

## **Evaluation of Sub-micellar Synthetic Surfactants versus Biosurfactants for Enhanced LNAPL Recovery**

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**Title:** Evaluation of Sub-micellar Synthetic Surfactants versus Biosurfactants for Enhanced LNAPL Recovery

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**Research Category:** Petroleum Environmental Technology – Recovery of free-phase LNAPL

**Keywords:** Biosurfactant, surfactant-enhanced subsurface remediation, LNAPL, interfacial tension, phase behavior, lipopeptide, rhamnolipid.

**Objective:** Surfactant-enhanced subsurface remediation (SESR) significantly reduces the time required to remove light non-aqueous phase liquids (LNAPLs) from contaminated aquifers by improving LNAPL solubility and/or mobility. The overall objectives of this research is to assess the relative technical and economic efficiency of using biosurfactants and their mixtures to recover free-phase LNAPL as compared to synthetic surfactants. Specific objectives of the research are: (1) to determine the optimum phase behavior of the lipopeptide biosurfactants made by *Bacillus* species and the rhamnolipid biosurfactant made by *Pseudomonas* species; (2) to determine the efficacy of mixtures of biosurfactants relative to single biosurfactants in producing low interfacial tension; and (3) to compare the efficacy of optimized biosurfactant formulations to that of synthetic surfactant formulations.

### **Progress Report/Accomplishments:**

***Biosurfactant Production and Characterization:*** To obtain purified biosurfactants for use as standards, we modified the method of Kim et. al. (1). When the maximum biosurfactant activity was reached, cells from 1-liter cultures were removed by centrifugation at 14,300 x g for 15 min at 4°C. Biosurfactant in the supernatant was precipitated with 40% w/v ammonium sulfate and overnight incubation at room temperature. The precipitate containing the biosurfactant along with other compounds was then collected by centrifugation at 14,300 x g for 30 min at 4°C. The precipitate was extracted with 250 µl of chilled acetone to remove most of the proteins. Further purification was achieved by preparative thin layer chromatography (TLC). The whole acetone extract (250 µl) was spotted on preparative silica gel TLC plates (Whatman, Clifton, NJ) and the plates were resolved with a solvent system of isopropanol: water: 28% w/v ammonium hydroxide (80:11:9). The TLC plates were developed with iodine vapor. Each fraction was scraped off the plate, dissolved in 250 µl water, and tested for surface activity with the oil spreading technique (ref. 3). Surface-active fractions were lyophilized. The oil spreading technique involved placing a sample of the biosurfactant

solution on top of a film of oil that is floating on the surface of water. The diameter of the clear zone is measured and used as an indication of biosurfactant activity.

To obtain larger amounts of the biosurfactant for phase behavior studies, 4-liter cultures were used and the biosurfactant was precipitated by acidification. When the maximum oil displacement diameter was obtained, cells from 4-liter cultures were removed by centrifugation at 14,300 *g* for 15 min at 4°C. The cell-free culture fluid was acidified by the addition of 2N HCl until a pH of 2 was obtained 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 containing the biosurfactant was then adjusted to pH 7 using NaOH and lyophilized.

The concentrations of biosurfactants obtained by acid precipitation were quantified by relating the peak areas obtained by high-pressure liquid chromatography (HPLC) to those of known standards. Biosurfactant from each strain was purified by ammonium sulfate precipitation, acetone extraction and TLC as discussed above and used to prepare standard solutions with concentrations ranging from 0.2 to 1 mg/ml. Standard solutions were then run on the HPLC using a RP-C18 column and a solvent system of 60% acetonitrile. Three peaks were obtained at retention times ranging from 1 to 4 minutes. The sum of the peak areas was determined and standard curves between the sum of the peak areas and the amount of the purified biosurfactant were constructed. To determine the concentration of the biosurfactant obtained by the acid precipitation method, the sum of the peak areas of a 1 mg/ml solution of the acid precipitated material was compared to the standard curve generated with the purified biosurfactant standard obtained from the same strain. The biosurfactant activity in the acid precipitate was determined by the oil spreading technique. Standard curves between the diameter of clear zones and the concentration of the biosurfactant standard obtained by ammonium sulfate precipitation, acetone extraction, and TLC in the range of 0.2 to 1 mg/ml were constructed. A 1 mg/ml solution of the acid precipitate was prepared and the diameter of clear zone was obtained using the oil spreading technique. The diameter was used to determine the biosurfactant activity from the standard curve. All measurements of biosurfactant concentration and activity were repeated 3 times. The mean, standard deviation, and percent variation were calculated. The values measured were accepted if the percent variation was less than or equal to 15%.

