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BACILLUS LIPOPEPTIDE BIOSURFACTANTS: PURIFICATION, STRUCTURE-ACTIVITY RELATIONSHIP, AND *IN-SITU* PRODUCTION IN OIL RESERVOIRS

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Noha Youssef
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BACILLUS LIPOPEPTIDE BIOSURFACTANTS: PURIFICATION, STRUCTURE-ACTIVITY RELATIONSHIP, AND *IN-SITU* PRODUCTION IN OIL RESERVOIRS

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY

Dr. Michael J. McInerney

Dr. David P. Nagle

Dr. Ralph S. Tanner

Dr. Kathleen E. Duncan

Dr. Roy M. Knapp

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Preface

Oil is an essential source of energy and one of the main factors that drives the economic development of the world. In an oil reservoir, primary and secondary stages of oil recovery recover less than 50% of the oil in the reservoir. The remaining oil is usually entrapped under capillary pressure and cannot be mobilized by conventional methods. To enhance its recovery, capillary pressure entrapping the oil has to be reduced. One approach to reduce capillary pressure is to reduce surface and interfacial tension. Biosurfactants are microbially-produced surface-active compounds that can partition at the oil-water interfaces lowering the interfacial tension and hence the capillary pressure and mobilizing the entrapped oil.

Biosurfactants produced by *Bacillus* species have very similar structure to surfactin, the lipopeptide produced by *Bacillus subtilis*. Lipopeptide biosurfactants have a heptapeptide polar head and a beta hydroxy fatty acid tail (C13 to C18). The activity of biosurfactants depends on their structural components. Biosurfactant activity has been manipulated previously by module swapping and changes in the amino acid composition. The main body of work presented in this dissertation focuses on manipulation of biosurfactant activity by changing the fatty acid composition either nutritionally or through the design of mixtures. Results from a field trial in a limestone petroleum reservoir conducted using injected *Bacillus* strains are also shown.

Chapter one focuses on the statistical comparison of three different methods to detect biosurfactant production; drop collapse, oil spreading, and blood agar lysis. The methods were compared for their ease of use and reliability in relation to the ability of the cultures to reduce surface tension. Results showed that both the oil spreading

technique and the drop collapse method are more reliable in detecting biosurfactant production compared to blood agar lysis. One more advantage of the oil spreading technique is that it can be used quantitatively. This work is published in the *Journal of Microbiological Methods*.

Chapter two reports on the importance of the 3-hydroxy fatty acid composition of lipopeptides for biosurfactant activity. A new protocol of biosurfactant purification that recovers most of the biosurfactant activity in the original culture was devised. Amino acid and fatty acid composition of different lipopeptides combined with multiple regression analysis showed the dependence of biosurfactant activity on fatty acid composition. This work is published in *Applied and Environmental Microbiology*.

In Chapter three, the fatty acid composition of lipopeptides was manipulated by mixing biosurfactants produced by different strains and the effect of the mixture on lowering interfacial tension against different hydrocarbons was studied. It was found that a more heterogeneous fatty acid composition is required for lowering interfacial tension. All the interfacial tension measurements were performed by Thu Nguyen in the department of Civil and Chemical Engineering. The chapter is written in the style recommended by the *Applied and Environmental Microbiology*.

Chapter four reports on the results of a field trial in a petroleum limestone formulation that was conducted using biosurfactant-producing *Bacillus* strains. It is shown that lipopeptide biosurfactants can be made in oil reservoirs at concentrations that far exceed that needed for substantial recovery of entrapped petroleum. The inoculum metabolized the substrate and produced the biosurfactant under the conditions of the reservoir. Molecular work was conducted by Dr. Randy Simpson.

The chapter is written in the style recommended by the *Applied and Environmental Microbiology*.

Abstract

To investigate the activity of lipopeptide biosurfactants produced by *Bacillus* strains, it was necessary to find a fast and reliable method to compare biosurfactant production in different strains. Three methods to detect biosurfactant production; drop collapse, oil spreading, and blood agar lysis were compared for their ease of use and reliability in relation to the ability of the cultures to reduce surface tension. There was a very strong, negative, linear correlation between the diameter of clear zone obtained with the oil spreading technique and surface tension (r_s= -0.959) and a weaker negative correlation between drop collapse method and surface tension (r_s= -0.82), suggesting that the oil spreading technique predicted biosurfactant production better than the drop collapse method. The large number of false negatives and positives obtained with the blood agar lysis method and its poor correlation to surface tension (r_s= -0.15) demonstrated that it is not a reliable method to detect biosurfactant production. The oil spreading technique was used as the method of choice to detect, quantify, and compare biosurfactant activities throughout the work in this thesis.

Biosurfactants produced by several *Bacillus* strains had different activities. In order to relate biosurfactant structural differences to activity, eight lipopeptide biosurfactants with different specific activities produced by various *Bacillus* species were purified by a new protocol. The amino acid composition of the 8 lipopeptides was the same (Glu/ Gln: Asp/ Asn: Val: Leu; 1:1:1:4), but the fatty acid composition differed. Multiple regression analysis showed that the specific biosurfactant activity depended on the ratios of both *iso* to normal, even-numbered fatty acids and *anteiso* to *iso*, odd-numbered fatty acids. Manipulation of biosurfactant fatty acid composition,

and hence specific activity, was possible through the addition of branched chain fatty acids to the growth medium.

Alternatively, biosurfactant activity can be manipulated by formulation of biosurfactant mixtures with different properties. The efficacy of biosurfactants from individual strains, and mixtures of biosurfactants from different strains with and without synthetic surfactants was tested for enhanced interfacial activity. To design (bio)surfactant formulations effective in lowering IFT, information about both the bio/surfactant structure and the nature of targeted non-aqueous phase liquids (NAPL) was required. The IFT against toluene was lowered by using lipopeptide biosurfactants with a heterogeneous fatty acid composition or by using mixtures of lipopeptide and rhamnolipid biosurfactants. Conversely, the IFT against hydrophobic NAPL was lowered by mixing lipopeptide biosurfactants with a more hydrophobic synthetic surfactants.

To investigate the feasibility of microbially enhanced oil recovery (MEOR), we conducted a controlled field experiment in a limestone formation. The feasibility of biosurfactant production *in-situ* by two biosurfactant-producing *Bacillus* species was studied. *In situ* metabolism and biosurfactant production were evident. The lipopeptide biosurfactant was produced at concentrations that far exceeded that needed to mobilize substantial amounts of entrapped oil. The data demonstrate the technical feasibility and cost-effectiveness of microbial processes for oil recovery and the success of inoculation.

Chapter One

Comparison of methods to detect biosurfactant production by diverse microorganisms

Abstract:

Three methods to detect biosurfactant production; drop collapse, oil spreading, and blood agar lysis were compared for their ease of use and reliability in relation to the ability of the cultures to reduce surface tension. The three methods were used to test for biosurfactant production in 205 environmental strains with different phylogenetic affiliations. Surface tension of select strains that gave conflicting results with the above three methods was also measured. Sixteen percent of the strains that lysed blood agar tested negative for biosurfactant production with the other two methods and had little reduction in surface tension (values above 60 mN/m). Thirty eight percent of the strains that did not lyse blood agar tested positive for biosurfactant production with the other two methods and had surface tension values as low as 35 mN/m. There was a very strong, negative, linear correlation between the diameter of clear zone obtained with the oil spreading technique and surface tension (r_s= -0.959) and a weaker negative correlation between drop collapse method and surface tension (r_s= -0.82), suggesting that the oil spreading technique better predicted biosurfactant production than the drop collapse method. The use of the drop collapse method as a primary method to detect biosurfactant producers, followed by the determination of the biosurfactant concentration using the oil spreading technique, constitutes a quick and easy protocol to screen and quantify biosurfactant production. The large number of false negatives and positives obtained with the blood agar lysis method and its poor

correlation to surface tension (r_s= -0.15) demonstrated that it is not a reliable method to detect biosurfactant production.

Introduction:

Biosurfactants are a diverse group of surface-active chemical compounds that are produced by a wide variety of microorganisms [Banat, 1995]. The types of biosurfactants include lipopeptides synthesized by *Bacillus* and other genera, glycolipids synthesized by *Pseudomonas* species and *Candida* species, phospholipids synthesized by *Thiobacillus thiooxidans*, polysaccharide-lipid complexes synthesized by *Acinetobacter* species, or even the microbial cell surface itself [Van Dyke et al., 1991, and Bodour and Miller-Maier, 2002]. Having both polar and non-polar domains, biosurfactants are able to partition at water-oil or water-air interfaces and thus reduce the interfacial or surface tension [Banat, 1995, Banat et al., 2002, and Georgiou et al., 1992]. Such surface properties made them good candidates for enhanced oil recovery (EOR) [Ron and Rosenberg, 2001, and Van Dyke et al., 1991]. Some biosurfactants are known to have therapeutic applications as antibiotics, and antifungal or antiviral compounds. Biosurfactants can also be used in bioremediation of soil or sand [Van Dyke et al., 1991] or in the cleanup of hydrocarbon contamination in groundwater [Ron and Rosenberg, 2001]. These diverse applications necessitate an easy, rapid, and reliable method to detect biosurfactant production with a minimum number of false positives and/or negatives.

Biosurfactant production is sometimes detected by measuring emulsification [Van Dyke et al., 1993, Makkar and Cameotra, 1997, and Makkar and Cameotra, 1998], hemolytic activity [Carrillo et al., 1996, Banat, 1993, Yonebayashi et al., 2000, and Mulligan et al., 1984], or cell surface hydrophobicity [Vander Mei et al., 1987, Mozes and Rouxhet, 1987, Neu and Poralla, 1990, Pruthi and Cameotra, 1997, and

Dillon et al., 1986]. The use of methods that measure properties other than the surface activity can be problematic. Although, a direct correlation was found between surface activity and emulsification activity [Cooper and Goldenberg, 1987, and Denger and Schink, 1995], and the emulsification index has been used as a screening method [Cooper and Goldenberg, 1987, Denger and Schink, 1995, Makkar and Cameotra, 1997, and Makkar and Cameotra, 1998], the ability of a molecule to form a stable emulsion is not always associated with surface tension lowering activity [Van dyke et al., 1993, Willumsen and Carlson, 1997, T. de Acevedo and McInerney, 1996, and Bosch et al., 1988]. Cell surface hydrophobicity is an important aspect in bacterial cell adhesion to surfaces [Vander Mei et al., 1987]. Since hydrophobic surfaces are usually associated with molecules with low surface energy [Mozes and Rouxhet, 1987], Neu and Poralla [Neu and Poralla, 1990] used this property to screen for biosurfactant production. Pruthi and Cameotra [Pruthi and Cameotra, 1997] found a direct correlation between hydrophobicity and biosurfactant production. However, it is not clear which method for measuring cell surface hydrophobicity is appropriate for general screening [Vander Mei et al., 1987, Mozes and Rouxhet, 1987, Neu and Poralla, 1990, Pruthi and Cameotra, 1997, and Dillon et al., 1986]. Seigmund and Wagner [1991] developed a colorimetric assay, based on the formation of insoluble ion pair between anionic surfactants, cationic cetyl trimethyl ammonium bromide (CTAB), and methylene blue. Since this approach is specific for anionic surfactants, it cannot be used as a general method of screening for biosurfactant producers.

There are a number of approaches that measure directly the surface activity of biosurfactants. These include surface and/or interfacial tension measurement

[McInerney et al., 1990, Haba et al., 2000, and Mercade et al., 1996], axisymmetric drop shape analysis profile (ADSA-P) [Vander Vegt et al., 1991, and Noordmans and Busscher, 1991], glass-slide test [Persson and Molin, 1987], drop collapse method [Jain et al., 1991, and Bodour and Miller-Maier, 1998], and the oil spreading technique [Morikawa et al., 2000]. The measurement of surface tension has traditionally been used to detect biosurfactant production and most of the other methods that measure the surface properties of biosurfactant use surface tension reduction as the standard [Noormans and Busscher, 1991, Persson and Molin, 1987, Willumsen and Carlson, 1997, Makkar and Cameotra, 1997, and Makkar and Cameotra, 1998, Neu and Poralla, 1990, and Bosch et al., 1988]. However, the measurement of surface tension is time-consuming which makes it inconvenient to use for screening of a large number of isolates. The drop collapse technique depends on the principal that a drop of a liquid containing a biosurfactant will collapse and spread completely over the surface of oil [Jain et al., 1991, and Bodour and Miller-Maier, 1998]. The method is easy and can be used to screen large number of samples [Bodour et al., 2003], but it has not been correlated to surface tension reduction to confirm its reliability. The oil spreading technique measures the diameter of clear zones caused when a drop of a biosurfactant-containing solution is placed on an oil-water surface [Morikawa et al., 2000]. Morikawa et al. used this method to compare the activity of both cyclic and linear forms of surfactin and arthrofactin. However, its ability to detect biosurfactant production in diverse microorganisms has not been tested.

