not shown). The ability to solubilize hydrocarbons was dependent on the concentration of the culture filtrate. When the culture filtrate was diluted 50%, recovery of all hydrophobic compounds except hexachlorobiphenyl decreased to control levels (Fig. 1). The results show that a variety of hydrophobic compounds may be solubilized by UG2 biosurfactants.

The effect of UG2 biosurfactants on removal of higher concentrations of a hydrocarbon mixture from soil was also investigated. Increasing effectiveness of the UG2 culture filtrate was observed when the hydrocarbon mixture was present at lower levels of soil saturation (Fig. 2). At 50% soil saturation, UG2 biosurfactants increased the amount of hydrocarbons recovered in the aqueous phase over H_2O by 0% (pyrene) to 28% (octadecane). The enhanced recovery of hydrocarbons using UG2 biosurfactants over H_2O at 25% saturation ranged from 128% (naphthalene) to 262% (octadecane), and at 10% saturation was from 206% (naphthalene) to 402% (undecane). The total amount of hydrocarbons removed from soil into the aqueous phase was increased at higher saturation levels.

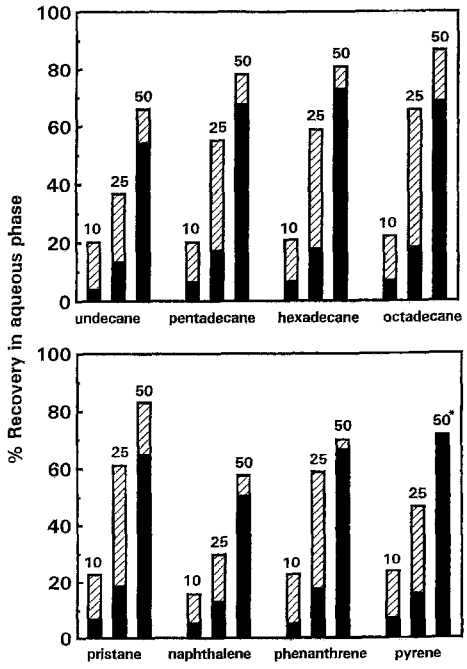

Fig. 2. Recovery of a selected hydrocarbon mixture from soil slurries into the aqueous phase at 10, 25 and 50% hydrocarbon holding capacity of soil. ■ H_2O, ▨ undiluted UG2 culture filtrate. * The recovery of pyrene was 7.4% higher using H_2O than with UG2 culture filtrate.

The basis for variability in solubilization may depend on factors discussed earlier, such as the binding of hydrophobic compounds and UG2 biosurfactants to soil, soil type and content, or prevailing environmental conditions. The partitioning results described here were lower than those using chemical surfactants, but further optimization of UG2 biosurfactant production and experimental conditions may increase the remediation potential of these biosurfactants for use with hydrocarbon contaminated soils either in situ or in a bioreactor. Additional research will be necessary to determine the usefulness of biosurfactants in these processes.

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