A methanolysis procedure, modified from the method of Yakimov et al. (2), was used to analyze the fatty acids present in the biosurfactant. Two hundred micrograms of the acid precipitated biosurfactant were hydrolyzed under vacuum for 16 hours at 90°C with 4 ml of 25% 12 N HCl in methanol in sealed tubes. The hydrolyzed fatty acids methyl esters (FAME) were then extracted with 7 ml of 1:1 v/v ethyl acetate: hexane (EAH solvent). The organic phase was concentrated under a stream of N<sub>2</sub> to 0.6 ml. The concentrated fractions were neutralized with 0.5 ml of 0.4 M phosphate buffer (pH 12) and incubated at room temperature for 10 min. The FAME in the organic layer were derivatized with BSTFA (Pierce, Rockford, IL) and analyzed by gas chromatography/mass spectrometry (GC/MS) (Agilent Technologies 6890N Network GC systems/ 5973 Network Mass Selective Detector, Willmington, DE). One microliter of each sample was used for injection. The oven temperature was set at 60°C for 5 min and then increased to 250°C over a 15-min interval. The column was a capillary column 0.25 mm X 30 m X 0.25 μm. The carrier gas was helium and the flow rate was 1.2 ml/min. The mass

spectrometer was operated at 400Hz. Peak areas obtained on the GC chromatogram were used to calculate the percentage of the FAME isomers compared to the area of all FAME.

*Biosurfactant / Oil Interactions:* The interaction between the biosurfactant and different oils was evaluated to find the optimal phase behavior of the biosurfactants. We assessed the oil-surfactant interaction by measuring the interfacial tension (IFT) between biosurfactant solutions and different oils using the Spinning Drop Tensiometer. The denser phase, which was the continuous surfactant solution, was used to fill the capillary tube and the less dense phase – the oil – formed a drop inside the capillary tube. Four oils (99%+ purity) were used: toluene, hexane, decane, and hexadecane. Two types of biosurfactants were studied: lipopeptides and rhamnolipids. The lipopeptide biosurfactants were produced by the acid precipitation method discussed above and stored in a freezer at -20°C and the rhamnolipid biosurfactants were purchased from Jeniel Biosurfactants Company and kept at room temperature. Since the biosurfactants showed the lowest IFT against toluene as compared with hexane, decane, and hexadecane, our studies focused primarily on toluene. Mixtures of two lipopeptide biosurfactants as well as mixtures of one lipopeptide and one rhamnolipid biosurfactant were also used to measure the IFT against toluene.

Figure 1 shows the results of IFTs against all four oils for the lipopeptide biosurfactant, called T89-42 (e. g., produced by *Bacillus subtilis* subspecies *subtilis* strain T89-42). From Figure 1, we observed that the IFT of T89-42 was lowest against toluene and that the toluene IFT was a minimum at salinity values lower than 8 wt%. Figure 2 shows the IFT values against toluene for different biosurfactants. The IFT of the rhamnolipid biosurfactant against toluene was much lower than those of any of the lipopeptide biosurfactants. Thus, the rhamnolipid biosurfactant was chosen for evaluation in subsequent phase studies, as shown in Table 1. Figures 3 and 4 show the IFT values against toluene for mixtures of two biosurfactants. Figure 3 represents the IFT values of mixtures of two different lipopeptide biosurfactants. We observed that for all three mixtures, the IFT values of the single biosurfactants ROB-2 and T89-42 (Fig. 2) were lower than those of the mixtures at any mixing ratios. Figure 4 shows the mixtures of a lipopeptide and a rhamnolipid biosurfactant. The rhamnolipid biosurfactant (JBR) alone produced the lowest IFT compared to any of the three mixtures. However, the mixture of JBR and T89-42 (val) (culture grown with valine) at the ratio of 0.008% to 0.002% produced an IFT as low as 0.02 mN/m. Therefore, this mixture will be of interest for future research.

