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Emulsification of hydrocarbons by subsurface bacteria

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SUMMARY

Biosurfactants have potential for use in enhancement of in situ biorestoration by increasing the bioavailability of contaminants. Microorganisms isolated from biostimulated, contaminated and uncontaminated zones at the site of an aviation fuel spill and hydrocarbon-degrading microorganisms isolated from sites contaminated with unleaded gasoline were examined for their abilities to emulsify petroleum hydrocarbons. Emulsifying ability was quantified by a method involving agitation and visual inspection. Biostimulated-zone microbes and hydrocarbon-degrading microorganisms were the best emulsifiers as compared to contaminated and uncontaminated zone microbes. Biostimulation (nutrient and oxygen addition) may have been the dominant factor which selected for and encouraged growth of emulsifiers; exposure to hydrocarbon was also important. Biostimulated microorganisms were better emulsifiers of aviation fuel (the contaminant hydrocarbon) than of heavier hydrocarbon to which they were not previously exposed. By measuring surface tension changes of culture broths, 11 out of 41 emulsifiers tested were identified as possible biosurfactant producers and two isolates produced large surface tension reductions indicating the high probability of biosurfactant production.

INTRODUCTION

In situ biorestoration is a process wherein microorganisms are stimulated to metabolize aquifer contaminants in place. One factor which often limits its effectiveness is the limited solubility of organic contaminants. A hydrophobic contaminant may sorb to organic fractions of subsurface materials or become trapped in pore spaces of the aquifer matrix.

The use of synthetic surfactants may enhance mobilization of contaminant hydrocarbons. Surfactants could enhance in situ biorestoration by: (1) aiding in release of hydrophobic contaminants sorbed to organic matter; (2) reducing interfacial tensions thereby releasing contaminant droplets trapped in pore spaces by capillary forces; and (3) increasing the surface area of the contaminant available for microbial attack. Surfactant soil washing increased recovery of gasoline in a model aquifer test system [21]; however, there was significant reduction in permeability. In addition, Ziegenfuss [26] found that soil washing with surfactants in static microcosms resulted in decreased mineralization of benzene and naphthalene due to the biological oxygen demand exerted by the surfactants.

An alternative to the use of synthetic surfactants for the enhancement of in situ biorestoration is the use of biosurfactants. This could be accomplished by stimulating indigenous biosurfactant-producing microorganisms to increase their production or by introducing specially adapted biosurfactant-producing bacteria into the contaminated subsurface. Biosurfactants are produced by a wide variety of microorganisms, and have been used for a variety of industrial applications, including microbial enhanced oil recovery and oil spill clean-up [6,20,24]. The enhancement of microbial biosurfactant production has been investigated for various applications and includes altering the type and/or amount of the nitrogen source [9,12,15], supplying growth additives [15] or trace elements [7,11], or altering the pH of the growth medium [14].

Little work has been conducted to assess the extent and importance of biosurfactant production by subsurface microorganisms and their potential role in contaminant degradation. Therefore, a study was conducted to characterize biosurfactant production by heterotrophs isolated from several subsurface samples of biostimulated, contaminated and uncontaminated materials collected from the site of an aviation fuel spill and of a group of hydrocarbon-degrading microorganisms isolated from an unleaded gasoline spill site. The first step in this process involved screening microbial isolates for their abilities to emulsify aviation fuel or unleaded gasoline, kerosene and diesel fuel. This was used to identify microorganisms with the potential to produce biosurfactants.

However, emulsification of the hydrocarbons in a

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screening procedure can be caused by other mechanisms in addition to the release of extracellular biosurfactants or bioemulsifiers by intact cells. The hydrophobicity of an organism is due to the presence of lipophilic and hydrophilic cell wall and cell membrane components, some of which are surface-active. Therefore, the cell itself can demonstrate significant emulsifying activity and act as a biosurfactant agent [25]. Secondly, the solvent action of the hydrocarbon on lipophilic cell surface components may cause the cell to lose its structural integrity [16] releasing surface-active compounds into the medium.