The hemolytic activity of biosurfactants was first discovered when Bernheimer and Avigad [Bernheimer and Avigad, 1970] reported that the biosurfactant produced

by *Bacillus subtilis*, surfactin, lysed red blood cells. Blood agar lysis has been used to quantify surfactin [Moran et al., 2002] and rhamnolipids [Johnson and Boese-Marrazo, 1980], and has been used to screen for biosurfactant production by new isolates [Carrillo et al., 1996, Banat, 1993, Yonebayashi et al., 2000, and Mulligan et al., 1984]. Carrilo et al. [1996] found an association between hemolytic activity and surfactant production, and they recommended the use of blood agar lysis as a primary method to screen for biosurfactant activity. However, only 13.5% of the hemolytic strains lowered the surface tension below 40 mN/m. Also, other microbial products such as virulence factors lyse blood agar and biosurfactants that are poorly diffusible may not lyse blood cells. Thus, it is not clear whether blood agar lysis should be used to screen for biosurfactant production.

In this study, we tested the hemolytic activity of 205 environmental isolates of different phylogenetic affiliations, and measured the surface activity of these isolates by using both the drop collapse and the oil spreading techniques. Surface tension was measured for cultures that gave conflicting results between these three methods. We found that the oil spreading and drop collapse methods were correlated with the ability of the cultures to reduce surface tension. However, blood agar gave a large number of false positives and negatives.

Materials and methods:

Media:

All cultures were grown aerobically in liquid medium (medium E) (pH 6.9) that contained (g/l): KH_2PO_4, 2.7; K_2HPO_4, 13.9; sucrose, 10; NaCl, 50; yeast extract, 0.5; and $NaNO_3$, 1. After autoclaving, 10 ml each of solutions A, B, and C were added to 1 liter of the above medium. Solution A contained 25 g/l of $MgSO_4$; solution B contained 100 g/l of $(NH_4)_2SO_4$; and solution C contained (g/l): EDTA, 0.5; $MnSO_4 \bullet H_2O$, 3; NaCl, 1; $CaCl_2 \bullet 2H_2O$, 0.1; $ZnSO_4 \bullet 7H_2O$, 0.1; $FeSO_4 \bullet 7H_2O$, 0.1; $CuSO_4 \bullet 5H_2O$, 0.01; $AlK(SO_4)_2$, 0.01; $Na_2MoO_4 \bullet 2H_2O$, 0.01; boric acid, 0.01; Na_2SeO_4, 0.005;$NiCl_2 \bullet 6H_2O$, 0.003. Solutions A and B were separately autoclaved while solution C was filter sterilized.

Blood agar plates contained 40 g of blood agar base (Becton Dickinson, Sparks MD), and 50 ml of sheep blood (Brown laboratory, Topeka KA, USA) per liter.

Inocula:

Two hundred and five strains of different phylogenetic affiliations were used for this study. Table 1.1 lists the number of strains of each species and their sources [Istock et al., 2001, Duncan et al., 1994, and Palmisano et al., 2001]. Cultures were grown aerobically in 25 ml of medium E, at 37ºC for 24 hours without shaking.

Screening methods:

Each strain was streaked onto blood agar plates and incubated for 48 hours at 37ºC. The plates were visually inspected for zones of clearing around the colonies, indicative of biosurfactant production. The diameter of the clear zones depends on the concentration of the biosurfactant [Mulligan et al., 1984]. The zones of clearing were

Table1.1. Number and types of strains used in this study.

Species	**Number of strains**	**Reference**
Bacillus mojavenesis	22	Istock et al., 2001 and Duncan et al., 1994
Bacillus subtilis subsp. *subtilis*	47	Istock et al., 2001 and Duncan et al., 1994
Bacillus subtilis subsp. *spizizenii*	43	Istock et al., 2001 and Duncan et al., 1994
Bacillus megaterium	15	Istock et al., 2001
Bacillus licheniformis	11	Duncan et al., 1994
Bacillus sonorensis	9	Palmisano et al., 2001
Bacillus species	14	Istock et al., 2001
Oil well isolates of unknown taxonomic affiliations	44	OU culture collection
Total number of strains	205	

scored as follows: '-', no hemolysis; '+', incomplete hemolysis; '++', complete hemolysis with a diameter of lysis <1 cm; '+++', complete hemolysis with a diameter of lysis >1cm but < 3cm; and '++++', complete hemolysis with a diameter of lysis > 3 cm and green colonies. Two plates for each strain were inoculated and clear zones in several different areas of each plate were analyzed.

For the drop collapse method, 2 μl of mineral oil was added to each well of a 96-well microtiter plate lid. The lid was equilibrated for 1 hour at room temperature, and then 5 μl of the culture was added to the surface of oil [Bodour and Miller-Maier, 1998]. The shape of the drop on the surface of oil was inspected after 1 minute. Biosurfactant-producing cultures gave flat drops with scoring system ranging from '+' to '++++' corresponding to partial to complete spreading on the oil surface. Those cultures that gave rounded drops were scored as negative '-' indicative of the lack of biosurfactant production. Aliquots from a culture of each strain were analyzed on two separate microtiter plates.

For the oil spreading technique, 50 ml of distilled water was added to a large Petri dish (25 cm diameter) followed by addition of 20 μl of crude oil to the surface of the water. Ten μl of culture was then added to the surface of oil [Morikawa et al., 2000]. The diameter of the clear zone on the oil surface was measured and related to the concentration of biosurfactant by using a standard curve prepared with a commercially available biosurfactant, surfactin (Sigma Chemicals Co., St. Louis, MO) at concentrations ranging from 50 to 2000 mg/l. The diameters of triplicate samples from the same culture of each strain were determined.

Surface tension was measured using a Du Nouy ring tensiometer [McInerney et al., 1990] for 60 different strains, which included representative strains that showed either positive or negative results with all the three methods and strains that showed positive results with one method and negative results with the other methods. Pure water and isopropanol were used as standards. Two milliliters of the culture were equilibrated for 15 minutes in a small weighing dish prior to measuring the surface tension. The surface tension value shown is the average of three readings from the same culture.

Statistical analysis of the correlation between different tests:

A statistical test of independence was used to verify the association between the different methods by using 2x2 contingency tables [Zar, 1999] in order to find correlations between dichotomous nominal scale data (e.g. observations that are either positive or negative). The correlation coefficient, ϕ_2 ranged between –1 (strong negative correlation) to 1 (strong positive correlation). Since this method did not differentiate between positive results of different magnitudes (e.g., +, ++, +++, ++++), a general rank correlation test according to Spearman [Zar, 1999] was conducted to determine the correlation between each of the three methods and surface tension as well as between the oil spreading technique and each of the other two methods. The Spearman rank correlation coefficient, r_s ranged between –1 (strong negative correlation) to 1 (strong positive correlation).

Results:

Oil spreading technique. Morikawa et al. [2000] showed that the area of displacement by a surfactant-containing solution is directly proportional to the concentration of the two biosurfactants tested. We tested whether the oil spreading technique could be used to detect biosurfactant production by diverse microorganisms. The diameter of the clear zone linearly increased with the concentration of surfactin over a concentration range of 50 to 400 mg/l (Figure 1.1). The coefficient of variation of the diameter of clear zones obtained with different concentrations of surfactin ranged from 0, for concentrations below 100 mg/l, to 4.7%, for concentrations above 100 mg/l. With different batches of surfactin, the coefficient of variation varied from 1.8, for concentrations below 100 mg/l, to 8.3%, for concentrations above 100 mg/l. When the degree of oil spreading of 60 strains was compared to the surface tension of the culture, an inverse linear relationship between the diameter of the clear zone and the surface tension of the culture was obtained (Figure 1.2). Cultures with large concentrations of biosurfactant as indicated by the oil spreading technique had low surface tension values. This, plus the fact that the diameter of the clear zone is proportional to the concentration of a standard biosurfactant, indicated that the oil spreading technique is a reliable method to detect biosurfactant production.

Blood agar method. As mentioned above, the lysis of sheep red blood cells has been recommended as a simple and easy method to test for biosurfactant activity [Banat, 1993, Yonebayashi et al., 2000]. There was a linear increase in the diameter of lysis on blood agar as the concentration of surfactin increased (Figure 1.3). The coefficient of variation of the diameters of lysis of blood agar for the same concentration of surfactin

Figure 1.1 The relationship between the diameter of the clear zone obtained by the oil spreading technique (in cm) and the concentration of surfactin (in mg/l). Error bars indicate the standard deviation of three independent measurements. The solid line is the least square fit with an r^2 of 0.997. Where the error bars are not visible, the standard deviation was within the area occupied by the symbol.

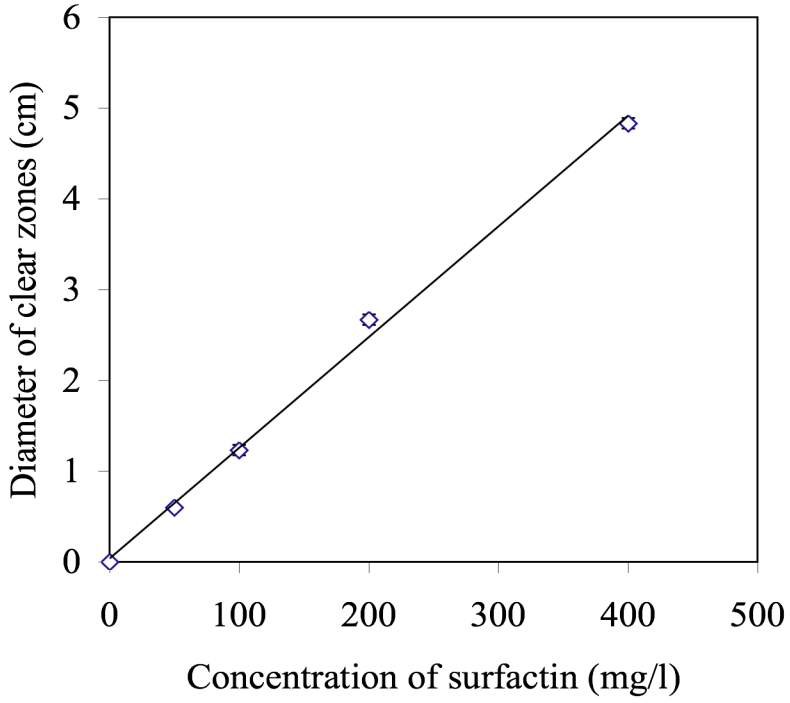

Diameter of clear zones (cm)
6
5
4
3
2
1
0
0
100
200
300
400
500
Concentration of surfactin (mg/l)

Figure 1.2. The relationship between the diameter of the clear zone obtained by the oil spreading technique (in cm) and surface tension of the culture (in mN/m). Each point represents a different culture. The solid line is the least square fit with an r^2 of 0.839.

