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ORIGINAL PAPER

Basis for formulating biosurfactant mixtures to achieve ultra low interfacial tension values against hydrocarbons

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Abstract Biosurfactants could potentially replace or be used in conjunction with synthetic surfactants to provide for more cost-effective subsurface remediation. The design of surfactant formulations that are effective in lowering interfacial tension (IFT), which is necessary to mobilize entrapped hydrocarbons, requires information about the surface-active agent (surfactant) and the targeted non-aqueous phase liquids (NAPL). We hypothesized that biosurfactant and synthetic surfactant mixtures can be formulated to provide the appropriate hydrophobic/hydrophilic conditions necessary to produce low IFT against NAPLs, and that such mixtures will produce synergism that make them more effective than individual biosurfactants or synthetic surfactants. Our work tested the interfacial activity of biosurfactants from individual strains and mixtures of biosurfactants from different strains with and without a synthetic surfactant. Multiple regression analysis showed that, for lipopeptide biosurfactants produced by various *Bacillus* species, the interfacial activity against toluene depended on the relative proportions of 3-OH-C_{14}, C_{15}, C_{16}, and C_{18} in the fatty acid tail. As the fatty acid composition became more heterogeneous the system produced lower IFT against toluene. In mixtures of lipopeptide biosurfactants with the more hydrophilic, rhamnolipid biosurfactant, the IFT against toluene decreased as the percentage of the 3-OH C_{14} fatty acid increased in the lipopeptide. Mixtures of lipopeptide biosurfactants with the more hydrophobic synthetic surfactant, C12, C13-8PO SO_4Na, were able to produce low IFT against hexane and decane. In general, we found that lipopeptide biosurfactants with a heterogeneous fatty acid composition or mixtures of lipopeptide and rhamnolipid biosurfactants lowered the IFT against hydrophilic NAPLs. Conversely, mixtures of lipopeptide biosurfactants with a more hydrophobic synthetic surfactant lowered the IFT against hydrophobic NAPLs.

Keywords Biosurfactant mixtures · Ultra low interfacial tension · LNAPL bioremediation

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Introduction

Subsurface contamination by light non-aqueous phase liquids (LNAPL) is a prevalent environmental problem at Superfund sites, refineries, pipelines and chemical/industrial facilities [5]. Subsurface LNAPL contamination exists in three zones: the source area where dissolution into the groundwater initiates, the concentrated plume that contains the center of mass of the dissolved contaminant, and the dilute contaminant plume [34]. Usually, the source area and the concentrated plume, where the majority of contaminants exist, are the most challenging to remediate. Conventional pump and treat methods have limited success due to the constant equilibration of hydrocarbons entrapped in the source area with the flowing ground water [5, 34].

Surfactant-enhanced subsurface remediation (SESR) has been identified as a promising technology for source area treatment [34, 36]. SESR has two general approaches.

Solubilization is the use of surfactants above their critical micelle concentration (CMC) to enhance the solubility of contaminants and thereby decrease the pore volumes of water flushing required for treatment. Mobilization is the use of surfactant concentrations above the critical microemulsion concentration (CμC) to reduce the interfacial tension (IFT) between LNAPL and water phases and mobilize the hydrocarbon as a separate phase. To overcome the capillary forces that entrap the LNAPL, large reductions in IFT are necessary. The IFT has to be in an ultra low range, e.g., below 0.1 mN/m, to release the trapped LNAPL and achieve significant mass removal [34, 36]. However, several factors limit the use of surfactants in subsurface remediation. The cost of SESR can be prohibitive when high concentrations of surfactants are required [17, 34]. In addition, persistence of surfactants or their metabolites can result in off site migration and thus potentially pose an environmental concern [34].

Biosurfactants may provide a more cost-effective approach for subsurface remediation when used alone or in combination with synthetic surfactants. The critical micelle concentration of many biosurfactants is much lower than synthetic surfactants [10, 20, 24, 26, 28, 35], suggesting that lower surfactant concentrations can be used. Sufficient amounts of biosurfactants can be produced during the growth of biosurfactant-producing microorganisms to produce low IFT values. IFT values less than 0.01 mN/m have been reported for lipopeptide biosurfactants at concentrations less than 100 mg/l [26, 27]. Lastly, biosurfactants are biodegradable [31], which reduces environmental concern.

Glycolipid biosurfactants, e.g., the rhamnolipids produced by *Pseudomonas* species [3, 19, 21], and trehalose lipids produced by *Rhodococcus* species [18] have been studied for their ability to mobilize, solubilize, and enhance the mineralization of alkanes such as hexadecane and octadecane and polycyclic aromatic hydrocarbons such as naphthalene and phenanthrene. Both batch [9, 44] and column studies [12] showed that biosurfactant addition increased the aqueous solubility of hydrocarbons. However, conflicting results were obtained regarding the effect of biosurfactants on the rate of hydrocarbon degradation. This may have been due to the pH, ionic strength, and biosurfactant concentration used for biodegradation studies. It has been shown that optimal pH for hydrocarbon solubilization might not be optimal for microbial growth and hydrocarbon degradation [37]. In some studies, biosurfactant concentrations above the CMC inhibited degradation [32]. In other studies, biodegradation was stimulated at biosurfactant concentrations above the CMC [38]. A few studies showed that biosurfactant addition mobilized entrapped hydrocarbons by lowering interfacial tension [6, 12]. The injection of over 40–70 pore volumes of the rhamnolipid solution was needed to recover 65% of the entrapped hydrocarbon [6]. Lipopeptide biosurfactants, on the other hand, recovered 20–80% of entrapped crude oil depending on the concentration (20–920 mg/l) using only two pore volumes of the lipopeptide solution [25].