Therefore, microbes with emulsification ability were further examined to identify the biosurfactant producers by: (1) determining the isolates which produced cell-free supernatant fluids with emulsifying abilities, thereby eliminating the isolates exhibiting emulsifying ability due solely to the presence of whole microbial cells; and (2) measuring surface tension changes produced by cells grown on glucose with and without hydrocarbon amendments. Surface tension reduction by actively growing cultures was used as an indirect indicator of biosurfactant production. Hydrocarbon was added because Duvnjak et al. [9] showed that a glucose-grown *Corynebacterium* produced a cell-bound surfactant which was subsequently released after the addition of hexadecane. Using this protocol, 13 isolates were identified as possible biosurfactant producers.

MATERIALS AND METHODS

Sampling sites and procedure

Subsurface materials were collected by a procedure developed by Dunlap et al. [8] and modified by Wilson et al. [23] which prevented contamination by surface microbes. Three subsurface samples were collected from a U.S. Coast Guard station in Traverse City, Michigan, at the site of an aviation fuel spill. The core materials were collected from uncontaminated, contaminated and biostimulated zones. The biostimulated sample was collected from the site of a field demonstration of patented process(es) [17,18] for in situ bioremediation of aquifers contaminated with petroleum hydrocarbons. At the time of sample collection, the indigenous subsurface microflora in the biostimulated zone was being stimulated to degrade the contaminants by the addition of a nutrient solution composed of nitrogen, phosphorus, and oxygen in the form of hydrogen peroxide [22]. The contaminated sample was obtained from the zone of contamination outside the biostimulated test plot. The uncontaminated sample was taken from a nearby location of similar depth and moisture content. All samples were taken at depths of approx. 194 to 201 inches and were composed of mixtures of sand and gravel.

Media and chemicals

The mineral salts solution used in this study was a modification of a mineral salts solution developed by Knetting and Zajic [15]. It contained per liter deionized water: KH_2PO_4, 2.0 g; K_2HPO_4, 5.0 g; $(NH_4)_2SO_4$, 3.0 g; NaCl, 0.1 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.01 g; and $MnSO_4 \cdot H_2O$, 0.002 g.

Four hydrocarbons were used in this study. Aviation fuel, similar to the fuel spilled at Traverse City, Michigan, was blended by Exxon Corporation. It contained 70% 2,2-dimethyl butane, 20% 2-methylbutane (IC5) and 10% toluene. Kerosene was obtained from Fisher Scientific, Houston, Texas. Number 2 Diesel Fuel Oil was obtained from Exxon Company, U.S.A., Baytown, Texas. Unleaded gasoline was purchased at a local service station. The hydrocarbons were filter-sterilized using filter assemblies (Gelman, Ann Arbor, MI) with 0.2-μm pore size polypropylene membranes.

Microbial cultures

Microorganisms from Traverse City, Michigan, were isolated by adding 1 g of subsurface material to 9.5 ml of 0.1% sodium pyrophosphate in screw-cap bottles. The mixture was placed on a reciprocal shaker for 1 h to produce a well-dispersed suspension. The suspension was diluted serially in phosphate-buffered saline (1.24 g Na_2HPO_4, 0.180 g $NaH_2PO_4 \cdot H_2O$ and 8.5 g NaCl per liter deionized water) and plated in triplicate on one-half strength Nutrient Broth (BBL, Cockeysville, MD) with 1.5% Bacto-Agar (Difco, Detroit, MI) to isolate heterotrophs. The plates were incubated at room temperature (approx. 22 °C). After the number of colonies stabilized, viable cell counts in the three subsurface samples were determined. Three successive streak plate isolations were performed on isolates selected for emulsification tests to ensure that pure cultures were obtained.

Nineteen hydrocarbon-degrading microorganisms were obtained from H.F. Ridgway of the Orange County Water District, Fountain Valley, California. These microorganisms were isolated on a mineral salts agar incubated in the presence of gasoline vapors. They were obtained from two subsurface sites contaminated with unleaded gasoline, at Seal Beach and San Diego, California.

Isolates from Traverse City and hydrocarbon-degrading microorganisms were maintained on one-half strength Nutrient Agar slants and R2A agar slants (Difco, Detroit, MI) respectively, and transferred to agar plates of the same compositions 7 days prior to use in emulsification tests.