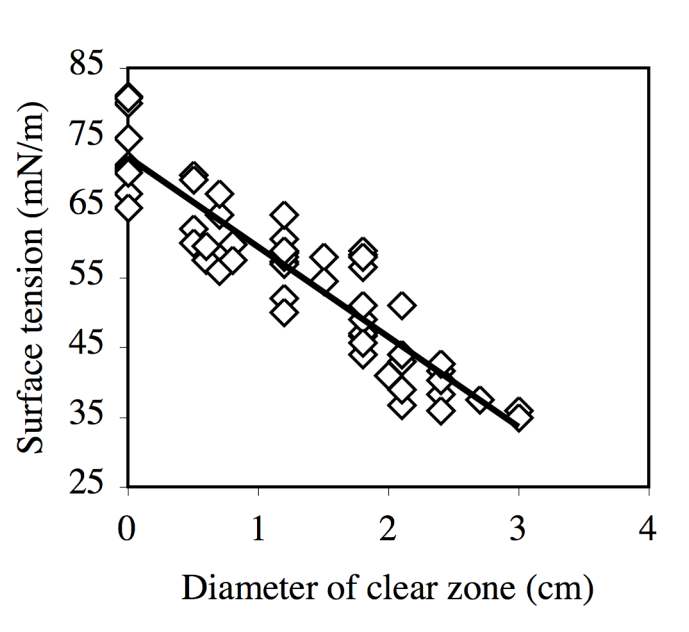
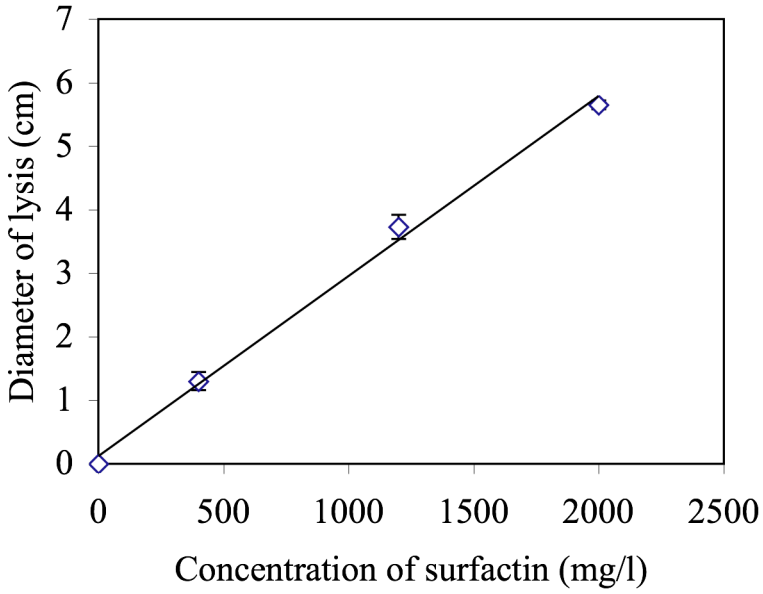

Surface tension (mN/m)
85
75
65
55
45
35
25
0
1
2
3
4
Diameter of clear zone (cm)

Figure 1.3. The relationship between the diameter of blood agar lysis (in cm) and the concentration of surfactin (in mg/l). Error bars indicate the standard deviation of three independent measurements. The solid line is the least square fit with an r^2 of 0.996. Where the error bars are not visible, the standard deviation was within the area occupied by the symbol.


Diameter of lysis (cm)
0
1
2
3
4
5
6
7
0
500
1000
1500
2000
2500
Concentration of surfactin (mg/l)

was below 11%. However, not all biosurfactants have a hemolytic activity and compounds other than biosurfactants may cause hemolysis. Of the 81 strains that did not lyse blood agar, 31 were positive for biosurfactant production both by oil spreading technique and drop collapse method, and 9 other strains were positive for biosurfactant production by the oil spreading technique (Table 1.2). Strains 7 to 16 in Table 1.3 did not lyse blood agar, but showed results ranging from ('++') corresponding to partial drop collapse on the oil surface to ('++++') corresponding to complete drop collapse on the oil surface with drop collapse method and gave diameters of clearing ranging from 1.2 to 3 cm by the oil spreading technique. Five of these strains had surface tension values below 40 mN/m consistent with their ability to produce biosurfactants. On the other hand, out of the 124 positive results obtained with the blood agar method, 20 gave negative results for biosurfactant production with oil spreading technique and drop collapse method (Table 1.2). Strains 1 to 6 in Table 1.3 lysed blood agar with scores ranging between ('++') corresponding to complete hemolysis with a diameter of lysis <1 cm, and ('++++') corresponding to complete hemolysis with a diameter of lysis > 3 cm and green colonies, but the results obtained with the oil spreading technique or drop collapse method for the same six strains were either negative or had low activity. These strains had surface tensions above 60 mN/m consistent with their inability to produce biosurfactants. Thus, the blood agar lysis gave a high percentage of both false positives and negatives.

Drop collapse method. Jain et al. [Jain et al., 1991] suggested the use of drop collapse method as a sensitive and easy method to test for biosurfactant production. The

Table 1.2. Comparison of methods for detection of biosurfactant production, 205 strains tested

Method	Positive by method			Negative by method		
	No. of positives	No. (%) of strains with identical response	Response level	No. of negatives	No. (%) of negatives that were positive with other	Method number
1- Blood agar lysis	124	10 (8%)	+	81	31 (38%)	2, 3
		21 (17%)	++		9 (11%)	3
		49 (39%)	+++			
		44 (35%)	++++			
2- Drop collapse	129	14 (11%)	+	76	27 (35.5%)	1
		29 (23%)	++		16 (21%)	3
		44 (34%)	+++			
		42 (33%)	++++			
3- Oil spreading	145	14 (10%)	+[a]	60	21 (35%)	1
		29 (20%)	++		0	2
		44 (30%)	+++			
		58 (40%)	++++			

a: For the oil spreading technique: '+' means a diameter of clearing between 0.5 and 0.9 cm, '++' means a diameter of clearing between 1 and 1.5 cm, '+++' means a diameter of clearing >1.5 and less than 2.1 cm, and '++++' means a diameter of clearing >2. 1 and <3 cm.

Table 1.3. Efficacy of the blood agar lysis in predicting biosurfactant production.

Strain	Blood agar lysis	Drop collapse	Oil spreading (cm)	Surface tension (mN/m)
1	+++[a]	-[b]	-	70.5
2	+++	+	0.8	60.5
3	++++	+	0.7	64
4	++	-	0.7	67
5	++	-	-	65
6	++	-	0.5	60
7	-	+++	1.8	44
8	-	++	1.2	52
9	-	++	1.2	54
10	-	+++	2.1	36.7
11	-	+++	1.8	47.5
12	-	++++	3	35
13	-	+++	1.8	51
14	-	++	1.2	52
15	-	++	1.2	50
16	-	+++	1.8	45.7

a: '-', no hemolysis; '+', incomplete hemolysis; '++', complete hemolysis with a diameter of lysis <1 cm; '+++', complete hemolysis with a diameter of lysis >1cm but < 3cm; and '++++', complete hemolysis with a diameter of lysis > 3 cm and green colonies.

b: flat drops with scoring system ranging from '+' to '++++' corresponding to partial to complete spreading on the oil surface. Rounded drops were scored as negative '-' indicative of the lack of biosurfactant production.

method can also be made quantitative [Bodour and Miller-Maier, 1998]. However, in this study, the drop collapse technique was only applied as a qualitative method to detect biosurfactant production. Table 1.2 shows that 76 strains tested negative by this method. Sixty of these strains were also negative for biosurfactant production by oil spreading technique. The other 16 strains were positive for biosurfactant production by the oil spreading technique with diameters of clearing on the oil surface ranging from 0.5 to 0.8 cm, which suggests low levels of biosurfactant concentration (50-63 mg/l). Consistent with this, surface tensions of five of these strains were high ranging from 60 to 69 dyne/cm. Thus, the drop collapse method may not be as sensitive as the oil spreading technique in detecting low levels of biosurfactant production.

Statistical comparison of the 4 methods used. Table 1.4 shows the coefficient of correlation between the four methods that were used to detect biosurfactant production by two different statistical analyses [Zar, 1999]. The Spearman rank correlation, (r_s= – 0.959) showed a strong negative correlation between oil spreading technique and surface tension (Figure 1.2). A weaker negative correlation, (r_s= – 0.82) was detected between drop collapse method and surface tension. However, there was a very weak negative correlation (r_s= – 0.15) between blood agar lysis method and surface tension. Oil spreading technique and drop collapse method were strongly correlated with Spearman rank correlation coefficient of r_s= 0.94. However, a weak correlation was detected between drop collapse method and blood agar lysis method (r_s= 0.528) and between oil spreading technique and blood agar lysis method (r_s= 0.497). The statistical test of independence (2x2 contingency table) gave similar conclusions regarding the relationships of the three methods.

Table 1.4. Statistical correlations between different methods.

	Spearman rank correlation coefficient (r_s)			Test of independence correlation coefficient (ϕ_2)		
	Oil spreading	Drop collapse	Blood agar	Oil spreading	Drop collapse	Blood agar
Oil spreading	1			1		
Drop collapse	0.94	1		0.8	1	
Blood agar	0.497	0.528	1	0.2	0.359	1
Surface tension	-0.959	-0.82	-0.15			

Discussion:

In this study, we tested the applicability of using the oil spreading technique to detect biosurfactant production in diverse microorganisms. The diameter of clear zones was linearly related to surfactin concentrations (Figure 1.1) and replicate analyses had low coefficients of variation (< 8.3%). Cultures that gave large diameter of clearing also had low surface tension (Figure 1.2). These analyses indicate that the oil spreading technique is reliable in detecting biosurfactant production as determined by surface tension measurements. Since the concentration of biosurfactant that exceeds the critical micelle concentration will not result in further decreases in surface tension, the oil spreading technique has a larger dynamic range than surface tension. It is also easy to perform and to standardize, and less time-consuming than surface tension measurements which makes it applicable for large screening studies.

The drop collapse method has been used to detect biosurfactant-producing microorganisms in natural environments [Bodour et al., 2003]. It was compared to surface tension [Bodour and Miller-Maier, 1998], but no correlation studies were conducted between the two methods to assess the reliability of the drop collapse technique. In this study, we found that the correlation coefficient between the drop collapse method and surface tension was strong (r_s= -0.82) where cultures showing a greater degree of collapse had low surface tension values. Comparing this method to the oil spreading technique suggests that it may not be as sensitive in detecting low concentrations of biosurfactants since the 16 strains that were negative by drop collapse method did have some clearing by the oil spreading technique (0.5-0.9 cm), which indicates low concentrations of biosurfactant. Despite this discrepancy, a

coefficient of correlation of r_s = 0.94 between the drop collapse method and the oil spreading technique indicates its reliability as a method of testing biosurfactant production especially since any false negatives would be those with low concentrations of biosurfactants. Because it can be performed in a microplate, it also had the added advantage of allowing the simultaneous analysis of 96 different samples.

Blood agar lysis method was included in this study since it is widely used to screen for biosurfactant production and in some cases it is the sole method used [Banat, 1993, Yonebayashi et al., 2000]. Mulligan et al. [Mulligan et al., 1984] recommended blood agar lysis method as a preliminary screening method. However, none of the studies mention the possibility of biosurfactant production without a hemolytic activity. In addition a number of false positives (16% in this study) were also obtained when using the blood agar lysis (Table 1.2). This method excluded many good biosurfactant producers. Forty nine percent of the strains that did not lyse blood agar were positive by one of the other two methods (Table 1.2). Blood agar lysis did not correlate to surface tension (r_s= -0.15) (Table 1.4) and thus is not recommended for use as screening method.

In conclusion, we suggest a simple protocol to screen and quantify biosurfactant production in large numbers of microorganisms. First, the cultures are analyzed by using the drop collapse method. Positive results obtained by this method would constitute cultures that produce either moderate or high amounts of biosurfactants (above 60 mg/l). The concentrations of biosurfactant produced can then be determined using the oil spreading technique. Cultures negative by the drop

collapse method could be screened by the oil spreading technique to detect those that produce low levels of biosurfactants. Surface tension can then be used to confirm the results if required.