Our previous work on lipopeptide biosurfactants showed that surfactant activity as measured by an oil spreading assay depended on the carbon chain length and the degree of branching of the fatty acid tail [40]. In this study, we sought to optimize biosurfactant formulations for LNAPL mobilization. Our hypothesis was that mixtures of biosurfactants are needed to achieve the ultra low IFT values required for LNAPL mobilization. To test this hypothesis, we first needed to determine how changes in the fatty acid composition of the lipopeptide influenced the IFT. Mixtures of synthetic surfactants have been shown to effectively mobilize perchloroethylene and LNAPL [34, 36], but the efficacy of biosurfactant mixtures has not been evaluated. In this study, lipopeptide biosurfactants from individual strains or mixtures from different strains, mixtures of lipopeptides and rhamnolipids, and mixtures of lipopeptides with synthetic surfactants were tested for their ability to lower interfacial tensions against LNAPL components with different hydrophobicities (toluene, hexane, decane, and hexadecane). The results provide a basis for formulating biosurfactant and synthetic surfactant formulations to achieve ultra low IFT against LNAPL components, which will be valuable not only to environmental remediation but also to other applications that rely upon reducing IFT or increasing the solubility of an oil.

Materials and methods

Sources of biosurfactants and the synthetic surfactant

The C_{12}, C_{13} alcohol propoxylated (PO) sulfate surfactant with 8 PO groups (C12, C13–8PO–SO_4Na) was donated by Sasol (Tucson, AR). Rhamnolipid is a mixture of mono- and di-rhamnolipids [19, 21]. Monorhamnolipid has the formula of α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate and dirhamnolipid has the formula of 2-*O*-α-L-rhamnopyranosyl α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate. The rhamnolipid was obtained from Jeneil Biosurfactants Co. (Saulkville, WI).

Lipopeptide biosurfactants were obtained from different biosurfactant-producing *Bacillus* species as described previously [40] (Table 1). Replicate cultures were grown aerobically at 37°C in a mineral salts medium with 5% NaCl and sucrose as previously described [40, 42]. When needed, 1 g/l L-valine or L-leucine was added to the growth medium before autoclaving [40]. Biosurfactant production was followed over time by using the oil spreading technique

Table 1 Bacterial strains used and the CMC of their lipopeptide biosurfactant

Species	Strain	CMC (mg/l)[a]
Bacillus subtilis subsp. *subtilis*	T89-42	10 ± 0.58
	ROGG-2	10 ± 0.58
Bacillus subtilis subsp. *spizizenii*	T89-3	10 ± 1.5
	ROB-2	17 ± 0
Bacillus mojevenesis	T89-14	17 ± 0.38
	ROG-4	7.8 ± 0.38

[a] The values are the average ± standard deviation of three measurements

[28, 40]. Maximum oil displacement diameter was observed after the culture had reached stationary phase and a pellicle had formed (between 40 and 44 h of growth). At this time, the cells from the 4 l cultures were removed by centrifugation at 14,300*g* for 15 min at 4°C. The cell-free culture fluid was acidified to pH 2 by the addition of 2N HCl and then incubated at 4°C overnight. The precipitate, which contained the biosurfactant, was collected by centrifugation at 14,300*g* for 30 min at 4°C. The pellet was then adjusted to pH 7 with 2N NaOH and lyophilized [40].

Acid-precipitated, lyophilized lipopeptide biosurfactant solutions were analyzed by high performance liquid chromatography (HPLC) using a reversed phase-C_{18} column and a solvent system of 60% acetonitrile in water [41]. Three peaks were obtained at retention times ranging from 1 to 4 min corresponding to the different fatty acid tails of the lipopeptide. The sum of each peak area was used to quantify the biosurfactant concentration in the acid precipitate in comparison to a standard curve prepared with a highly purified biosurfactant preparation obtained from the same strain by a modified TLC method [15, 40]. The surface-active fractions obtained from TLC plates were lyophilized and used to prepare standard solutions with concentrations ranging from 0.2 to 1 mg/ml.

Structural analysis of lipopeptide biosurfactants

The fatty acid composition of each purified biosurfactant was determined by a methanolysis procedure, modified from the method of Yakimov et al. [39, 40].

The amino acid composition of lipopeptide biosurfactant was determined in the Molecular Biology Research Facility of the William K. Warren Research Institute (Oklahoma City, OK) as described previously [40]. The method did not differentiate between acid and amide forms of glutamic and aspartic acids [40]. To clarify which amino acid was present, direct electrospray-mass spectrometry was used. Samples were run in the negative ion mode and the resulting ion fragments were used to determine the exact amino acid composition of two lipopeptides.

Preparation of biosurfactant mixtures

Lipopeptide biosurfactants from different strains were prepared by the acid precipitation method [39, 40] and mixed in different proportions. Mixtures of lipopeptides with rhamnolipids or with C12, C13–8PO–SO_4Na were also prepared. The final surfactant concentration of each mixture was 1 g/l.

Surface and interfacial tension measurement

The surface tension of biosurfactant solutions, with final concentrations ranging from 0 to 1 g/l, was measured with a Du Nuoy ring tensiometer [20]. The tensiometer was calibrated with water as the standard for high range surface tension and isopropanol as the standard for low range surface tension. The critical micelle concentration (CMC) was the concentration at which a sharp increase in surface tension was observed (Table 1).

The IFT between surfactant solutions and different hydrocarbons was determined by using a spinning drop tensiometer [8]. The surfactant solution was used to fill the capillary tube and then the hydrocarbon was added to form a drop inside the capillary tube. The hydrocarbons used were toluene, hexane, decane, and hexadecane, each with 99% purity. The IFT of a 1 g/l surfactant solution was measured against each of the above hydrocarbons with NaCl additions ranging from 0 to 9% (w/v).

Surface and interfacial tension measurements were done in triplicate for each treatment. Most experiments were repeated 2 or 3 times. Averages and standard deviations were calculated for each analysis.