Emulsifying capacity

Emulsifying capacity was determined by a modification of a technique used by Broderick and Cooney [4]. Several colonies of a pure culture were suspended in test tubes containing mineral salts amended with 0.03% yeast extract and 0.03% glucose after which the mixture was overlaid with 10% hydrocarbon. The heavy inoculum allowed assessment of emulsification by isolates unable to grow in the amended mineral salts with 10% hydrocarbon (non-growth conditions) whereas the addition of yeast extract and glucose allowed assessment of emulsification by isolates producing growth-associated surface-active compounds. The tubes were vortexed for 5 s to obtain emulsion formation. To eliminate unstable emulsions, readings were taken 2 h after mixing. The resulting emulsion was estimated visually from 0 through 4 (Fig. 1) on the 3rd day of incubation. Each experiment was performed in triplicate on three different trial dates.

Emulsification of aviation fuel by isolates from Traverse City and unleaded gasoline by the hydrocarbon-degrading microorganisms was assessed to evaluate fuels to which the microorganisms were previously exposed. To assess the ability of both sets of isolates to emulsify fuels to which they were not previously exposed, emulsification of kerosene and diesel fuel was also assessed.

Identification of biosurfactant producers

Several colonies of each isolate which emulsified aviation fuel (isolates from Traverse City) or unleaded gasoline (hydrocarbon-degrading microorganisms) with a mean emulsification rating from three trials of 1.6 or higher, were suspended in 24 ml of mineral salts amended with 0.03% yeast extract and 0.03% glucose. The resulting turbid suspension of cells was overlaid with 10% aviation fuel or unleaded gasoline and vortexed daily for 3 days. After 3 days incubation, 12 ml of the suspension was removed by pipette and centrifuged at 12060 × *g* for 20 min at 4 °C. Then, 4.0 ml of the resulting cell-free supernatant fluid was overlaid with 10% hydrocarbon, vortexed for 5 s and the extent of emulsification recorded after 2 h of settling time.

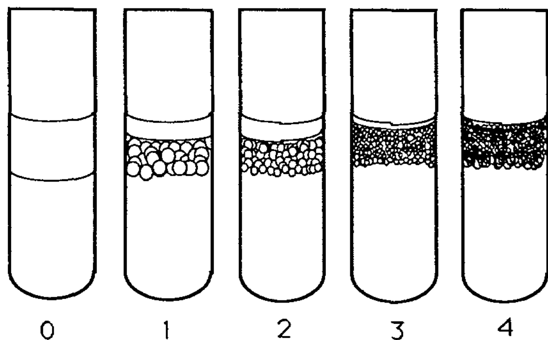

Fig. 1. Hydrocarbon emulsifying capacity was ranked from 0 to 4 (low to high).

Isolates producing cell-free supernatant fluids which retained emulsifying ability were grown in liquid culture to assess surface tension reductions. Several colonies of each pure culture were inoculated into 250-ml flasks containing 100 ml of mineral salts emended with 0.1% glucose. For many isolates, additional or alternative nutrients (0.03% yeast extract, 0.1% acetate, or 0.1% Nutrient Broth) were required for growth. Each flask was incubated at room temperature (22 °C) on a wrist action shaker (Burrell Corporation, Pittsburgh, PA). Un-inoculated controls contained the same nutrient formulation and were treated identically.

After 48 h, if a moderately turbid suspension of cells was obtained, triplicate surface tension measurements were taken of the whole culture broth and un-inoculated control at room temperature using a duNouy Interfacial Tensiometer (CSC Scientific Company, Fairfax, VA). Before measurement, the cell suspension was allowed to stand for 30 min to allow for equilibration. If the surface tension of a cell suspension was significantly lower than the un-inoculated control, the surface tension of the cell-free supernatant fluid was also measured.

To the flasks containing isolates which had surface tensions which were equal to or greater than the un-inoculated control while grown on glucose, one drop of either aviation fuel (isolates from Traverse City) or unleaded gasoline (hydrocarbon-degrading microorganisms) was added to each test flask and control to determine if hydrocarbon addition caused a lowering of the surface tension. The flasks were then returned to the shaker and two drops and three drops of hydrocarbon were added at 72 and 96 h, respectively. After 96 h incubation, surface tension measurements of the whole broths and the cell-free supernatant fluids were again recorded.