To find optimal phase behavior, several series of solutions were evaluated. For each solution series, the biosurfactant concentration was kept constant and the salt concentration was varied. As the concentration of salt increased at a given surfactant concentration, three types of microemulsions were observed, changing from Winsor Type I (micelles in the aqueous phase) to Type III (middle phase microemulsion) to Type II (reverse micelles in the oil phase). The optimal phase behavior is believed to be Winsor Type III (middle-phase) microemulsion where the IFT is minimum and the solubilization are maximum. The results for phase behavior are summarized in Tables 1 and 2. In some oil-biosurfactant systems, the optimal phase behavior could not be visibly determined due to the very low surfactant concentrations used (less than 100 ppm); thus, equilibrium IFTs were measured to find the optimum formulation. Each IFT measurement was done

three times to determine the standard deviation, which was required to have a deviation within  $\pm 15\%$  of the average value to be accepted. Triplicate samples were prepared in capillary tubes for each oil contaminant and each biosurfactant formulation. To date, we found the best formulation to be the rhamnolipid biosurfactant alone at 0.1% concentration and 7% NaCl. At this salinity and surfactant concentration, the Winsor Type I microemulsion approached a Winsor Type III microemulsion and the IFT was  $0.012 \pm 0.00037$  mN/m. The best biosurfactant mixture was the rhamnolipid and lipopeptide biosurfactant mixture: 0.008 wt% JBR and 0.002 wt% T89-42 (val) at 3.0 wt% NaCl. The phase study with this mixture has not been done yet, but it is expected to be difficult to see any phase behavior due to the low surfactant concentration of 0.01 wt%. The IFT was measured to be 0.020 mN/m.

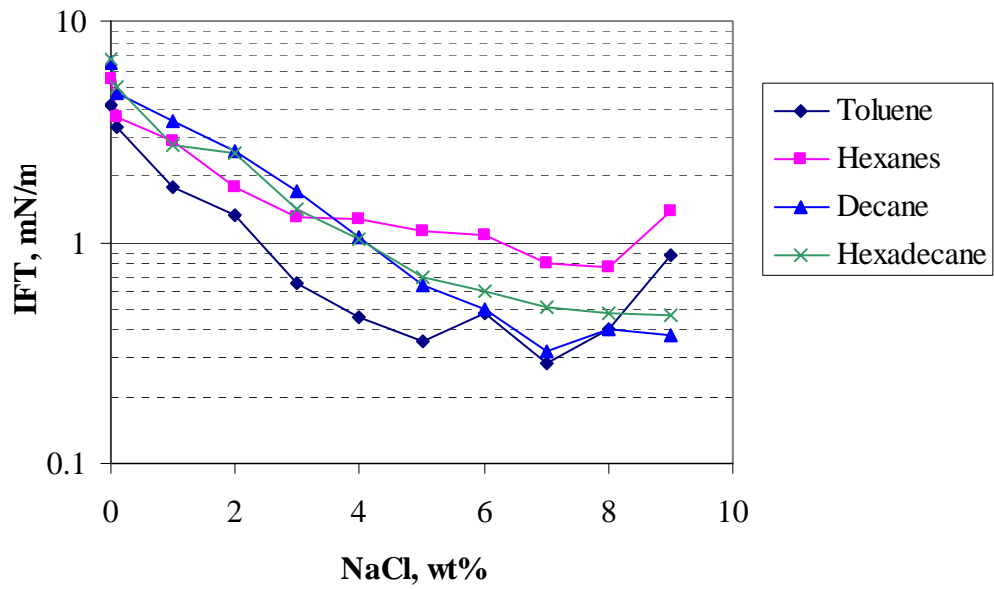
Future work: In the future, the following tasks will be conducted:

- The oil-surfactant interactions will be studied.
- The solid-surfactant interactions will be evaluated by adsorption studies.
- The surfactant-water interactions, such as foaming, precipitation, and phase separation properties, will be studied.
- The column studies will be performed using the optimum formulations to determine the oil removal efficiency of biosurfactant formulations.