Regression analysis

Multiple regression analysis [43] was used to assess how variability in the fatty acid isomers of lipopeptide biosurfactants contributed to variation in the IFT against toluene. All fatty acid isomers, the sums of the tridecanoate, tetradecanoate, pentadecanoate, hexadecanoate, and octadecanoate isomers, ratios of even *iso* to normal isomers and other combinations were tested.

Results

Determination of the relative hydrophobicity/hydrophilicity of biosurfactants/synthetic surfactants

Interfacial tension values against hydrocarbons with different equivalent alkane carbon numbers (EACN) [1] were used to determine the relative hydrophobicity/hydrophilicity of biosurfactants and a synthetic surfactant. The

hydrophobicity of hydrocarbons increases with the EACN [1]. A surfactant that has its lowest IFT against a hydrocarbon with a low EACN is considered to be relatively hydrophilic [7]. The lipopeptides and the rhamnolipid preparations had their lowest IFT values against toluene (Table 2). The IFT values obtained with these biosurfactants increased as EACN increased (Table 2). The IFT values against toluene (EACN = 1, see Table 2) for the lipopeptides obtained from strains T89-42, T89-3, ROB-2, and the rhamnolipid were slightly lower than those obtained with the T89-14, and ROGG-2 biosurfactant preparations (Table 2), suggesting that the T89-42, T89-3, ROB-2, and the rhamnolipid biosurfactants were more hydrophilic that the latter biosurfactants. C12, C13–8PO–SO_4Na had its lowest IFT against decane (EACN = 10) suggesting that it was more hydrophobic than both the lipopeptide and rhamnolipid biosurfactant preparations.

By calculating the coefficients of variations (CV) from the data in Table 2, it is evident that there were variations in IFT values against toluene, hexane, decane, and hexadecane for different batches of T89-42 and T89-3 biosurfactant preparations (CV ranging from 30 to 63%) and against hexane for different batches of the ROGG-2 biosurfactant preparations (CV of 31%). The same cultivation conditions, medium formulation, and time of collection were used for each batch. The cause for the variations is not known, but the variations in IFT were correlated with changes in the fatty acid composition as discussed below. In general, however, the biosurfactants produced their lowest IFT with the lower EACN hydrocarbons, which supports the conclusion that rhamnolipids and lipopeptides were more hydrophilic than C12, C13–8PO–SO_4Na.

Effect of fatty acid composition of lipopeptide biosurfactants on interfacial activity

Amino acid analysis showed that all of the lipopeptides listed in Table 2 were heptapeptides with the same amino acid composition (mean ± SD of the mole ratio): glutamate or glutamine: aspartate or aspargine: valine: leucine (E or Q: D or N: V: L) (0.99 ± 0.04: 0.99 ± 0.04: 1 ± 0.04: 3.6 ± 0.12). The acid hydrolysis method used to determine the above amino acid compositions did not differentiate between glutamate and glutamine or aspartate and asparagine. Direct electrospray-mass spectrometry was also used to elucidate the amino acid composition of the biosurfactants produced by strains T89-3 and T89-42. The ion molecular weights of 1,020, 1,042, and 1,064 obtained with the T89-42 biosurfactant preparation correspond to M-1, M-2 + Na, M-3 + 2Na ions fragments where *M* is the molecular weight of the lipopeptide (Table 3). These molecular weights are consistent with a lipopeptide that contains 3-hydroxy tetradecanoate, glutamate, aspartate, valine, and three leucines (an amino acid composition of 1E, 1D, 1V, 3L). Similar analyses show that the T89-3 biosurfactant preparation had an amino acid composition of 1E, 1D, 1V, and 3L (Table 3) with 3-hydroxy fatty acids of different carbon lengths. These data suggest that the lipopeptides are surfactin A [28, 30].

As mentioned above, the IFT values against toluene for different batches of the biosurfactant from the same strain varied. The fatty acid composition of the different preparations also varied. Multiple regression analysis [43] was used to determine the changes in fatty acids isomers that contributed to the variation in IFT against toluene. The best

Table 2 IFT of different biosurfactants and a synthetic surfactant against hydrocarbons with a range of equivalent alkane carbon number (EACN)

Surfactant	Strain[a]	IFT (mN/m) with different hydrocarbons			
		Toluene (1)[b]	Hexane (6)[b]	Decane (10)[b]	Hexadecane (16)[b]
Lipopeptide[a]	T89-42 (4)	0.63 ± 0.26[c]	1.22 ± 0.49[c]	0.84 ± 0.5[c]	0.86 ± 0.41[c]
	T89-3 (3)	0.3 ± 0.19	1.17 ± 0.65	1.29 ± 0.57	1.49 ± 0.45
	ROB-2 (1)	0.66 ± 0.03	1.27 ± 0.09	1.22 ± 0.07	2.75 ± 0.05
	T89-14 (1)	1.05 ± 0.4	1.19 ± 0.5	1.46 ± 0.4	1.91 ± 0.25
	ROGG-2 (2)	2.17 ± 0.17	3.27 ± 1.03	3.9 ± 0.34	4.27 ± 0.55
Rhamnolipid		0.31 ± 0.01	0.65 ± 0.02	0.69 ± 0.1	0.8 ± 0.02
C12, C13–8PO–SO_4Na		2.19 ± 0.01	0.2 ± 0.02	0.02 ± 0.001	0.04 ± 0.001

[a] The lipopeptides are designated by the name of the strain from which the lipopeptide was purified. The numbers in parentheses refers to the number of replicate batches analyzed

[b] The equivalent alkane carbon number (EACN) of each hydrocarbon is given in parentheses

[c] Values are the average ± standard deviation of three IFT measurements for each 1 g/l surfactant solution. When more than one batch of a lipopeptide was analyzed, all of the measurements were used to compute the average ± standard deviation. As is commonly done in surfactant formulation work, the salinity was varied in these samples to produce a minimum in IFT—the resulting salinity values varied from 3.5 to 10 wt%