RESULTS

Microbial isolates

Viable cell counts per gram of subsurface material from the three subsurface samples from Traverse City (with standard deviations) were as follows: biostimulated zone, 3.27×10^5 (0.35); contaminated zone 1.02×10^6 (0.11) and uncontaminated zone, 3.13×10^4 (0.35). Representative plates from each zone containing approximately 100 colonies were selected for emulsification studies. Colonies on each of the three plates were placed in groups labelled a–z based on colonial morphology. Colony type designations indicate different colonial forms within each sample but do not differentiate between colony types in

TABLE 1

Colony types of isolates of Traverse City

Area	Colony type[a]	Designated isolates	Number of group members
Uncontaminated	a	BF4.1	1
	b	BF4.2	3
	c	BF4.3–6	19
	d	BF4.7	5
	e	BF4.8–19	57
	f	BF4.20	1
	g	BF4.21	1
	h	BF4.22	1
	i	BF4.23	3
	j	BF4.24	1
	k	BF4.25	1
	l	BF4.26	1
Biostimulated	m	AV6.1–13	64
	n	AV6.14–15	6
	o	AV6.16–18	11
	p	AV6.19	1
	q	AV6.20–22	14
Contaminated	r	BE2.1–2	9
	s	BE2.3–5, 28	17
	t	BE2.6–13	33
	u	BE2.14–22	44
	v	BE2.23	2
	w	BE2.24	3
	x	BE2.25	1
	y	BE2.26	5
	z	BE2.27	1

[a] Colony type designations indicate different colonial forms within each sample but do not differentiate between colony types in different samples.

different samples. Similarities of colony types between subsurface samples were not determined. For every five colonies belonging to the same group, one colony was randomly selected and purified to represent the group members in subsequent tests for emulsifying capacity. Hydrocarbon emulsifying capacity was determined on 70 designated isolates; 22, 26 and 28 from biostimulated (AV6), uncontaminated (BF4) and contaminated (BE2) zones, respectively. The colony types, designated isolates and numbers of group members are shown in Table 1.

Emulsifying ability

To depict emulsification capacity of the isolates from Traverse City accurately, the emulsification test results on each designated isolate were multiplied by the number of colonies the designated isolate represented. The results tabulated in this manner on aviation fuel emulsification are shown in Fig. 2. The greatest proportion of excellent microbial emulsifiers was obtained from the biostimulated zone. The contaminated zone contained organisms with the greatest diversity of emulsifying ability, whereas the isolates from the uncontaminated zone were the poorest emulsifiers.