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Chapter 2

Importance of the 3-hydroxy fatty acid composition of lipopeptides for biosurfactant activity

Abstract

Biosurfactant production may be an economic approach to improve oil recovery. To obtain candidates most suitable for oil recovery, 207 strains, mostly belonging to the genus *Bacillus*, were tested for growth and biosurfactant production in medium with 5% NaCl under aerobic and anaerobic conditions. All strains grew aerobically with 5% NaCl and 147 strains produced a biosurfactant. Thirty-five strains grew anaerobically with 5% NaCl and two produced a biosurfactant. In order to relate structural differences to activity, eight lipopeptide biosurfactants with different specific activities produced by various *Bacillus* species were purified by a new protocol. The amino acid composition of the 8 lipopeptides was the same (Glu/ Gln: Asp/ Asn: Val: Leu; 1:1:1:4), but the fatty acid composition differed. Multiple regression analysis showed that the specific biosurfactant activity depended on the ratios of both *iso* to normal, even-numbered fatty acids and *anteiso* to *iso*, odd-numbered fatty acids. The multiple regression model accurately predicted the specific biosurfactant activity of 4 newly purified biosurfactants (r^2 = 0.91). The fatty acid composition of the biosurfactant produced by *Bacillus subtilis* subsp. *subtilis* strain T89-42 was altered by the addition of branched-chain amino acids to the growth medium. The specific activities of biosurfactants produced in cultures with the different amino acid additions were accurately predicted by the multiple regression model from the fatty acid composition (r^2 = 0.95). Our work shows that many strains

of *Bacillus mojavensis* and *Bacillus subtilis* produce biosurfactants and that the fatty acid composition is important for biosurfactant activity.

Introduction:

Biosurfactants are compounds produced by a variety of microorganisms (3) that are capable of lowering surface and/ or interfacial tension (3, 4, 13) by partitioning at the water/air and water/oil interfaces (34, 39). They can have a variety of structures including fatty acids, neutral lipids, phospholipids, glycolipids, and lipopeptides (13). Biosurfactants aid in the tertiary stage of oil recovery from low production oil reservoirs by releasing oil trapped by capillary pressure (34).

The activity of biosurfactants depends on their structural components, e.g. the types of hydrophilic and hydrophobic groups and their spatial orientation (9). Most lipopeptide biosurfactants have been shown to have a structure similar to surfactin, the biosurfactant produced by *Bacillus subtilis* (2, 14, 20, 32). Surfactin is a cyclic lipopeptide with β-hydroxy fatty acids linked to a heptapeptide (L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu) (2, 14). Solubility and surface activity of surfactin depend on the arrangement of the amino acid residues to produce two domains, a minor hydrophilic domain and a major hydrophobic domain (9). Changes in the amino acids in position 2, 4, and/ or 7 of surfactin to a more hydrophobic residue increased the surface activity and decreased the critical micelle concentration (8, 31, 32, 35, 36). In contrast, Yakimov et al. (40) changed the fatty acid composition of lichenysin A, a lipopeptide produced by *Bacillus licheniformis* BAS50, by addition of branched-chain amino acids to the growth medium. The increase in the percentage of branched-chain fatty acids in lichenysin A decreased the activity of the biosurfactant.

Candidate microorganisms for enhanced oil recovery should produce biosurfactants at low oxygen tension, slightly elevated temperatures, and high salt

concentrations since these are the conditions encountered in many domestic oil reservoirs. The lipopeptide produced by *Bacillus mojavenesis* strain JF-2 generates low interfacial tension (less than 0.01 mN/m) needed for substantial oil recovery (24, 26). It grows and produces the lipopeptide anaerobically at salt concentrations up to 8% and temperatures up to 45ºC (19, 24). However, most of the activity is lost after extended incubations (N. Youssef, and M. J. McInerney, unpublished data), and complex nutrients are required for its anaerobic growth (25).

In an attempt to find better candidates for microbially enhanced oil recovery, a number of bacterial strains, mostly *Bacillus* strains, were screened for anaerobic growth and stable biosurfactant production (28, 42) in the presence of 5% NaCl. Biosurfactant activity varied markedly among the strains. To understand the factors that influence biosurfactant activity, the biosurfactant concentration and amino acid and fatty acid compositions of a number of lipopeptide biosurfactants produced by strains of *Bacillus subtilis* and *Bacillus mojavensis* were determined.

Materials and methods:

Bacterial strains and cultivation:

The taxonomic affiliation and the number of strains used in this study are shown in Table 2.1. All cultures were grown at 37ºC in presence and absence of O_2 in a mineral salts medium with 5% NaCl and sucrose as the energy source as previously described (42). For screening, duplicate, 25-ml cultures were used, while duplicate or triplicate, 1-liter cultures were used for biosurfactant extraction and purification. Each culture was grown until maximal activity was obtained (usually between 42 and 44 h of incubation). When needed, amino acids (L-valine, L -alanine, L -leucine, and L -isoleucine) were added to the medium at 1 g/l before autoclaving.

Screening for biosurfactant production:

Biosurfactant activity was measured by the oil spreading technique (28, 42). Fifty milliliters of distilled water were added to a large Petri dish (25 cm in diameter) followed by the addition of 20 μl of crude oil to the surface of the water. Ten microliters of a culture grown in mineral medium was added to the surface of oil. The diameter of the clear zone on the oil surface was measured for triplicate samples from each replicate culture. Biosurfactant activity, defined as diameter of clearing on the oil surface in centimeters, ranged from 0 to 3 cm. The coefficient of variation ranged from 0 to 17% for replicates of the same strain. To compare biosurfactant stability, duplicate cultures of strains with the highest biosurfactant activity were sampled over a period of 14 days and tested for biosurfactant activity. The surface activity relative to that of the biosurfactant produced by *B. mojavensis* strain JF-2 was obtained by dividing the oil displacement

Table 2.1: Numbers, taxonomic affiliations, growth properties, and biosurfactant production by bacterial strains used in this study.

Species	# of strains	Growth		Biosurfactant production		Number of strains with biosurfactant activity relative to JF-2 ranging from[b]			References[c]
		$+O_2$	$-O_2$	$+O_2$	$-O_2$	0.42-0.75	0.83-1.7	1.75-2.5	
B.mojavensis	23	23[a]	1[a]	11[a]	1[a]	4	5	2	(33)
B.subtilis subsp. subtilis	47	47	0	39	0	14	20	5	(10, 12, 18)
B.subtilis subsp. spizizenii	43	43	0	38	0	10	20	8	(10, 12, 18)
B. megaterium	15	15	0	7	0	4	3	0	(18)
B. licheniformis	12	12	12	8	1	7	1	0	(12)
B. sonorensis	9	9	9	4	0	4	0	0	(29)
B. species	14	14	0	5	0	4	1	0	(18)
Oil well isolates	44	44	13	35	0	15	19	1	OU culture collection

[a] Number of strains showing growth or biosurfactant production under the conditions indicated in the column heading. All the strains were grown with 5% salt; duplicate cultures of each stain were analyzed for each condition.

[b] Relative activity was measured by dividing diameter of clearing of different strains by that of JF-2 (diameter of clearing =1.2 ± 0.17 cm). The activity was measured in triplicates for each culture and the coefficient of variation for duplicates of the same strain ranged from 0 to 17%.

[c] Reference provides the origin of the strains.

diameter obtained with a given strain by the value obtained for *B. mojavensis* strain JF-2, (1.2 ± 0.17 cm).

Biosurfactant extraction and purification:

The method used for biosurfactant extraction and purification was modified from Kim et al. (23). When the maximum oil displacement diameter was obtained, cells from duplicate or triplicate, 1-liter cultures were removed by centrifugation at 14,300 x *g* for 15 min at 4ºC. The pellet was dried at 110ºC overnight and the dry weight determined. Biosurfactant in the supernatant was precipitated with 40% w/v ammonium sulfate and incubated overnight 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 4ºC. The precipitate was extracted with 250 μl of chilled acetone to remove most of the proteins. Instead of the column chromatography steps used by Kim et al. (23), further purification was achieved by preparative thin layer chromatography (TLC) of the acetone extract. The whole acetone extract (250 μl) was spotted on preparative silica gel TLC plates (Whatman, Clifton, NJ) and separated by a solvent system of isopropanol: water: 28% w/v ammonium hydroxide (80:11:9). The TLC plates were visualized 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. Surface-active fractions were lyophilized. The weight of the lyophilized biosurfactant was determined and used to calculate the biosurfactant yield (biosurfactant weight/ dry weight of cells). Biosurfactant yields of different strains varied from 0.9 to 3.1 $mg \bullet g^{-1}$ dry weight of

cell. The coefficient of variation of biosurfactant yield between different batches of the same strain ranged from 4.9 to 27%.

To compare surface activities of different biosurfactants, 1$\mu g \bullet \mu l^{-1}$ solutions of purified biosurfactants were prepared and tested by oil spreading technique. The specific activity of the purified biosurfactant was expressed as the diameter of the clear zone in millimeters per microgram of the purified biosurfactant. Biosurfactant specific activity of different strains varied from 0.7 to 4.5 $mm \bullet \mu g^{-1}$. Triplicate samples were done for each culture. The coefficient of variation of specific activity between different batches of the same strain ranged from 4 to 26%, while that for the same batch of the same strain was less than 5%.

Amino acid analysis:

The amino acid composition of each purified biosurfactant was determined by the molecular biology research facility of the William K. Warren Research Institute (Oklahoma City, OK). Purified biosurfactants were acid hydrolyzed under vacuum in sealed tubes with 6N HCl at 110ºC for 18-24 hours. Each hydrolyzed sample was vacuum dried, dissolved in 0.01N HCl and filtered through a 0.45 μm nylon filter before analysis. Amino acid analysis was performed by cation exchange chromatography. Amino acid elution was accomplished with a two-buffer system. The sample was injected onto the column equilibrated with 0.2 N sodium citrate, pH 3.28. This buffer eluted the first 9 amino acids. The remaining amino acids were eluted by1N NaCl in 0.2 N sodium acetate, pH 7.4. Amino acids were detected by an on-line post-column reaction with ninhydrin (Tritone, Pickering Lab., Inc). Derivatized amino acids were quantified by their absorption at 570 nm, except for glutamic acid and

proline, which were detected at 440 nm. The procedure was performed with a totally automated Beckman System Gold Model 126 HPLC Amino Acid Analyzer.

Fatty acid analysis:

A methanolysis procedure, modified from the method of Yakimov et al. (40), was used to analyze the fatty acids. Two hundred micrograms of the purified biosurfactant were hydrolyzed under vacuum for 16 hours at 90ºC 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_2 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; triplicate injections were made for each biosurfactant preparation. The oven temperature was set at 60ºC for 5 min and then increased to 250ºC 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 400 Hz. Peak areas obtained on the GC chromatogram were used to calculate the percentage of the FAME isomers compared to the area of all FAME. The electron ionization mass spectra were dominated by fragment ions specific for trimethylsilyl (TMS) derivatives. The fragment ion at 175, which is specific for TMS derivatized hydroxyl groups in the beta position, was used to extract

the chromatogram to detect peaks corresponding to 3-hydroxy fatty acids. The M-15 fragments (loss of methyl group) on the MS spectra were used to identify the carbon chain length of the fatty acid isomers. These corresponded to 301 for 3-OH-C13, 315 for 3-OH-C14, 329 for 3-OH-C15, 343 for 3-OH-C16, and 357 for 3-OH-C17. M-31 fragments, characteristic of fatty acids and corresponding to the loss of methanol, were also detected. *Iso* isomers of fatty acids were identified by the presence of M-43 fragment (loss of an isopropyl group) and the absence of M-29 fragment (loss of an ethyl group) from the MS spectra. *Anteiso* isomers were identified by the presence of M-57 fragment (loss of a secondary butyl group) and the absence of M-43 fragment (loss of an isopropyl group) from the MS spectra. Fragments M-29, M-43, and M-57 were very small in comparison to others specific for trimethylsilyl (TMS) derivatives, but were discernable by magnifying the mass spectrum. Retention times and mass spectra were compared to authentic standard methyl 3-hydroxy tetradecanoate (Larodan Fine Chemicals, Malamö, Sweden).

Statistical analyses:

SPSS for windows (release 11.5.0, SPSS Inc., Chicago, IL) and Microsoft excel for Mac (version 11.1.1) were used to calculate Pearson's correlation coefficients and test regression models.

Results:

Screening of microorganisms.

Thirty-five strains mostly belonging to *Bacillus licheniformis* and *Bacillus sonorensis* grew anaerobically with 5% NaCl (Table 2.1), two of which produced a biosurfactant. One hundred and forty seven strains mostly belonging to *Bacillus subtilis* subsp. *subtilis* and *B. subtilis* subsp. *spizizenii* produced a biosurfactant under aerobic conditions (Table 1). Sixteen strains produced biosurfactants with activities 1.75 to 2.5 times that of JF-2 biosurfactant. Sixty-nine strains had biosurfactant activity comparable to JF-2 (0.83 to 1.7 times) (Table 2.1). Some *Bacillus mojavensis* strains (2 out of 5 tested) maintained their biosurfactant activity over a 14-day incubation period compared to *B. mojavensis* strain JF-2 that lost 50% of its activity in 7 days.