Table 3 Electrospray mass spectrometry data for two lipopeptide biosurfactants

Biosurfactant[a]	Ion fragment[b]	Fragment molecular weight	β-OH Fatty acid tail length	Amino acid composition
T89-42	M-1	1,006	C13	1D, 1E, 1V, 3L
		1,020	C14	1D, 1E, 1V, 3L
		1,034	C15	1D, 1E, 1V, 3L
	M-2 + Na	1,028	C13	1D, 1E, 1V, 3L
		1,042	C14	1D, 1E, 1V, 3L
		1,056	C15	1D, 1E, 1V, 3L
	M-3 + 2Na	1,064	C14	1D, 1E, 1V, 3L
		1,078	C15	1D, 1E, 1V, 3L
T89-3	M-2 + Na	1,014	C12	1D, 1E, 1V, 3L
		1,028	C13	1D, 1E, 1V, 3L
		1,042	C14	1D, 1E, 1V, 3L
		1,056	C15	1D, 1E, 1V, 3L
	M-3 + 2Na	1,050	C13	1D, 1E, 1V, 3L
		1,064	C14	1D, 1E, 1V, 3L
		1,078	C15	1D, 1E, 1V, 3L

[a] The name of the lipopeptide biosurfactant refers to the name of the strain from which it was purified

[b] Ion fragments were obtained in the negative ion mode. M is the molecular weight of the biosurfactant. M-1 corresponds to the loss of one hydrogen ion from the molecule. M-2 + Na corresponds to the loss of 2 hydrogen ions and the addition of one sodium ion. M-3 + 2Na corresponds to the loss of 3 hydrogen ions and the addition of 2 sodium ions. All ion fragments are negatively charged

model that correlated the interfacial tension of lipopeptide biosurfactants against toluene was dependent on the sum of the 3-OH C_{14} isomers, the 3-OH C_{15} isomers, 3-OH C_{16}, and 3-OH C_{18} fatty acids. When the values expected for interfacial tension (obtained by using the multiple regression equation from the fatty acid composition) were plotted against the values of interfacial tensions obtained experimentally (for one biosurfactant purified from four replicate cultures, another biosurfactant purified from three replicate cultures, and a third biosurfactant purified from duplicate cultures) (Fig. 1a), the linear correlation coefficient (r^2) was 0.986 [11]. F-statistic showed that the effect of the fatty acid composition on IFT was significant ($P < 0.05$) and stepwise testing using Student's t test showed that each of the coefficients in the multiple regression equation significantly influenced the IFT ($P < 0.05$) [11]. The multiple regression model also accurately predicted the interfacial tension against toluene from the fatty acid composition for five other lipopeptide biosurfactants produced by four strains of *B. mojavensis* and one strain of *B. subtilis* subsp. *subtilis* strains and for 20 biosurfactant mixtures (Fig. 1b).

Comparing the coefficients in the multiple regression equation (Fig. 1a legend), it did not appear that one of the fatty acid isomers was more important in determining IFT than the others. However, it was observed that low IFT values against toluene (<0.5 mN/m) were obtained only when the percentages of 3-OH C14 and 3-OH C15 constituted less than 70% of the total fatty acids, the percentage of 3-OH C15 was higher than or equal to that of 3-OH C14, and the ratio of 3-OH C16 to 3-OH C18 was more than 8. In cases where the percentage of 3 OH C14 comprised more than 70% of the total fatty acids, the IFT against toluene was high (>1.5 mN/m).

Formulating lipopeptide biosurfactant mixtures for low IFT against toluene

Because the interfacial activity of the biosurfactant depended on the fatty acid composition of the lipopeptide, we hypothesized that lipopeptide biosurfactant mixtures could be formulated to obtain low interfacial tension against toluene based on the fatty acid composition. To test this hypothesis, biosurfactants from strains T89-42 and T89-3 were mixed in different proportions and the IFT was measured against toluene. Each biosurfactant had the same amino acid composition (Table 3). Table 4 shows the fatty acid composition of two separate batches of T89-42 and T89-3 biosurfactants. The first batch of T89-42 biosurfactant had an IFT of 0.27 ± 0.04 mN/m and the second batch had an IFT of 0.71 ± 0.04 mN/m against toluene. Similarly, the first batch of T89-3 biosurfactant had an IFT of 0.12 ± 0.01 mN/m and the second batch had an IFT of 0.48 ± 0.02 mN/m against toluene. The differences in IFT between the first and second batches for each strain were explained by using the multiple regression model. In the first batch, the percentage of 3-OH C14 was less than that of 3-OH C15, their sum was less than 70% of the total fatty acids, and the ratio of 3-OH C16 to 3-OH C18 was more