Results of emulsification tests conducted on the 19

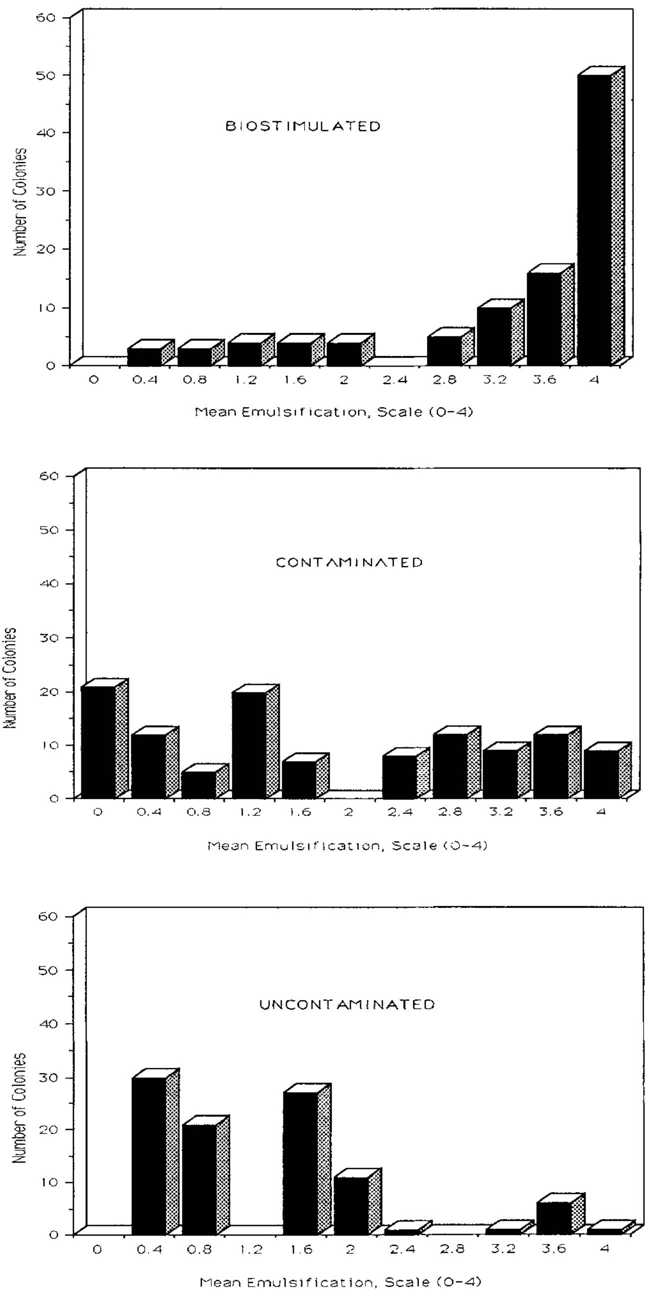

Fig. 2. Aviation fuel emulsification by isolates from Traverse City.

hydrocarbon-degrading microorganisms are shown in Fig. 3. The greatest proportion of excellent microbial emulsifiers was obtained from emulsification of unleaded gasoline, the contaminant present at the isolation site. Emulsification of kerosene and diesel by hydrocarbon-degrading microorganisms was generally weaker.

Descriptive statistics quantifying the observations displayed in the histograms (Figs. 2 and 3) and on kerosene and diesel emulsification by isolates from Traverse City are shown in Table 2. The interquartile range is the difference between the upper quartile (75th percentile) and lower quartile (25th percentile) and quantifies the spread of the data. The highest median reported (4.0) was demonstrated by microbes from the biostimulated zone on aviation fuel; the low interquartile range, 0.66, indicated the data were skewed to the higher values. Similarly, the response by the hydrocarbon-degrading microorganisms for emulsification of unleaded gasoline had a relatively high median, 2.67, and low interquartile range, 0.67. Microbes from the biostimulated zone and hydrocarbon-degrading microorganisms were also good emulsifiers of kerosene and diesel although the medians were lower and the interquartile ranges larger. A large interquartile range (2.66) was noted for emulsification of aviation fuel and kerosene by contaminated microbes, indicating that this population had a wide range of emulsifying abilities. In addition, microorganisms from the contaminated zone were only fair emulsifiers of the three fuels as shown by relatively low medians (1.34). In contrast, low medians and low interquartile ranges indicated the majority of the microorganisms from the uncontaminated zone were poor emulsifiers.