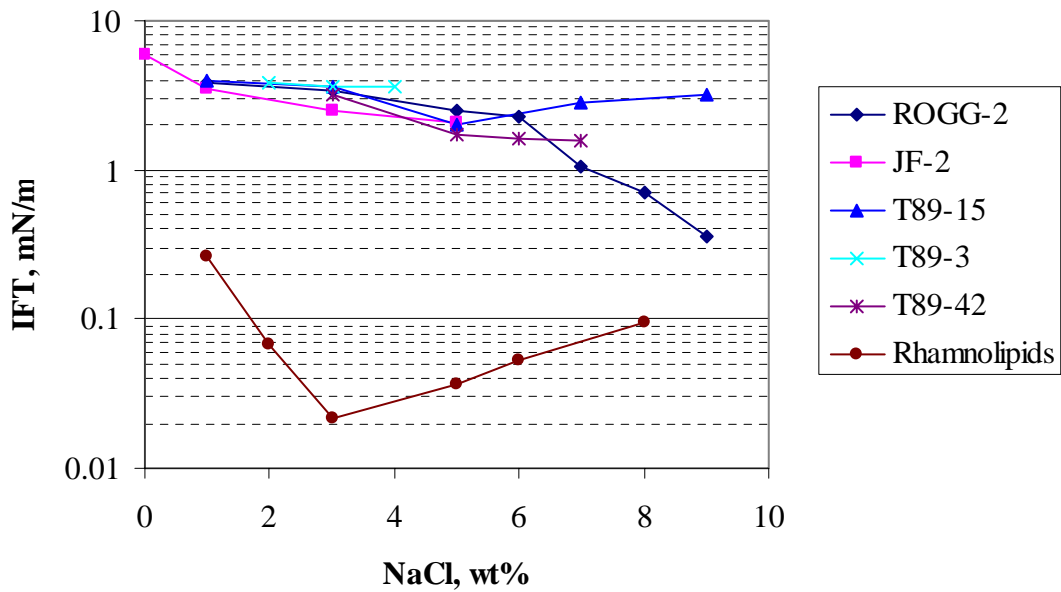
**References:**

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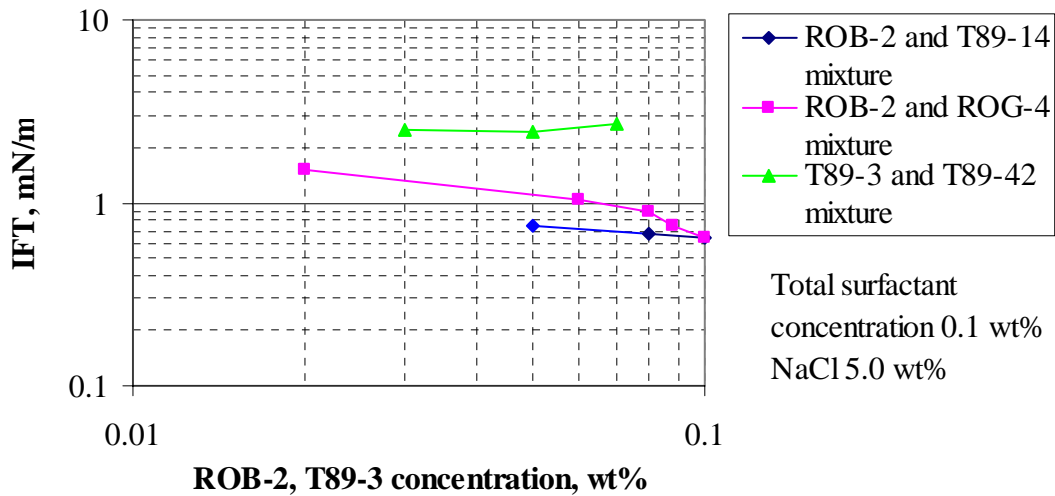
**Figure 1. The IFT of T89-42 Lipopeptide Biosurfactants (0.1wt%) versus Salinity for Four Oils**