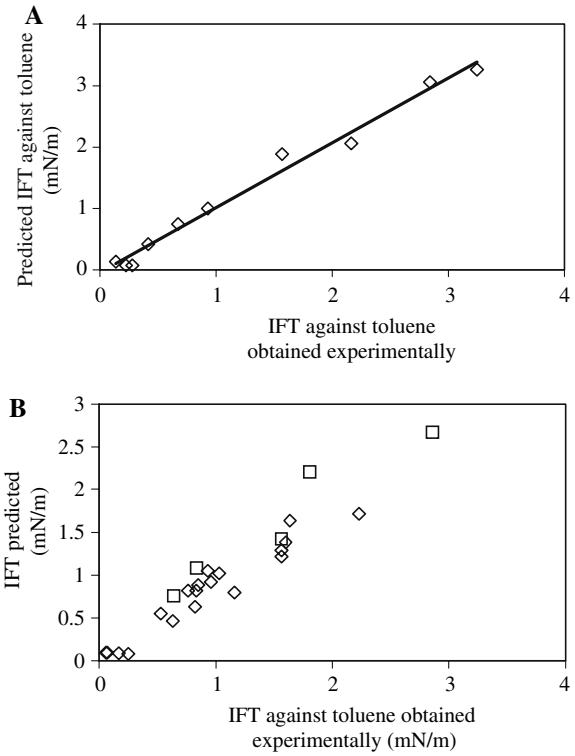

Fig. 1 Multiple regression analysis for the fatty acid predictors of interfacial activity against toluene for lipopeptide biosurfactants. **a** Values on the *x*-axis are the experimentally obtained IFT against toluene for lipopeptides produced by three different strains (4 replicate cultures for one strain, 3 replicate cultures for the second strain, and duplicate cultures for the third strain). Values on the *y*-axis were obtained by using the multiple regression equation: *y* (IFT against toluene) = 0.09 (percentage of 3-OH C_{14}) + 0.06 (percentage of 3-OH C_{15}) + 0.05 (percentage of 3-OH C_{16}) + 0.09 (percentage of 3-OH C_{18}) –5.7. The equation of the straight line was $y = 1.1x - 0.053$. The coefficient of linear regression was $r^2 = 0.986$. **b** The multiple regression equation above was used to predict IFT against toluene for five other individual biosurfactants (*open squares*), and 20 biosurfactant mixtures (*open diamonds*). The coefficient of linear regression (r^2) between the predicted and actual IFT for the five individual biosurfactants was 0.92 ($y = 0.9x + 0.29$), and that for biosurfactant mixtures was 0.93 ($y = 0.84x + 0.04$)

than 8, consistent with the predictions of the multiple regression model (Table 4). However, in the second batch, the ratio of 3-OH C16 to 3-OH C18 was low (1.2 and 0.25 for T89-42 and T89-3 biosurfactants, respectively), which explained the relatively higher IFT values (Table 4) (i.e., the surfactant was more hydrophobic).

The T89-42 and T89-3 biosurfactants from the first batch were mixed in different proportions to test the predictions of the multiple regression model. The fatty acid composition of the mixture was calculated from the percentage of each fatty acid in the mixture by using the following equation:

$$FA_{M,i} = [f_1 \times \%FA_{1,i}] + [f_2 \times \% FA_{2,i}],$$

where $FA_{M,i}$ is the percentage of fatty acid *i* in the mixture, f_1 and f_2 are the fractions of biosurfactant 1 and 2 in the mixture, respectively, and $FA_{1,i}$ and $FA_{2,i}$ are the percent of fatty acid *i* in biosurfactants 1 and 2, respectively. Table 4 shows the calculated fatty acid percentages in the mixtures of T89-42 and T89-3 biosurfactants and the predicted IFT values. Ultra low IFT values (<0.1 mN/m) were predicted with mixtures with 20 and 40% of the T89-42 biosurfactant. When the mixture contained 20% of the T89-42 biosurfactant, the predicted IFT value was 0.09 mN/m and the experimentally obtained IFT value was 0.06 ± 0.02 mN/m. With a mixture containing 40% of the T89-42 biosurfactant, the predicted IFT value was 0.09 mN/m and the experimentally obtained IFT value was 0.07 ± 0.01 mN/m. In each case, ultra low values could be explained by the high 3-OH C16 to 3-OH C18 ratio, which was greater than 9.

Based on the low percentage of 3-OH C16 (<3%) in the T89-42 and T89-3 biosurfactants from the second batch, the multiple regression model did not predict ultra low IFT (<0.1 mN/m) for any combination of these two biosurfactants. IFT values against toluene for mixtures of the two biosurfactants from the second batch were similar to IFT values obtained with the individual biosurfactants. The predicted IFT value for the 50/50 mixture was 0.53 mN/m and the experimentally obtained IFT value was 0.68 ± 0.15 mN/m (Table 4). The relatively high IFT value was expected since the sum of percentages of 3-OH C14 and 3-OH C15 was more than 70% of the total fatty acids, and the ratio of 3-OH C16 to 3-OH C18 was 0.5 (much less than the above cited value of 8).

Similar results were obtained when the ROB-2 and the T89-14 biosurfactants were mixed in different proportions. The multiple regression model did not predict ultra low IFT values for either of the two biosurfactant preparations alone or for any combination of the two biosurfactant preparations due to the low 3-OH C16 to 3-OH C18 ratio for each of the biosurfactants preparation and the various mixtures of the two preparations (data not shown). Experimentally obtained IFT values against toluene were in agreement with the model predictions (data not shown).

Collectively, these results argued for the validity of the multiple regression model and suggested that the fatty acid composition of lipopeptide biosurfactants is an accurate predictor of the IFT against toluene and could be used to formulate mixtures to achieve ultra low IFT.

Mixtures of lipopeptide biosurfactants with rhamnolipids lower IFT against toluene

Due to the similarity in amino acid composition among lipopeptide biosurfactants, differences in hydrophobicity/

Table 4 Predicted versus experimentally determined IFT values against toluene for different biosurfactant formulations

Biosurfactant[a]	Percentage of different fatty acids (% mass values)[b]						Predicted IFT (mN/m)[c]	Experimentally obtained IFT (mN/m)[d]
	3-OH C_{14}	3-OH C_{15}	3-OH C_{16}	3-OH C_{18}	Sum of 3-OH C14 and C15	3-OH C16/3-OH C18		
T89-42 (1)	22.2	45.8	17	2	68	8.5	0.08	0.27 ± 0.04
T89-3 (1)	27.5	27.4	28.4	2.9	54.9	9.8	0.13	0.12 ± 0.01
0.2 T89-42 (1) + 0.8 T89-3 (1)[e]	26.4[f]	31.1[f]	26.12[f]	2.72[f]	57.5	9.6	0.09	0.06 ± 0.02
0.4 T89-42 (1) + 0.6 T89-3 (1)[e]	19.9[f]	29.3[f]	18.2[f]	1.96[f]	49.2	9.3	0.09	0.07 ± 0.01
T89-42 (2)	29.1	56.3	3.27	2.83	85.4	1.2	0.74	0.71 ± 0.04
T89-3 (2)	33	45	1	4	78	0.25	0.42	0.48 ± 0.02
0.5 T89-42 (2) + 0.5 T89-3 (2)[e]	31.1[f]	50.7[f]	1.69[f]	3.4[f]	81.8	0.5	0.53	0.68 ± 0.15