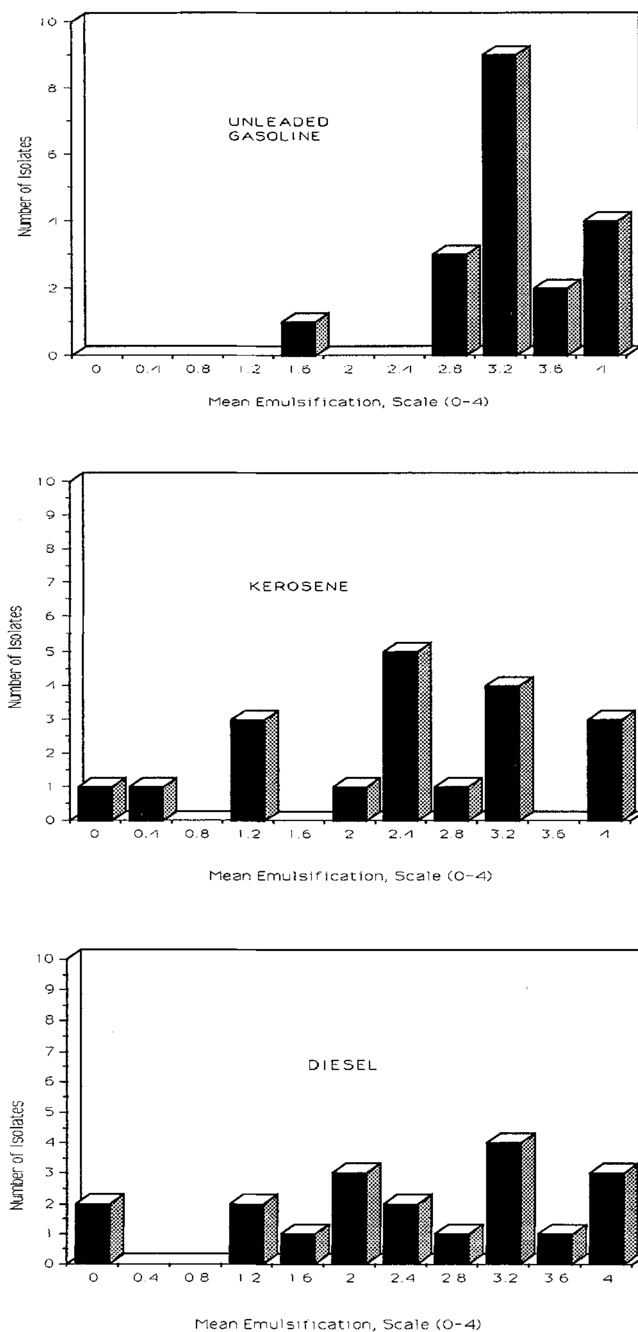

Fig. 3. Emulsification by hydrocarbon degrading microorganisms.

TABLE 2

Descriptive statistics on emulsification capacity

Subsurface sample/ hydrocarbon	Median	Interquartile range
Biostimulated/aviation	4.00	0.66
Contaminated/aviation	1.34	2.66
Uncontaminated/aviation	0.67	1.33
HC degraders/gasoline	2.67	0.67
Biostimulated/kerosene	2.00	1.03
Contaminated/kerosene	1.34	3.00
Uncontaminated/kerosene	1.00	1.35
HC degraders/kerosene	2.37	1.66
Biostimulated/diesel	2.67	2.33
Contaminated/diesel	1.34	1.41
Uncontaminated/diesel	1.00	0.67
HC degraders/diesel	2.37	1.67

Differences in the emulsifying capacity of microorganisms from samples of biostimulated, contaminated and uncontaminated subsurface materials were further quantified statistically by performing Mann Whitney U Tests, a nonparametric analogue to the *t*-test. Aviation gasoline emulsification by isolates from the biostimulated zone was significantly different from that of the contami-

TABLE 3

Comparisons of emulsification capacity between Traverse City subsurface samples

Subsurface samples	Hydrocarbon	*P* value[a]	95% CI
Biostimulated vs. uncontaminated	Aviation	0.0000	2.00, 2.50
Biostimulated vs. contaminated	Aviation	0.0000	1.33, 2.09
Contaminated vs. uncontaminated	Aviation	0.0464	0.00, 0.91
Biostimulated vs. uncontaminated	Kerosene	0.0000	0.67, 1.03
Biostimulated vs. contaminated	Kerosene	0.0508	0.00, 0.75
Contaminated vs. uncontaminated	Kerosene	0.2024	0.00, 0.67
Biostimulated vs. uncontaminated	Diesel	0.0003	0.33, 1.08
Biostimulated vs. contaminated	Diesel	0.0001	0.33, 1.16
Contaminated vs. uncontaminated	Diesel	0.0590	− 0.42, 0.00

[a] Based on Mann Whitney U Test, $\alpha = 0.05$.

nated and uncontaminated isolates (Table 3). Similar differences were obtained with diesel fuel and kerosene by isolates from biostimulated versus uncontaminated samples. However, the emulsifying capacity of kerosene by microorganisms from the biostimulated zone versus contaminated microorganisms showed no significant difference. In addition, in comparing emulsification by contaminated with uncontaminated zone microorganisms, aviation fuel was slightly different, while kerosene and diesel emulsification were the same by isolates from both zones.

Identification of biosurfactant producers

Cell-free supernatant fluids were prepared for those isolates able to emulsify aviation fuel (Traverse City samples) or unleaded gasoline (hydrocarbon-degrading microorganisms). In many cases, emulsification was significantly reduced in the cell-free supernatant fluids. The majority of the biostimulated microbes (73.4%) and hydrocarbon-degrading microorganisms (84.2%) retained at least some emulsifying ability in the supernatant fluids. In contrast, 37.5% of contaminated and 33.3% of pristine zone microbes lost all emulsifying ability after removal of microbial cells.

Forty-one isolates produced cell-free supernatant fluids with the ability to emulsify hydrocarbon with a visual rating of at least one. These isolates were grown in liquid culture to measure changes in surface tension. Surface tension values of whole culture broths, cell-free supernatant fluids and gasoline-treated cells were determined.