Evaluation of a new protocol for biosurfactant purification:

The lipopeptide produced by triplicate cultures of *Bacillus mojavensis* strain ROB-2 was used to compare the efficiency of two purification methods. Method 1 involved acid precipitation (using 1N HCl to adjust the pH of the cell-free culture fluid to 2) (41) followed by thin layer chromatography (TLC). Method 2 used ammonium sulfate precipitation followed by acetone extraction and thin layer chromatography. Seventy five percent of the biosurfactant activity remained in the cell-free culture fluid after cell removal. The surface-active fraction obtained from the TLC plate by method 1 had 23 ± 7% of the activity originally present in the culture, while the surface-active fraction obtained from the TLC plate by method 2 had 63 ± 11% of the activity originally present in the culture. The specific biosurfactant activity of the surface-

active fraction from the TLC plate for 12 different strains was 0.65 ± 0.07 mm• μg^{-1} by method 1 and 1.9 ± 0.7 mm• μg^{-1} by method 2. Method 2, being more efficient, was used to purify the biosurfactants.

Relationship between biosurfactant yield and activity:

The biosurfactant yields of seven different *Bacillus* strains (duplicate cultures for each strain) with activities ranging from 0.5 to 4.25 times that of *B. mojavenesis* strain JF-2 were determined. Biosurfactant activity was poorly correlated with biosurfactant yield (linear correlation coefficient $r^2 = 0.09$, and Pearson correlation coefficient (15) = -0.29). The biosurfactant activity did not always increase with an increase in biosurfactant yield, i.e. some biosurfactants were produced in high yields, but had relatively low activity, while others were produced in low yields, but had high activity.

Biosurfactants structure-activity relationship:

The lack of correlation between biosurfactant yield and surface activity prompted us to study the effect of variation in structural components of different biosurfactants on activity. Amino acid analysis of eight purified biosurfactants showed that they contained the same amino acid composition (mean ± std dev of the mole ratio): Glu/ Gln: Asp/ Asn: Val: Leu (0.99 ± 0.04: 0.99 ±0.04: 1 ± 0.04: 3.6 ± 0.12). Since the acid hydrolysis method used for amino acid analysis does not differentiate between acid or amide forms of the acidic amino acids (i.e. glutamate and glutamine, or aspartate and asparagine), the peptide portion of these biosurfactants may differ in their Glu/ Gln and/ or their Asp/ Asn content.

The fatty acid portion of the biosurfactants contained 3-hydroxy tridecanoate (3-OH-C13), tetradecanoate (3-OH-C14), pentadecanoate (3-OH-C15), and hexadecanoate (3-OH-C16) (Table 2.2). The 3-OH-C13, and 3-OH-C15 fatty acids were present as mixtures of *iso* and *anteiso* isomers while 3-OH-C14 was comprised of normal and *iso* isomers. The 3-OH-C16 fatty acid contained only the normal isomer. In some cases, the 3-OH-C14 and 3-OH-C15 fatty acids together constituted the majority of the fatty acids of the lipopeptide. However, in other cases, the 3-OH-C14 alone was the major fatty acid isomer. When the fatty acids of the biosurfactant purified from duplicate cultures of the same strain were analyzed, the fatty acid composition varied from one batch to another along with the specific activity of the biosurfactant. Multiple regression analysis was used to determine the fatty acid isomers that contributed to activity (43). All fatty acid isomers, the sums of the tridecanoate, tetradecanoate, pentadecanoate, hexadecanoate, ratios of even *iso*/normal isomers and other combinations were used to construct multiple regression models. There was a significant positive correlation between the % mass of the *iso* 3-OH C14 fatty acid and biosurfactant specific activity (Pearson's bivariate correlation coefficient, $r=0.813$, $p<0.001$) and a significant positive linear correlation between ratio of *iso* to normal, even-numbered fatty acids and biosurfactant specific activity (Pearson's bivariate correlation coefficient, $r=0.953$, $p<0.001$). No fatty acid other than the *iso* 3-OH C14 showed a significant positive linear correlation with biosurfactant specific activity. We found that the best model of specific activity of lipopeptide biosurfactants depended on both the ratio of *iso* to normal, even-numbered fatty acids (positive dependence) and the ratio of *anteiso* to *iso,* odd-numbered fatty

Table 2.2: Comparison of biosurfactant activity and fatty acid ratios of different biosurfactants

Species	Strain	Specific activity mm•μg^{-1}	Fatty acid composition			
			C13	C14	C15	C16
B. mojavensis	JF-2	3 ±2[a]	1.3 ±2.5[b]	49.6 ±85.4[b]	11.3 ±14.9	24.4 ±45
B. subtilis subsp. *spizizenii*	TG6-19	2.4 ±0.8	3.6 ±7.1	58.4 ±54.8	30.3 ±54.8	3.5 ±0.2
B. subtilis subsp. *subtilis*	T89-15	3 ±2	2.4 ±4.8	67.7 ±47.2	22.7 ±29.4	4.3 ±3.8
B. subtilis subsp. *subtilis*	T89-42	1.63 ± 0.3	4.7 ±9.4	67.1 ±29.4	24.6 ±47.2	4.3 ±38
B. subtilis subsp. *spizizenii*	T88-8	3 ±2.95	2.4 ± 4.8	61 ±52.8	27.3 ±52.8	6.3 ±88
B. subtilis subsp. *spizizenii*	CL1-14	2.55 ±2.1	4.2 ±8.4	67.3 ±56.9	25.8 ±44.4	4.4 ±4.9
B. mojavensis	T89-14	2 ±2	6.4 ± 12.8	57 ±69.5	32.8 ±55.7	2.8 ±1.2
B. subtilis subsp. *spizizenii*	T89-3	1.35 ±1.3	6.8 ±13.6	49.7 ±44.3	35.5 ±29	6.2 ±0.7

[a] Mean ± std dev of triplicate cultures. The specific activity of purified biosurfactant is expressed as diameter of spreading in mm per μg of the biosurfactant purified.

[b] Mean ± the range of duplicate cultures. The numbers refer to 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 area of all FAME.

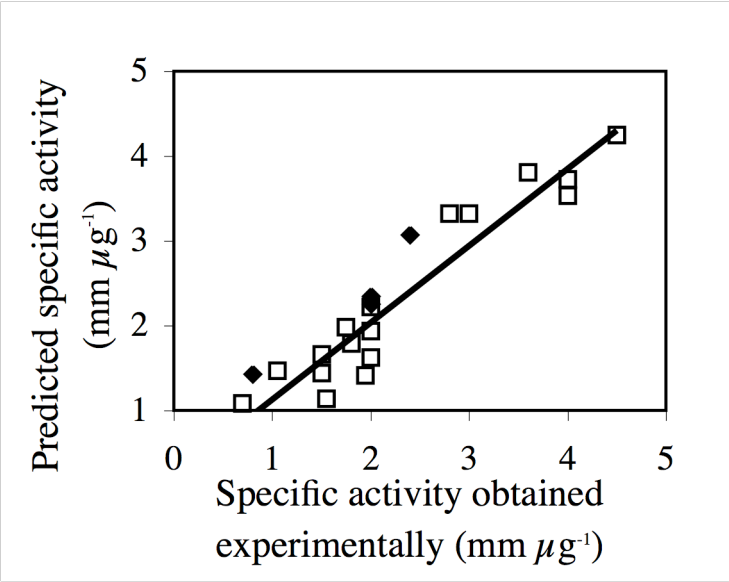
acids (negative dependence). When the values expected for specific activity (obtained by using the multiple regression equation) were plotted against the values of specific activity obtained experimentally (Fig. 2.1, open squares) (43), the linear correlation coefficient (r^2) was 0.91 (15) and the Pearson correlation coefficient (r) was 0.94 (15). The variation not explained by the multiple regression model might be due to the probability of the presence of a different amino acid in the peptide portion (Glu/ Gln and/ or Asp/ Asn content) of the lipopeptides.

The multiple regression model accurately predicted the specific biosurfactant activity from the ratios of both *iso* to normal, even-numbered fatty acids and *anteiso* to *iso,* odd-numbered fatty acids for four other lipopeptide biosurfactants produced by *B. mojavensis* and *B. subtilis* subsp. *spizizenii* strains (Fig. 2.1, closed diamonds).

Effect of amino acid addition:

Precursors of branched chain fatty acids (21) were added to the growth medium of *B. subtilis* subsp. *subtilis* strain T89-42 to change the fatty acid composition and test the predictions of the multiple regression model (Table 2.3). When the strain was grown with 1g/l valine, the specific activity increased 3.2-fold, and the yield almost doubled compared to un-amended cultures. The ratio of *iso* to normal 3-OH even-numbered fatty acids increased 2.8-fold (Table 2.3). The increase in both the specific activity and the ratio of *iso* to normal even-numbered fatty acids when valine was added to the growth medium supports the finding that the specific activity is positively correlated to this ratio. When strain T89-42 was grown with alanine, the specific activity increased 1.7-fold, the ratio of *iso* to normal even-

Figure 2.1: Multiple regression analysis for the fatty acid predictors of specific activity of lipopeptide biosurfactants. Values on the X-axis are the experimentally obtained specific activities of the different lipopeptide biosurfactants. Values on the Y-axis were obtained by using the multiple regression equation: y (specific activity) = 0.39 (ratio of *iso* to normal even-numbered fatty acids) – 0.09 (ratio of *anteiso* to *iso* odd-numbered fatty acids) + 0.73. The equation of the straight line was y = 0.908 x + 0.214. The coefficient of linear regression was r^2 = 0.908. The multiple regression equation above was used to predict the specific activity of four other strains. The coefficient of linear regression (r^2) between the predicted and actual specific activity for these four strains was 0.9134 (y = 0.925x + 0.611). Squares: values for the experimentally obtained versus calculated specific activities for seven biosurfactants purified from duplicate cultures and 1 biosurfactant purified from triplicate cultures. Closed diamonds: values for the experimentally obtained versus calculated specific activities of four other purified biosurfactants.


Predicted specific activity (mm μg^{-1})
5
4
3
2
1
0
1
2
3
4
5
Specific activity obtained experimentally (mm μg^{-1})

numbered fatty acids increased 1.2-fold, while the ratio of *anteiso* to *iso* odd-numbered fatty acids was about the same as in the control without amino acid addition (Table 2.3). When leucine was present, the specific activity doubled (Table 2.3). The increase in the specific activity with leucine addition could not be accounted for by an increase in the ratios of *iso* to normal even-numbered fatty acid isomers since the *iso* and normal isomers of even-numbered fatty acids with leucine addition comprised only 3.8% of the total fatty acids compared to 48 % of the total fatty acids in the control without amino acid addition. However, the decrease in the ratio of *anteiso* to *iso* odd-numbered fatty acids may explain the increase in specific activity since this ratio is negatively correlated to specific activity. When isoleucine was added to the growth medium, the specific activity was similar to the unamended control. An increase in the ratio of *iso* to normal even numbered fatty acids (1.7-fold) might have counteracted the increase in the ratio of *anteiso* to *iso* odd numbered fatty acids (2.7-fold) to keep the specific activity close to that of the control without amino acid addition.

Figure 2.2 shows that there was a strong linear correlation ($r^2 = 0.95$) (15) between the values of specific activities of biosurfactants produced in cultures with the different amino acid additions and the specific activities predicted from the multiple regression equation based on the fatty acid composition. Pearson correlation coefficient (r) was 0.98 (15).

Table 2.3: Yields, surface activities, and fatty acid ratios of biosurfactants from *Bacillus subtilis* subsp. *subtilis* strain T89-42 grown in presence and absence of exogenous amino acids in the medium.