[a] The name of the lipopeptide biosurfactant refers to the name of the strain from which it was purified. The number in parentheses refers to the batch number. The T89-42 and T89-3 biosurfactant were obtained from two different batches of cultures

[b] The percentage of different fatty acids (mass values%) in the lipid portion of the purified biosurfactant. The percentage was calculated by dividing the peak areas of individual fatty acids by the total peak areas of all FAME

[c] The IFT was calculated by using the multiple regression equation in the legend of Fig. 1a

[d] The IFT values are averages ± standard deviation of three measurements

[e] Components of lipopeptide mixture and fraction of each biosurfactant in the mixture are given

[f] Fatty acid composition of the mixture calculated using the equation: $FA_{M,i} = [f_1 \times \%FA_{1,i}] + [f_2 \times \% FA_{2,i}]$, where $FA_{M,i}$ is the percentage of fatty acid i in the mixture, f_1 and f_2 are the fractions of biosurfactant 1 and 2 in the mixture, respectively, and $FA_{1,i}$ and $FA_{2,i}$ are the percent of fatty acid i in biosurfactants 1 and 2, respectively

hydrophilicity between individual lipopeptide biosurfactants might not be pronounced, making it difficult to formulate lipopeptide biosurfactant mixtures to achieve ultra low IFT especially with the variable fatty acid composition. The IFT values against different hydrocarbons showed that the rhamnolipid was more hydrophilic than T89-42 and T89-3 biosurfactants (Table 2). Mixtures of lipopeptides with rhamnolipid will be more hydrophilic than those with only lipopeptides. We hypothesized that mixtures of lipopeptide and rhamnolipid biosurfactants will be more effective than individual biosurfactants in achieving ultra low IFT values against toluene, a hydrocarbon with low EACN. To test this hypothesis, the rhamnolipid was mixed with T89-42 biosurfactants produced under different culture conditions to manipulate the 3-OH fatty acid tail of the lipopeptide and hence the hydrophilicity of the lipopeptide biosurfactant. When strain T89-42 was grown in a medium without amino acid addition, the lipopeptide biosurfactant contained mainly 3-OH C14, and 3-OH C15. The sum of these comprised 67% of the total fatty acid. According to the multiple regression model, the IFT against toluene was predicted to be 0.92 mN/m and the experimentally obtained value was 0.95 ± 0.01 mN/m. The IFT against toluene for the rhamnolipid alone was 0.31 ± 0.01 mN/m (Table 2). When the T89-42 biosurfactant was mixed with the rhamnolipid in different proportions, the IFT against toluene decreased from 0.95 ± 0.01 mN/m for T89-42 biosurfactant alone to 0.09 mN/m when only 20% of the mixture was the lipopeptide (Table 5). These data support the hypothesis that the addition of the more hydrophilic rhamnolipid to lipopeptides lowers the IFT against hydrocarbons with low EACN.

To further test the hypothesis, the fatty acid composition of T89-42 biosurfactant was changed by growing the strain with 1 g/l of valine, a precursor of *iso* even-numbered fatty acids, or 1 g/l leucine, a precursor of *iso* odd-numbered fatty acids [40]. When the lipopeptide produced with valine addition to the growth medium (62% of the fatty acids was 3-OH-C14) was mixed with the rhamnolipid in different proportions, the IFT of the mixture was 0.02 mN/m when 20% of the mixture was the lipopeptide (Table 5). However, when the lipopeptide produced with leucine addition (only 5% of the fatty acids was 3-OH C14) was mixed with the rhamnolipid in different proportions, the IFT of the mixture with rhamnolipid slightly increased (Table 5). Thus, when the percentage of more hydrophobic fatty acids (3OH C15 and 3OH C17) in the lipopeptide increased with leucine addition, the biosurfactant mixture was less effective in lowering IFT against a hydrophilic hydrocarbon (toluene) compared to mixtures that contained lipopeptides with a high percentage of more hydrophilic fatty acids, e.g., those obtained with valine addition.

Mixtures of lipopeptide biosurfactants with C12, C13–8PO–SO_4Na lower IFT against hexane and decane

As shown above, mixing lipopeptide biosurfactants with the more hydrophilic rhamnolipid biosurfactant was an

Table 5 IFT (mN/m) against toluene for mixtures of the T89-42 biosurfactant with different percentages of the rhamnolipid biosurfacant

Amino acid added to cultivation medium	Percentage of 3-OH C14 (% mass value)in the T89-42 biosurfactant	IFT (mN/m) against toluene for mixtures of the T89-42 biosurfactant with the indicated percent of the rhamnolipid biosurfactant		
		0%	50%	80%
Leucine	5	0.23	0.52	0.56
None	33	0.95 ± 0.01[a]	0.32 ± 0.12[a]	0.09 ± 0.02[a]
Valine	62	1.33 ± 0.04[a]	0.14	0.02