Eleven isolates produced moderate or large surface tension reductions of either whole broth and/or cell-free supernatant fluids by lowering the surface tension at least 10 dynes/cm less than the applicable control (Table 4).

TABLE 4

Isolates producing moderate or large surface tension reductions

Isolate	Medium[a]	Gasoline[b]	Mean surface tension (SD) in	
			Sample	Control
AV6.1	G + Y, CF	+	44.7 (0.49)	66.4 (0.85)
AV6.2	G + Y, W	−	50.0 (0)	60.7 (0.92)
AV6.5	G + Y, W	−	45.7 (0.07)	60.7 (0.92)
BF4.24	G + Y, CF	+	49.7 (0.27)	60.3 (0.42)
BF4.26	G, CF	−	26.4 (0.35)	69.4 (0.49)
3GC[c]	G, CF	+	47.7 (0.5)	70.7 (0.36)
11GA[c]	G, CF	+	57.4 (0.36)	70.7 (0.36)
11GB[c]	G, CF	+	53.7 (0.36)	70.7 (0.36)
12G[c]	G, CF	+	55.6 (0.12)	70.7 (0.36)
27G[c]	G, CF	−	46.4 (0.56)	61.2 (0.97)
71G[c]	G, CF	−	25.0 (0)	55.9 (0.64)
MF13[c]	G, CF	−	57.0 (0.81)	71.8 (0.29)
MF13[c]	G, CF	+	51.6 (0.68)	70.7 (0.36)

[a] W, whole broth; CF, cell-free supernatant fluid. Zajic mineral salts amended with: G, 0.03% glucose; Y, 0.03% yeast extract.
[b] Aviation fuel or unleaded gasoline added after moderate turbidity obtained.
[c] Hydrocarbon-degrading microorganisms obtained from H.F. Ridgway.

The remainder of the isolates failed to lower surface tensions values or lowered them to values less than 10 dynes/cm as compared to controls. Two isolates, BF4.26 and a hydrocarbon-degrading microorganism, 71G, produced large surface tension reductions with growth on glucose, lowering the surface tensions of the culture broths to 26.4 and 25.0 dynes/cm, respectively.

DISCUSSION

The intent of this study was to isolate general microflora from various subsurface locations and determine its emulsifying capacity. For enhancement of in situ biorestoration, the biosurfactant-producing microorganisms do not necessarily have to be the same organisms degrading the contaminant once the contaminant is mobilized. To date, chemoheterotrophic bacteria have been found to be the numerically predominant forms present in many aquifer environments [2]; in addition, counts for chemoheterotrophs are usually higher on dilute media than on concentrated media [13]. Therefore, a general microbial population from Traverse City, Michigan was isolated on dilute nutrient agar and, realizing the limitations of obtaining organisms from one type of media, emulsifying capacity of a group of microorganisms isolated from a different location on medium supplemented with gasoline was also investigated.

Among the chemoheterotrophs isolated from biostimulated, contaminated and uncontaminated samples, those from the biostimulated zone were the best emulsifiers of aviation fuel. These data suggest that exposure to the aviation fuel and/or exposure to nutrient addition (biostimulation) selected for microorganisms which can emulsify compounds found in the fuel mixture. One effect of a pollutant on the microbial environment is the enrichment of particular successful populations resulting in a decline in diversity [1]. Indeed, biostimulated zone microbes showed the least diversity, being represented by only five groups (Table 1). The microorganisms able to emulsify the aviation fuel, thereby increasing surface area and making the hydrocarbon droplets more available for microbial attack, may have been better able to proliferate under conditions of added nutrients and oxygen.

Although the differences were not as great as that between biostimulated and contaminant zone microbes, microorganisms from the contaminated zone were better emulsifiers than those from the uncontaminated zone. Therefore, it appears that exposure to compounds in aviation fuel selected for growth of emulsifiers in the contaminated zone over the uncontaminated zone. Selection pressure was due to exposure to the contaminants only; the contaminated zone received no nutrient addition. Another effect of a toxic pollutant, such as aviation fuel, on the microbial ecology is to eliminate the more sensitive species allowing the tolerant species to proliferate [1]. Perhaps in the contaminated zone, the emulsifiers were able to proliferate due to their ability to disperse the pollutant and reduce its toxicity.