Amino acid added	Biosurfactant yield	Specific activity	Ratio of *iso*/n even-numbered fatty acid[c]	Ratio of *anteiso*/*iso* odd-numbered fatty acids[c]
None	1.2 ± 0.25 [a]	2 ± 0.05 [b]	2.25 (±0.5)[d]	0.55 (±0.1)[d]
Alanine	0.28 ± 0.006	3.1 ± 0.4	2.7 (±0.6)	0.45 (±0.3)
Valine	2.3 ± 0.06	5.7 ± 0.6	6.33 (±0.06)	0.62 (±1.05)
Leucine	0.25 ± 0.04	4.1 ± 0.6	3.9 (±0.38)	0.037 (±0.07)
Isoleucine	0.37 ±0.09	2.2 ±0.4	3.9 (±3.2)	2.7 (±0.6)

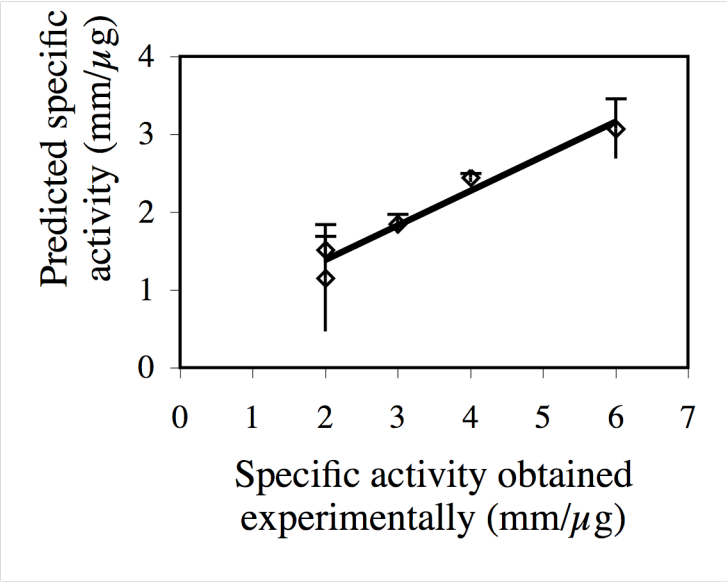
[a] Mean ± std dev of triplicate cultures. The biosurfactant yield is expressed as mg biosurfactant • g^{-1} dry weight of cells.

[b] Mean ± std dev of triplicate cultures. Specific activity of biosurfactant is expressed as mm of clearing • μg^{-1} of biosurfactant.

[c] Even-numbered fatty acids are *iso*-3-OH-C14, normal 3-OH-C14 and normal 3-OH-C16. Odd-numbered fatty acids are *anteiso* 3-OH-C13, 3-OH-C15 (and 3OH-C17 only in case of isoleucine addition), and *iso* 3-OH-C13, 3-OH-C15 (and 3-OH-C17 only in case of isoleucine and leucine additions).

[d] Mean ±(range) of the fatty acid ratios of the biosurfactant purified from duplicate cultures.

Figure 2.2: Correlation between the experimentally obtained specific biosurfactant activities and those predicted by the multiple regression equation based on known fatty acid composition. The predicted specific activity was calculated from the fatty acid ratios given in Table 2.3 with the multiple regression equation given in the legend to Figure 2.1. Data points represent each culture condition shown in Table 2.3. The error bars represent range of duplicate cultures for each growth condition. The equation of the straight line was $y = 0.323\ x + 1.12$. The coefficient of linear regression was $r^2 = 0.902$.


Predicted specific activity (mm/μg)
0
1
2
3
4
0
1
2
3
4
5
6
7
Specific activity obtained experimentally (mm/μg)

Discussion:

The majority of strains examined in this study were members of *Bacillus subtilis (*subspecies *subtilis* and *spizizenii*), *Bacillus licheniformis*, or species closely related to them. Although many of these strains had originally been isolated for other studies (10, 12, 18, 33) and therefore not selected to be biosurfactant producers, 68% produced biosurfactant, as compared to 80% of isolates from oil wells (Table 2.1). Approximately 14% of these strains that were examined with an oil-spreading technique had relative activities 1.75 to 2.5 times that of JF-2 (15 out of 110; Table 2.1), while 1 in 44 strains isolated from oil wells (2%) had such a high relative activity. Therefore, in accordance with recent findings (7), we found that biosurfactant-producing microbes can be readily isolated from uncontaminated, undisturbed arid soils. However, our percentage of biosurfactant-producing isolates was quite high (e.g. 68% vs. 3.4%), reflecting our focus upon *Bacillus* species known to contain strains that produce biosurfactants rather than screening more broadly for novel biosurfactant producers. Strains varied greatly in biosurfactant yield (mg biosurfactant produced per gram dry weight of cells) and in the surface activity of active fractions collected from TLC plates. There was only a weak correlation between yield and surface activity.

The lack of correlation between yield and activity suggested that the variability in surface activity of biosurfactants produced by the closely related strains used for this study was due to structure differences rather than a result of changed gene expression. The three dimensional structure of surfactin, the biosurfactant produced by *B. subtilis* (9), showed that the carboxylic groups of both glutamate and aspartate form

a minor hydrophilic domain, and the non-polar residues in position 4 and, to a lesser extent, in positions 2 and 7 form major hydrophobic domains with the lipid tail. The presence of these 2 domains was found to be important for surface activity. Since then, structural variants of surfactin were obtained via chemical modification (38), cultural modification, or genetic recombination (8, 14, 31, 32, 35, 36) to obtain a biosurfactant molecule with higher surface activity. A substitution of valine to isoleucine in position 4 decreased the CMC by two-fold and increased the surface activity possibly due to the expansion of the major domain by the incorporation of the more hydrophobic isoleucine (8). Monoanionic biosurfactants (e.g. lichenysin A with asparagine in position 5) had higher surface activity compared to dianionic biosurfactants (e.g. surfactin with aspartate in position 5) (14, 41). In this study, all the biosurfactants tested had 1 valine and 4 leucines. Glutamine and/ or asparagine could replace glutamate and aspartate in positions 1 and 5, respectively, since these amino acids could not be distinguished by the method used for amino acid analysis. The presence of glutamine or asparagine in the peptide chain would mean that the biosurfactant is monoanionic and hence should have higher activity. A more detailed study on the presence of the amide form of acidic amino acids is required to rule out their effect on the biosurfactant activity.

The fatty acid composition of the lipopeptide also affects activity. Yakimov et al. (40) found that an increase in the percentage of branched chain fatty acids in lichenysin A of *Bacillus licheniformis* strain BAS50 decreased surface activity and an increase in the percentage of straight chain 3-hydroxy tetradecanoate (n-3OH-C14) increased surface activity. This is in contrast to our results that the percentage of 3-

hydroxy *iso* even-numbered fatty acids (in our case *iso*-3-OH-C14 was the only even-numbered branched-chain fatty acid) was correlated to surface activity. However, Yakimov et al. (40) studied only one lipopeptide, lichenysin A. Lichenysin A is a monoanionic lipopeptide with a heptapeptide (Glu: Asn: Val: Leu: Ile; 1:1:1:3:1). The presence of the amide form asparagine and the more hydrophobic isoleucine residue results in a lipopeptide with different properties than the lipopeptides compared in this study.

Kaneda (21) showed that biosynthesis of branched chain fatty acids proceeds from the corresponding acyl CoA esters derived from branched-chain amino acids (L-valine, L-isoleucine, and L-leucine). Since the fatty acid composition of the biosurfactant, is controlled by the abundance of fatty acids precursors in the cell (1, 5, 6, 11, 16, 17), we added exogenous branched-chain amino acids to the growth medium to determine the effect of changes of fatty acid composition on biosurfactant activity. The results of the exogenous amino acid addition to the growth medium (Table 2.3) suggests that altering the ratios of even-numbered fatty acids has a more pronounced effect on specific activity than does altering the ratios of odd-numbered fatty acids. A 2.8-fold and 1.2-fold increase in the ratio of *iso* to normal even-numbered fatty acids (with valine, and alanine addition, respectively) led to a 3.2-fold and 1.7-fold increase in specific activity. However, a 15-fold decrease in the ratio of anteiso to iso odd numbered fatty acids only led to a 2-fold increase in the specific activity when leucine was added to the medium. A 2.7-fold increase in the latter ratio with isoleucine addition did not change the specific activity much compared to the control without amino acid addition. We hypothesize that branched even numbered fatty acids (in this

case *iso* C14 was the only branched even numbered fatty acid) might give the optimum hydrophilic-lipophilic balance required for optimum surface activity. A more definitive conclusion could be drawn if the lipopeptide with only the *iso* C14 fatty acid could be purified from the mixture of lipopeptides. A higher activity in this case would support this hypothesis.

This work shows that fatty acid of lipopeptide biosurfactants is important for activity and that manipulation of the medium composition to change the lipopeptide fatty acid composition may result in biosurfactants with higher specific activities. This may be a more useful approach than the molecular engineering of the lipopeptide (27, 35, 37) since the various regulatory policies make it difficult to use recombinant strains for in situ applications.

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Chapter 3

Basis for formulating biosurfactant mixtures effective in achieving ultra low interfacial tension values against hydrocarbons

Abstract

Biosurfactants could potentially replace or be used in conjunction with synthetic surfactants to provide for more cost-effective subsurface remediation. To design biosurfactant/ synthetic surfactant formulations that are effective in lowering interfacial tension (IFT) needed to mobilize entrapped hydrocarbons, information about the surface-active agent and the targeted non-aqueous phase liquids (NAPL) are required. We hypothesized that biosurfactant/synthetic surfactant mixtures can be formulated to provide the appropriate hydrophobic/hydrophilic conditions to generate low IFT against NAPLs and that such mixtures will be more effective than individual biosurfactants or synthetic surfactants. Our work tested the efficacy of biosurfactants from individual strains and mixtures of biosurfactants from different strains with and without synthetic surfactants for enhanced interfacial activity. 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. A more heterogeneous fatty acid composition was more effective in lowering the 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 sulfate, produced lower 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 more hydrophobic synthetic surfactants lowered the IFT against hydrophobic NAPLs.

Introduction

Subsurface light non-aqueous phase liquid (LNAPL) contamination 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 contaminant, and the dilute contaminant plume (33). 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 dissolution of hydrocarbons entrapped in the source area into the passing ground water (5, 33).

Surfactant-enhanced subsurface remediation (SESR) has been identified as a promising technology for source area treatment (33, 35). SESR has two general approaches. Solubilization is the use of surfactants to enhance the aqueous solubility of contaminants thereby decreasing the volumes of water flushing required for treatment. Mobilization is the use of surfactant concentrations far above the critical micelle concentration to reduce the interfacial tension (IFT) between LNAPL and water phases. Capillary forces entrap the LNAPL, and large reductions in IFT are necessary to reduce the capillary forces and mobilize the LNAPL and thus achieve high LNAPL mass removal efficiencies (33, 35). However, several factors limit the use of surfactants in subsurface remediation. The cost of SESR can be high due to the high concentrations of surfactants required to achieve ultra low IFT (17, 33). The

persistence of surfactants or their metabolites in groundwater and their off site migration may also pose a problem (33).

Biosurfactants may provide a more cost-effective approach for subsurface remediation when used alone or in combination with synthetic surfactants. Sufficient amounts of biosurfactants can be produced during the growth process of microorganisms to produce IFT values less than 0.01 mN/m, as reported for lipopeptide biosurfactants at concentrations less than 100 mg/l (26, 27). The critical micelle concentration of many biosurfactants is much lower than synthetic surfactants (10, 20, 24, 26, 28, 34). Lastly, biosurfactants are biodegradable (30).

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 mineralization of alkanes such as hexadecane and octadecane, and polycyclic aromatic hydrocarbons such as naphthalene and phenanthrene. Both batch (9, 43) and column studies (12) showed that biosurfactant addition increased the aqueous solubility of hydrocarbons. However, mixed 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 (36). The use of improper biosurfactant concentration may also impede biodegradation. In some studies, biosurfactant concentrations above the CMC inhibited degradation (31). In other studies, biodegradation was stimulated at biosurfactant concentrations above the CMC (37). A

few studies showed that biosurfactant addition mobilized entrapped hydrocarbons by lowering interfacial tension (6, 12). The injection of over 40 to 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 to 80% of entrapped crude oil depending on the concentration (20 to 920 mg/l) and only two pore volumes of the lipopeptide solution were needed (25).