[a] IFT values against toluene are the average ± standard deviation of three measurements

effective approach to obtain low IFT values against hydrocarbons with low EACN, e.g., toluene. To obtain an effective biosurfactant formulation against hydrocarbons with higher EACN, e.g., hexane and decane, the hydrophobicity of the mixture should increase relative to that which was effective with low EACN hydrocarbons. We hypothesized that mixtures of lipopeptide biosurfactants with the more hydrophobic, synthetic surfactant C12, C13–8PO–SO_4Na would be able to produce low IFT against hydrophobic hydrocarbons such as hexane and decane. To test this hypothesis, lipopeptide biosurfactants from three different strains that differed in hydrophobicity were mixed with C12, C13–8PO–SO_4Na in different proportions at 5% NaCl. At this salt concentration, the IFT against toluene was 0.48 ± 0.02 mN/m for the T89-3 biosurfactant, 0.95 ± 0.01 mN/m for the T89-42 biosurfactant, and 2.18 ± 0.01 mN/m for the ROGG-2 biosurfactant. These data indicate that the T89-3 biosurfactant was more hydrophilic than the T89-42 biosurfactant, which was more hydrophilic than the ROGG-2 biosurfactant. In mixtures of lipopeptides with C12, C13–8PO–SO_4Na, the hydrophobicity of the mixture will increase as the amount of C12, C13–8PO–SO_4Na increases. Secondly, the mixture will be more hydrophilic with the T89-3 biosurfactant than with the ROGG-2 biosurfactant. Considering these two factors, we expected that the lowest IFT against hexane (a hydrocarbon with moderate hydrophobicity and an EACN of 6) will be obtained with mixtures of the T89-3 biosurfactant (most hydrophilic) and a small percentage of C12, C13–8PO–SO_4Na. Similarly, the lowest IFT against decane (a hydrophobic hydrocarbon with an EACN of 10) will be obtained with a mixture of the ROGG-2 biosurfactant (most hydrophobic) and a high percentage of C12, C13-8PO sulfate. As predicted, an ultra low IFT against hexane of 0.014 ± 0.004 mN/m was obtained with a mixture of the T89-3 biosurfactant and 25% of C12, C13–8PO–SO_4Na and an ultra low IFT against decane of 0.013 ± 0.001 mN/m was obtained with a mixture of the ROGG-2 biosurfactant and 50% of C12, C13–8PO–SO_4Na (Table 6). The IFT of each component alone against the different hydrocarbons is given in the footnote to Table 6. These results supported the hypothesis that low IFT values against hydrocarbons with high EACN are obtained when the hydrophobicity of the biosurfactant mixture increases.

Discussion

Surfactant-enhanced subsurface remediation (SESR) technology significantly reduces the time required to remove LNAPL from subsurface by removing the entrapped mass of hydrocarbon by mobilization with surfactants [34, 36]. While advances in surfactant chemistry have dramatically improved LNAPL removal efficiencies, the key to further improvements in the economic competitiveness of surfactant-based technologies is to reduce the mass of surfactant needed to recover the entrapped LNAPL [17]. Interestingly, Knapp et al. [16] found that lipopeptide biosurfactants can remove a large percentage of residual hydrocarbon from sand-packed columns at biosurfactant concentrations about 10 to 100-fold lower than typically used for surfactant-enhanced LNAPL mobilization [16]. Other studies with lipopeptide biosurfactants showed oil recoveries of 56–90%, but with higher lipopeptide concentrations (1 g/l) [4, 22, 23, 29]. The reason for the discrepancy in the lipopeptide concentration needed for larger residual oil recoveries was not clear. In order to mobilize LNAPL, a significant reduction in the oil-water interfacial tension is required to reduce the capillary forces that trap the oil [33, 36]. Until now, the interfacial activity and the efficacy of recovering residual hydrocarbon have only been studied with individual biosurfactant compounds. These studies show that solubilization and biodegradation are the main mechanisms for oil removal by biosurfactants [2, 9, 14, 37, 44]. Only a few studies showed mobilization of entrapped hydrocarbons [6, 12, 13]. Here, we show that ultra low IFT can be achieved by altering the hydrophilic/hydrophobic balance of the formulation by selective addition of biosurfactants or surfactants.

Previously, we showed that specific biosurfactant surface activity against crude oil, as measured by an oil spreading assay, depended on the ratios of *iso* to normal even-numbered fatty acid and of *anteiso* to *iso* odd-numbered fatty acids of the lipid tail [40]. While the study

Table 6 IFT (mN/m) against different hydrocarbons for mixtures of lipopeptides with different percentages of C12, C13–8PO–SO_4Na

Lipopeptide[a]	IFT (mN/m) against the indicated hydrocarbon for mixtures of lipopeptides and C12, C13–8PO–SO_4Na			
	25% of C12, C13–8PO–SO_4Na[b]		50% of C12, C13–8PO–SO_4Na[b]	
	Hexane	Decane	Hexane	Decane
T89-3	0.014 ± 0.004[c]	0.04 ± 0.001[c]	0.08 ± 0.006[c]	0.02 ± 0.001[c]
T89-42	0.03 ± 0.01	0.04 ± 0.006	0.06 ± 0.01	0.02 ± 0.002
ROGG-2	0.05 ± 0.01	0.03 ± 0.001	0.05 ± 0.01	0.013 ± 0.001

[a] The name of the lipopeptide biosurfactant refers to the name of the strain from which it was purified. IFT values (mN/m) for the lipopeptides are: T89-3 (hexane) 0.38 ± 0.04, T89-3 (decane) 0.67 ± 0.03, T89-42 (hexane) 0.88 ± 0.09, T89-42 (decane) 0.46 ± 0.05, ROGG-2 (hexane) 2.25 ± 0.42, ROGG-2 (decane) 3.77 ± 0.16