We are not aware of any studies regarding the effect of contamination and nutrient addition on emulsifying capacity; however, there is a plethora of work on the effects of contamination on hydrocarbon-degrading microorganisms. The distribution of hydrocarbon degraders correlated highly with the degree of oil pollution [1]. In a recent study at the same aviation fuel spill site in Traverse City, Michigan, used in this study, Fiorenza [10] found both the heterotrophic and hydrocarbon-degrading populations to be one or two orders of magnitude greater in subsurface materials collected from contaminated levels than from uncontaminated levels. Cook and Westlake [5] observed slightly increased bacterial populations in samples of taiga soil contaminated with crude oil as compared to uncontaminated samples and significantly higher increases after the addition of fertilizer. The numbers of hydrocarbon degraders were not quantified. In the present study, a slight increase in the emulsifying capacity was observed in contaminated subsurface materials in comparison to uncontaminated materials, whereas emulsifying capacity was significantly higher in subsurface materials receiving nutrients.

There appears to be some substrate specificity for emulsification by microbes isolated from the biostimulated zone. The degree of emulsification of kerosene and diesel by the biostimulated zone microbes was less than emulsification of aviation fuel. In contrast, the emulsifying ability of the contaminated and uncontaminated zone microbes for kerosene and diesel was similar to that for aviation fuel. For a yeast cultivated on various hydrocarbons, Roy et al. [19] also observed high emulsifying ability for the alkane used as a growth substrate and decreased emulsification of shorter-chain alkanes. Bannerjee et al. [3] isolated a strain of *Pseudomonas cepacia* which produced an extracellular emulsifier growing on 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). The emulsifier was most active for 2,4,5-T, emulsified other chlorinated compounds and showed no activity towards unrelated compounds.

Therefore the data on the isolates from Traverse City suggest that nutrient addition may be the dominant factor which selected for and encouraged growth of organisms with the ability to emulsify hydrocarbons, although the importance of nutrient addition on emulsification capacity of subsurface microflora from the uncontaminated zone was not assessed. In addition, because microorganisms from the contaminated zone were poorer emulsifiers of aviation fuel than microbes from the biostimulated zone and better emulsifiers than microbes from the pristine zone, exposure to hydrocarbon was also important.

The emulsifying ability of a group of hydrocarbon degraders was compared with that of the three Traverse City populations. Of interest were the similar patterns of emulsification by the biostimulated zone microbes, heterotrophs isolated on Nutrient Agar, and the hydro-

carbon-degrading microorganisms. Selection pressures were once again in force; it was advantageous for both hydrocarbon-utilizing organisms and biostimulated microorganisms isolated from a hydrocarbon-contaminated site to possess the ability to emulsify the contaminant.

The data on the emulsifying ability of cell-free supernatant fluids indicated that many of the isolates had emulsifying ability due, at least in part, to the whole cells themselves; emulsification was often reduced after removal of the cells. However, the majority of the biostimulated zone microbes and the hydrocarbon degraders, unlike contaminated and pristine zone microbes, retained emulsifying ability after removal of cells indicating that extracellular surface-active compounds were excreted into the medium. Those isolates retaining emulsifying ability were the most promising biosurfactant producers.

The isolates were then grown in liquid culture and surface tension reductions were measured. The majority of the isolates achieved either no or small surface tension reductions (< 10 dynes/cm); these microorganisms were not identified as biosurfactant producers under the culture conditions defined herein. However, they may have been stimulated to produce biosurfactants given the proper nutrients and growth conditions. For example, Gerson and Zajic [11] found that the addition of Nutrient Broth resulted in increased surfactant production by *Corynebacterium lepus*. The most promising isolates for biosurfactant production were those with moderate to large surface tension reductions (> 10 dynes/cm). Two of these isolates produced large surface tension reductions indicating the high probability of biosurfactant production. Further work in this laboratory will concentrate on identifying methods to enhance biosurfactant production by these isolates.

Our results suggest that emulsification of hydrocarbons by microorganisms in the subsurface can be an important mechanism in bioremediation of contaminated aquifers. The extent and importance of biosurfactant production was not assessed. However, since biosurfactants increase emulsion stability and lower surface and interfacial tensions, the production of microbial surfactants for optimization of in situ biorestoration merits further attention.

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DISCLAIMER

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