**Figure 2. The IFTs at Different Biosurfactants (0.1 wt%) versus Salinity for Toluene**

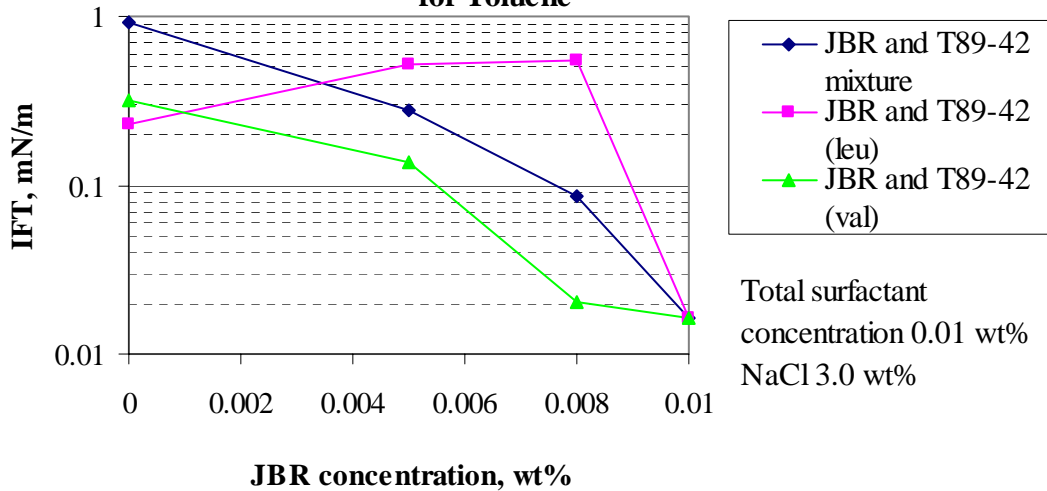


**Figure 3. The IFT of Mixtures of Two Lipopeptide Biosurfactants versus Biosurfactant Concentration for Toluene**



ROB-2, T89-42, ROG-4, and T89-3 are lipopeptide biosurfactants  
 The x-axis represents the concentration of ROB-2 and T89-3 in each of the 3 mixtures.

**Figure 4. The IFT of Mixture of a Lipopeptide and a Rhamnolipid Biosurfactant versus Biosurfactant concentration for Toluene**



T89-42: T89-42 biosurfactant that was grown without any amino acid in the growth medium  
 T89-42 (leu): T89-42 that was grown with the presence of the amino acid leucine  
 T89-42 (val): T89-42 biosurfactant that was grown with the presence of the amino acid valine  
 JBR: Rhamnolipid biosurfactant  
 The x-axis represents the concentration of JBR in each mixture.

**Table 1.** Phase Study Results for JBR 0.1 wt% with Toluene

<b>JBR, wt %</b>	0.1%											
<b>NaCl, wt%</b>	3.0%	4.0%	5.0%	6.0%	7.0%	8.0%	9.0%	10.0%	11.0%	12.0%	13.0%	14.0%
<b>Microemulsion</b>	none	none	I	I	I	III	III	III	III	III	II	II

**Table 2.** Phase Study Results for JBR 1.0 wt% with Toluene

<b>JBR, wt %</b>	1.0%											
<b>NaCl, wt%</b>	3.0%	4.0%	5.0%	6.0%	7.0%	8.0%	9.0%	10.0%	11.0%	12.0%	13.0%	14.0%
<b>Microemulsion</b>	I	I	I	I	I	I	I	I	I	I + III	III	III

<b>JBR, wt %</b>	1.0%		
<b>NaCl, wt%</b>	15.0%	16.0%	17.0%
<b>Microemulsion</b>	III	III	II