[b] Percentage of C12, C13–8PO–SO_4Na in the surfactant mixture

[c] IFT of the mixture against the hydrocarbon in the table header. Values are the average ± standard deviation of three measurements

illustrated the importance of the fatty acid composition for surface activity, the relationship was not useful to predict the efficacy of lipopeptides in mobilizing entrapped LNAPL because the mobilization depends on IFT reduction [33]. Although most of the characterized lipopeptide biosurfactants studied have very similar structures, especially in the peptide portion of the molecule, a wide variation in the IFT against toluene was observed with different lipopeptides (Table 2). In order to explain this, experiments were conducted to delineate the structural features important for interfacial activity. Here, multiple regression analysis showed that interfacial tension against toluene is correlated to the percentages of 3-OH C_{14}, 3-OH C_{15}, 3-OH C_{16}, and 3-OH C_{18} fatty acids in the lipid tail of lipopeptides. Low IFT values against toluene are obtained when the percentages of 3-OH C14 and 3-OH C15 constitute less than 70% of the total fatty acids, the percentage of 3-OH C15 is higher than or equal to that of 3 OH C14, and the ratio of 3-OH C16 to 3-OH C18 is more than 8. Low IFT values against toluene were obtained with lipopeptide biosurfactants that had this fatty acid composition, e.g., the T89-3 biosurfactant (IFT of 0.12 ± 0.01 mN/m) and the T89-42 biosurfactant (0.27 ± 0.04 mN/m) (Table 4). However, ultra low IFT values (<0.1 mN/m) were not observed with lipopeptide biosurfactants produced by individual strains. Using the information from the multiple regression model, we predicted that ultra low IFT against toluene could be obtained by mixing lipopeptide biosurfactants in different proportions such that the fatty acid composition of the mixture is 50–60% 3-OH C14 and 3-OH C15 fatty acids with a 3-OH C16 to 3-OH C18 ratio of at least 8. This prediction proved correct when T89-42 and T89-3 biosurfactants were mixed in different proportions to obtain formulations where the sum of 3-OH C14 and 3-OH C15 fatty acids was in the 50–60% range and the 3-OH C16 to 3-OH C18 ratio was 9.3 and 9.6. With these mixtures, ultra low IFT values (0.06 ± 0.02 and 0.07 ± 0.01 mN/m) were obtained against toluene (Table 4). Sometimes, the above fatty acid balance may be hard to achieve with binary mixtures (biosurfactants from two strains) and the addition of a third component to the mixture may be required. It was shown previously that nutritional manipulation by the addition of branched-chain amino acids to the culture medium leads to the production of lipopeptide biosurfactants with 70–90% of their total fatty acid composition as a single fatty acid, e.g., 3-OH C14 with valine addition or 3-OH C15 with leucine addition [40]. The biosurfactants produced under these conditions could certainly be used as the third component in the mixtures to increase the percentage of a certain fatty acid to achieve the appropriate fatty acid composition required for ultra low IFT values.

Although rhamnolipid biosurfactants have been investigated for subsurface remediation, most of the studies have focused on hydrocarbon removal by solubilization (increase in the aqueous solubility of the hydrocarbon) [9, 37, 44] rather than mobilization (lowering IFT between aqueous and LNAPL phases to reduce the capillary pressure that traps the oil) [12]. Here, we found that the rhamnolipid biosurfactant had a low IFT against toluene (0.31 ± 0.01 mN/m) and was more hydrophilic than all of the lipopeptides studied. We hypothesized that mixtures of rhamnolipids with lipopeptides would alter the hydrophilic/hydrophobic balance and achieve the ultra low IFT values against toluene needed for hydrocarbon mobilization. Our results showed that ultra low IFT values against toluene were obtained with rhamnolipid-lipopeptide mixtures when the percentage of 3-OH C14 fatty acid in the lipopeptide tail was 33% or greater (Table 5). Although these mixtures were not tested against hydrocarbons with higher EACN, we predict that ultra low IFT against hydrocarbons with high EACN can be achieved by adding a lipopeptide with a

more hydrophobic tail. These results would be important in formulating biosurfactant mixtures to remove hydrocarbons attached to particulate matter where solubilization is difficult unless the capillary forces are reduced [13].

Mixtures of lipopeptide biosurfactants with the more hydrophobic C12, C13–8PO–SO_4Na were the most effective in lowering the IFT against hexane and decane. Because lipopeptide biosurfactants had varying degrees of hydrophilicity, it was possible to formulate mixtures of different lipopeptides with C12, C13–8PO–SO_4Na that varied in hydrophilicity/hydrophobicity. Varying the percentage of C12, C13–8PO–SO_4Na in the mixtures was also used to increase the hydrophobicity of the mixture. Low IFT values against hexane and decane were obtained with C12, C13–8PO–SO_4Na alone (0.2 ± 0.02 and 0.02 ± 0.001 mN/m, respectively) (Table 2). The addition of lipopeptides to C12, C13–8PO–SO_4Na lowered the IFT values against hexane (0.014 ± 0.004 mN/m) and decane (0.013 ± 0.001 mN/m) (Table 6). More importantly, the addition of lipopeptides lowered the amount of C12, C13–8PO–SO_4Na required to achieve these ultra low IFT values. An ultra low IFT value against hexane was obtained with the T89-3 biosurfactant and 250 mg/l of with C12, C13–8PO–SO_4Na compared with 1 g/l for C12, C13–8PO–SO_4Na alone. Reducing the amount of the surfactant is important from an economic point of view. However, further information on the economics of biosurfactant production will be needed to determine if the use of biosurfactants will provide an economic advantage compared to synthetic surfactants.

This work focused on biosurfactant/synthetic surfactant interfacial behavior against single hydrocarbons with varying EACN. We found that knowledge about biosurfactant fatty acid composition and its relationship to hydrophobicity/hydrophilicity can be used to formulate biosurfactant/surfactant mixtures to achieve ultra low IFT against hydrocarbon with different EACN. The use of biosurfactant mixtures increased the likelihood for achieving the optimum interfacial behavior compared to individual biosurfactants. Previous work often paid little attention to the fatty acid composition since this can be quite variable, making it difficult to correlate changes in the fatty acid composition to changes in interfacial activity. Our work provides guidelines to reduce the trial and error approach often used to find optimum formulations for mobilizing entrapped hydrocarbons. Future work focusing on mobilization of hydrophobic hydrocarbons is certainly required before going from the well-controlled laboratory experiments to designing field scale technology.

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