Previous work on lipopeptide biosurfactants showed that activity against crude oil depended on the carbon chain length and the degree of branching of the fatty acid tail (39). In this study, we sought to optimize biosurfactants formulations for LNAPL mobilization. Our hypothesis was that mixtures of biosurfactants are needed to achieve ultra low IFT required for LNAPL mobilization. Mixtures of synthetic surfactants have been shown to be effective in mobilizing perchloroethylene and LNAPL (33, 35), but the efficacy of biosurfactant mixtures is not known. 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/synthetic surfactant formulations to achieve ultra low IFT against LNAPL components.

Materials and methods

Sources of biosurfactants/surfactants

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 a-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 (39) (Table 3.1). Replicate cultures were grown aerobically at 37ºC in a mineral salts medium with 5% NaCl and sucrose as previously described (39, 41). When needed, 1 g/l L-valine or L-leucine was added to the growth medium before autoclaving (39). Biosurfactant production was followed over time by using the oil spreading technique (28, 39). 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 4ºC. The cell-free culture fluid was acidified to pH 2 by the addition of 2N HCl and then incubated at 4ºC overnight. The precipitate, which contained the biosurfactant, was collected by centrifugation at 14,300 *g* for 30 min at 4ºC. The pellet was then adjusted to pH 7 with 2N NaOH and lyophilized (39).

Table 3.1: 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
	T89-15	ND
Bacillus subtilis subsp. *spizizenii*	T89-3	10 ± 1.5
Bacillus mojavenesis	ROB-2	17.4 ± 0
	T89-14	17.4 ± 0.38
	ROG-4	7.8 ± 0.38

a: values are average ± standard deviation of 3 measurements

ND: not determined.

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 (40). Three peaks were obtained at retention times ranging from 1 to 4 minutes 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, 39). 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.

Structure 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. (38, 39).

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 (39). The method did not differentiate between acid and amide forms of glutamic and aspartic acids (39). 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 prepared by the acid precipitation method were 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

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 3.1).

The interfacial tension (IFT) between surfactant solutions and different hydrocarbons was determined 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 1g/l surfactant solution was measured against each of the above hydrocarbons with NaCl additions ranging from 0 to 9% (w/v). The optimal salinity of a given surfactant is defined as the salt concentration at which the lowest value for IFT was obtained.

Surface and interfacial tension measurements were done in triplicates 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 (42) 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) and against a single hydrocarbon at different salt concentrations were used to determine the relative hydrophobicity/ hydrophilicity of biosurfactants and synthetic surfactants. The hydrophobicity of hydrocarbons increases with the EACN (1). Biosurfactants or synthetic surfactants that have their lowest IFT against hydrocarbons with high EACN are considered to be relatively hydrophobic (7). Table 3.2 shows the IFT values against different hydrocarbons. Comparing the IFT values against toluene, the biosurfactants/synthetic surfactants can be ordered from the most hydrophilic to the most hydrophobic as follows, rhamnolipid> T89-42> T89-3>ROB-2>T89-14> ROGG-2> C12, C13 – 8PO – SO_4Na. However, the IFT values against toluene for T89-42 and T89-3 biosurfactants were very similar as were those for ROGG-2 biosurfactant and C12, C13 – 8PO – SO_4Na. To distinguish these compounds further, the IFT values against hydrocarbons with different EACN were analyzed. The T89-42 biosurfactant had its lowest IFT against decane while the T89-3 biosurfactant had its lowest IFT value against hexane. This indicates that the T89-3 biosurfactant was more hydrophilic than the T89-42 biosurfactant. Similarly, the ROGG-2 biosurfactant had its lowest IFT against toluene and C12, C13 – 8PO – SO_4Na had its lowest IFT against decane. This indicates that the ROGG-2 biosurfactant was more hydrophilic than C12, C13 – 8PO – SO_4Na. Incorporating all of the above analyses gave the order of

Table 3.2: EACN scans of different biosurfactant obtained at their optimal salinity

	Surfactant	Hydrocarbon (EACN)			
		Toluene (1)	Hexane (6)	Decane (10)	Hexadecane (16)
Lipopeptide	T89-42	0.54±0.04 (3.7%)[a]	0.79 (8%)[a]	0.5 ± 0.07(9%)[a]	0.6 ±0.06 (8%)[a]
	T89-3	0.51 (5%)	0.42 (9%)	0.63 (9%)	0.97 (9%)
	ROB-2	0.66 ±0.03	1.27 ±0.09	1.22 ±0.07	2.75 ± 0.05
	T89-14	1.05 ±0.4	1.19 ±0.5	1.46 ±0.4	1.91 ± 0.25
	ROGG-2	2.17	2.55	3.65	3.89
Rhamnolipid	JBR 515	0.31 ± 0.01	0.65 ± 0.02	0.69 ± 0.1	0.8 ± 0.02
Synthetic surfactant	C12, C13-8PO sulfate	2.19 ± 0.01	0.2 ± 0.02	0.02 ± 0.001	0.04 ± 0.001

a: values are average interfacial tensions ± standard deviation of 3 measurements of 1g/l biosurfactant solutions.

Optimal salinity in g% shown between parentheses for some biosurfactants.

hydrophilicity as rhamnolipid> T89-3> T89-42>ROB-2>T89-14> ROGG-2> C12, C13 – 8PO – SO_4Na.

The differences between the highest and the lowest IFT values against different hydrocarbons for each surfactant were compared (Table 3.2). Three different patterns emerged. A relatively small difference was observed for T89-3, T89-42, and rhamnolipid biosurfactants (0.4-0.7 mN/m), a moderate difference was observed for ROB-2, T89-14, and ROGG-2 biosurfactants (0.9-2 mN/m), and a large difference was observed for C12, C13 – 8PO – SO_4Na (over a 100-fold change as the EACN increased from 1 to 10). These analyses support the conclusion that rhamnolipids and lipopeptides were more hydrophilic than C12, C13 – 8PO – SO_4Na.

Additional information about the relative hydrophilicity/ hydrophobicity of the biosurfactants/synthetic surfactants was inferred from the salt concentration that gave the lowest IFT value (optimal salt concentration) against each of the four hydrocarbons. High optimum salt concentration indicates that the surfactant is hydrophilic. Table 3.2 shows the optimum salt concentration in parentheses for T89-42 and T89-3 biosurfactants against the four hydrocarbons. The higher optimal salt concentration against toluene and hexane for the T89-3 biosurfactant compared to the T89-42 biosurfactant supported the previous observation that the T89-3 biosurfactant was more hydrophilic than the T89-42 biosurfactant.

Effect of fatty acid composition of lipopeptide biosurfactants on interfacial activity

Amino acid analysis showed that all of the lipopeptides listed in Table 3.2 were heptapeptides with the same amino acid composition (mean ± std dev of the mole ratio): E/ Q: D/ 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 composition 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 two biosurfactants produced by strains T89-3 and T89-42. Since electrospray MS was run in the negative ion mode, the ion fragments were negatively charged and corresponded to M-1, M-2+Na, and M-3+2Na (M is the molecular weight). Comparison of the molecular weights of the ions allowed determination of the amino acid composition. For example, if the lipopeptide contained a 3-hydroxy tetradecanoate, glutamate, and aspartate (an amino acid composition of 1E, 1D, 1V, 3L), the molecular weight would be 1021 and the M-1, M-2+Na, M-3+2Na ion molecular weights would be 1020, 1042, and 1064, respectively. With one amide and one acidic amino acid, the molecular weight would be 1020 and the M-1, M-2+Na, M-3+2Na ion molecular weights would be 1019, 1041, and 1063, respectively. With 2 amides in the peptide head, the molecular weight would be 1019 and the M-1, M-2+Na, M-3+2Na ion molecular weights would be 1018, 1041, and 1062, respectively. The analysis of the molecular weights of the ions supported the following amino acid composition: 1E, 1D, 1V, and 3L (Table 3.3). This is, the T89-42 and T89-3 biosurfactants each contained 1 glutamate and 1 aspartate in their peptide heads. Although electrospray MS was not conducted for the rest of biosurfactants in Table 3.2, we expect that the amino acid composition of these biosurfactants to be the same as the T89-42 and T89-3 biosurfactants since they are produced by strains of *Bacillus subtilis* and the closely related species, *Bacillus mojavensis*.

Table 3.3. Electrospray mass spectrometry data for two lipopeptide biosurfactants

Biosurfactant	Ion fragment	Fragment molecular weight	β-OH Fatty acid tail length	Amino acid composition
T89-42	M-1[a]	1006	C13	1D, 1E, 1V, 3L
		1020	C14	1D, 1E, 1V, 3L
		1034	C15	1D, 1E, 1V, 3L
	M-2+Na[a]	1028	C13	1D, 1E, 1V, 3L
		1042	C14	1D, 1E, 1V, 3L
		1056	C15	1D, 1E, 1V, 3L
	M-3+2Na[a]	1064	C14	1D, 1E, 1V, 3L
		1078	C15	1D, 1E, 1V, 3L
T89-3	M-2+Na[a]	1014	C12	1D, 1E, 1V, 3L
		1028	C13	1D, 1E, 1V, 3L
		1042	C14	1D, 1E, 1V, 3L
		1056	C15	1D, 1E, 1V, 3L
	M-3+2Na[a]	1050	C13	1D, 1E, 1V, 3L
		1064	C14	1D, 1E, 1V, 3L
		1078	C15	1D, 1E, 1V, 3L

a: ion fragments 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.

Lipopeptide biosurfactants shown in Table 3.2 contained 3-hydroxy tridecanoate (3-OH-C13), tetradecanoate (3-OH-C14), pentadecanoate (3-OH-C15), hexadecanoate (3-OH-C16), and octadecanoate (3-OH C18). The 3-OH-C13, and 3-OH-C15 fatty acids were each present as mixtures of *iso* and *anteiso* isomers while 3-OH-C14 was comprised of normal and *iso* isomers. Only the normal isomer was detected for 3-OH-C16 and 3-OH C18 fatty acids. In some cases, the isomers of the 3-OH-C14 and 3-OH-C15 fatty acids constituted the majority of the fatty acids of the lipopeptide. However, in other cases, isomers of 3-OH-C14 were the major fatty acid component.

When the fatty acids of the biosurfactant purified from replicate cultures of the same strain were analyzed, the fatty acid composition varied along with the interfacial tension against toluene. The variation in fatty acid composition of lipopeptide biosurfactants was correlated to variations in the IFT against toluene. Multiple regression analysis (42) was used to determine the changes in fatty acids isomers that contributed to the variation in activity (interfacial tension against toluene). The best model that explained the interfacial tension of lipopeptide biosurfactants against toluene depended 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. 3.1a), the linear correlation coefficient (r^2) was 0.986 (11) and the Pearson correlation

Figure 3.1: 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 = 1.1 x - 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 twenty 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 = 0.9 x + 0.29), and that for biosurfactant mixtures was 0.93 (y=0.84 x + 0.04).

Figure 3.1A

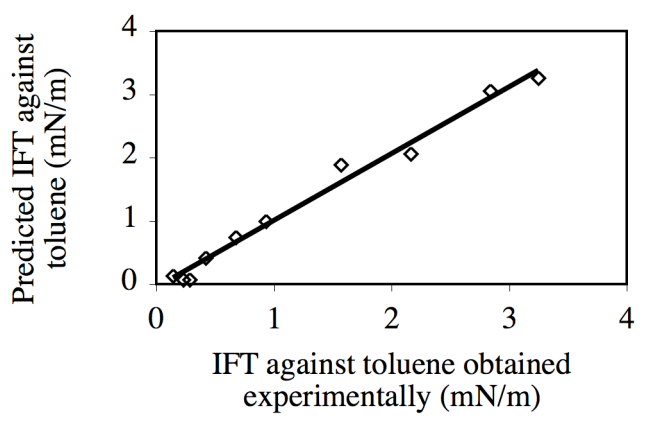
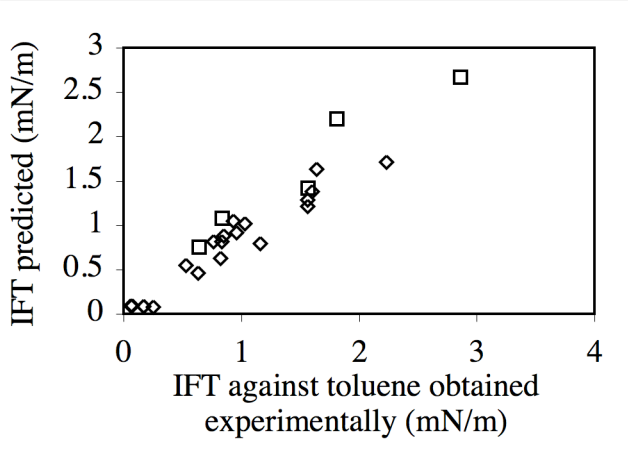


Figure 3.1B



coefficient (r) was 0.99 (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 twenty biosurfactant mixtures (Fig. 3.1b).

Comparing the coefficients in the multiple regression equation (Figure 3.1a 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 (< 0.5mN/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

Since 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 3.3). Table 3.4 shows the fatty acid composition of two separate batches of T89-42 and T89-3 biosurfactants. The IFT against toluene for the biosurfactants from each strain differed from one batch to another. The first batch of T89-42 biosurfactant had low IFT (0.27 ± 0.04 mN/m). The second batch had a relatively higher IFT (0.71 ± 0.04 mN/m) against

toluene. Similarly, the first batch of T89-3 biosurfactant had low IFT (0.12 ± 0.01 mN/m). The second batch had a relatively higher IFT (0.51 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 than 8, consistent with the predictions of the multiple regression model (Table 3.4). 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 3.4).

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 using the following equation: [(fraction of the first biosurfactant in the mixture) x (percentage of the fatty acid in the first biosurfactant)] + [(fraction of the second biosurfactant in the mixture) x (percentage of the fatty acid in the second biosurfactant)]. Table 3.4 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 in mixtures with 20% and 40% 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

Table 3.4: Predicted versus experimentally determined IFT values against toluene for different biosurfactant formulations.

Biosurfactant	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	Predicted IFT [e]	Experimentally obtained IFT [f]
T89-42 (1) [a]	22.2 [b]	45.8 [b]	17 [b]	2 [b]	68	8.5	0.08	0.27 ± 0.04
T89-3 (1) [a]	27.5 [b]	27.4 [b]	28.4 [b]	2.9 [b]	54.9	9.8	0.13	0.12 ± 0.01
0.2 T89-42 (1) + 0.8 T89-3 (1) [c]	26.4 [d]	31.1 [d]	26.12 [d]	2.72 [d]	57.5	9.6	0.09	0.06 ± 0.02
0.4 T89-42 (1) + 0.6 T89-3 (1) [c]	19.9 [d]	29.3 [d]	18.2 [d]	1.96 [d]	49.2	9.3	0.09	0.07 ± 0.01
T89-42 (2) [a]	29.1 [b]	56.3 [b]	3.27 [b]	2.83 [b]	85.4	1.2	0.74	0.71 ± 0.04
T89-3 (2) [a]	33 [b]	45 [b]	1 [b]	4 [b]	78	0.25	0.42	0.51
0.5 T89-42 (2) + 0.5 T89-3 (2) [c]	31.1 [d]	50.7 [d]	1.69 [d]	3.4 [d]	81.8	0.5	0.53	0.78
ROB-2	25 [b]	13 [b]	36 [b]	18 [b]	38	2	0.75	0.66 ± 0.03
T89-14	49 [b]	36 [b]	4 [b]	0.1 [b]	85	40	1.08	1.05 ± 0.39
0.5 ROB-2 + 0.5 T89-14 [c]	37 [d]	24.5 [d]	20 [d]	9.6 [d]	61.5	2	0.96	0.85 ± 0.1
0.8 ROB-2 + 0.2 T89-14 [c]	29.8 [d]	17.6 [d]	29.6 [d]	14.4 [d]	47.4	2	0.83	0.78 ± 0.2

a: Numbers between parentheses refer to the batch number. T89-42 and T89-3 were grown in 2 separate batches.
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 area of all FAME.
c: Components of lipopeptide mixtures and fractions of each biosurfactant in the mixture
d: Fatty acid composition of the mixture calculated using the equation [(fraction of the first biosurfactant in the mixture) x (percentage of the fatty acid in the first biosurfactant tail)] + [(fraction of the second biosurfactant in the mixture) x (percentage of the fatty acid in the second biosurfactant tail)].
e: IFT calculated using the multiple regression equation in Figure 1a legend.
f: IFT values are averages ± standard deviation of 3 measurements

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 data for the 50/50 mixture is shown in Table 3.4. The predicted IFT value was 0.53 mN/m and the experimentally obtained IFT value was 0.78 mN/m. The relatively high IFT values were 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 needed 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 due to the low 3-OH C16 to 3-OH C18 ratio for both the biosurfactants produced by individual strains and the mixtures (Table 3.4).

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/ 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 3.2). 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 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 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 3.2). 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 3.5). 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 (39). 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 3.5). 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 3.5). 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 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 will be

Table 3.5. Mixtures of lipopeptide biosurfactants with rhamnolipid JBR515. Percentage of 3-OH C14 fatty acid in the lipopeptide tail and the IFT of the mixture against toluene.

Biosurfactant	Amino acid added	% of 3-OH C14	100% lipopeptide [a]	50% rhamnolipid in the mixture [a]	80% rhamnolipid in the mixture [a]
T89-42	Leucine	5	0.23	0.52	0.56
	None	33	0.95 ± 0.01 [b]	0.28	0.09
	Valine	62	1.33 ± 0.04 [b]	0.14	0.02

a: IFT values against toluene in mN/m

b: IFT values against toluene are average ± standard deviation of 3 measurements.

needed to obtain 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 0.51 mN/m for T89-3biosurfactant, 0.95 ± 0.01 mN/m for the T89-42 biosurfactant, and 2.17 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 3.6). The IFT of each component alone against the different hydrocarbons is given in Table 3.2. These

Table 3.6. Mixtures of lipopeptides with C12, C13 – 8PO – SO_4Na.

Lipopeptide	25% C12, C13 – 8PO – SO_4Na [a]		50% C12, C13 – 8PO – SO_4Na [a]	
	Hexane	Decane	Hexane	Decane
T89-3	0.014± 0.004 [b]	0.04± 0.001 [b]	0.08± 0.006 [b]	0.02± 0.001 [b]
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; percentage of C12, C13 – 8PO – SO_4Na in the mixture

b: IFT of the mixture against the hydrocarbon in the table header. Values are average ± standard deviation of 3 measurements.

The IFT of each component alone with the different hydrocarbons is given in Table 3.2.

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 (33, 35). 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, McInerney et al. 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% with 1 mg/ml solutions of lipopeptides (4, 22, 23, 29). 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 (32, 35). 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, 36, 43). 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.

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 3.2). In order to explain this, a structure-interfacial activity study was conducted to delineate the structural features important for interfacial activity. Previous work has shown that specific biosurfactant surface activity against crude oil depended on both the ratios of *iso* to normal even-numbered fatty acid, and *anteiso* to *iso* odd-numbered fatty acids of the lipid tail (39). 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 were 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). 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 maximum 3-OH C16 to 3-OH C18 ratio. 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 3.4). 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 (39). The biosurfactants produced under these conditions could certainly be used as the third component 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, 36, 43) 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 3.5). 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 C12, C13 – 8PO – SO_4Na were the most effective in lowering the IFT against hexane and decane. Since 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 3.2). 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 3.6). 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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Chapter 4

In-situ biosurfactant production by injected *Bacillus* strains in a limestone petroleum reservoir.

Abstract

Biosurfactant-mediated oil recovery may be an economic approach to recover significant amounts of oil entrapped in reservoirs, but evidence that biosurfactants can be produced *in situ* at concentrations needed to mobilize oil is lacking. We tested whether two *Bacillus* strains that produce lipopeptide biosurfactants can metabolize and produce their biosurfactants in an oil reservoir. Five wells that produce from the same Viola limestone formation were used. Two wells received an inoculum (a mixture of *Bacillus* strain RS-1 and *Bacillus subtilis* subsp. *spizizenii* NRRL B-23049) and nutrients (glucose, sodium nitrate, and trace metals), two wells received just nutrients, and one well received only formation water. Results showed *in situ* metabolism and biosurfactant production. The average concentration of lipopeptide biosurfactant in the produced fluids of inoculated wells was about 90 mg/l. This concentration is ~ 9-times the minimum concentration required to mobilize entrapped oil form sandstone cores. Carbon dioxide, acetate, lactate, ethanol, and 2,3-butanediol were detected in the produced fluids of the inoculated wells. Only CO_2 and ethanol were detected in the produced fluids of the nutrients only treated wells. Microbiological and molecular data showed that the microorganisms injected into the formation were retrieved in the produced fluids of the inoculated wells. We provide essential data for modeling microbial oil recovery processes *in situ* including, growth rates (0.06 ± 0.01 h^{-1}), carbon balance (107 ± 34 %), biosurfactant production rates

($0.02 \pm 0.001\ h^{-1}$), and biosurfactant yields (0.015 ± 0.001 mole biosurfactant/ mole glucose). The data demonstrate the technical feasibility of microbial processes for oil recovery.

Introduction

Oil is an essential source of energy and one of the main factors that drives the economic development of the world (16). Current oil production technologies only recover about one-third to one-half of the oil originally present in an oil reservoir (16, 26, 37). Future predictions are that world oil demand will be met by an increasingly smaller number of countries, mainly located in West Africa, Persian Gulf, and the former Soviet Union (16). The exploitation of oil resources in existing mature reservoirs is essential to avoid the political, economic, and strategic consequences that will result if the number of oil exporting countries dwindles. A key to exploiting this untapped resource is to overcome the capillary forces that entrap oil in small pores within the reservoir. Enhanced oil recovery (EOR), e.g., the use of heat, chemicals such as surfactants, microbial processes, and miscible gas injection (15), has the potential to recover a significant portion of this entrapped oil. However, oil recovered by EOR constitutes less than 10% of the total oil produced in the United States (http://www.fe.doe.gov/programs/oilgas/eor/). Interfacial tension between the hydrocarbon and aqueous phases is largely responsible for trapping the hydrocarbon in the porous matrix and several orders of magnitude reduction in interfacial tension are needed for hydrocarbon mobilization (1, 15, 47). To achieve large reductions in interfacial tension, surfactant concentrations significantly above that needed to form micelles (e.g., the critical micelle concentration) are required (7, 41, 42). The high chemical costs have prevented the widespread use of surfactants for enhanced oil recovery.

Microbially enhanced oil recovery (MEOR) processes employ the use of microbial metabolites such as biosurfactants to lower interfacial tension between brine and oil and hence mobilize entrapped oil (2, 8, 24, 32). Several biosurfactants, in particular the lipopeptides made by *Bacillus* species, generate the low interfacial tensions between the hydrocarbon and the aqueous phases required to mobilize residual hydrocarbon (25, 30). MEOR has several advantages compared to other EOR processes in that it does not consume large amounts of energy, as do thermal processes, nor does it depend on the price of crude oil, as do many chemical processes (2, 31, 32). MEOR can also be cost-effective, since microbial products can be produced from inexpensive, and renewable resources, and several MEOR processes have been shown to produce incremental oil for about \$19 per m^3 (\$3 per barrel) (6, 9, 31, 32). Nevertheless, microbial processes have always been viewed with considerable skepticism for a number of reasons. First, the lack of quantitative information regarding reaction rates, stoichiometries, product concentration, and yields needed to simulate the performance of microbial processes makes it difficult to extrapolate results from a given field test to other reservoirs (31, 32). Second, it is not clear whether microbial processes can generate the necessary metabolites in sufficient quantities and rates needed to mobilize entrapped oil in oil reservoirs (10, 31, 32). Third, technical performances of many field trials have been inconsistent (14, 23). Lastly, it is unclear whether the microbial strains used as inocula actually grow and metabolize in the reservoir (31, 32).

Here, we show in a well-controlled field experiment that biosurfactants are produced *in-situ* in amounts sufficient to mobilize substantial amounts of entrapped

oil. We provide, for the first time, data on *in-situ* product concentrations and yields, rates of growth, substrate utilization, and metabolites formation, and an excellent carbon mass balance. This quantitative information shows that biosurfactant-mediated oil recovery is technically feasible and will facilitate the use of computer simulations to determine the efficacy of MEOR in different reservoirs.

Materials and Methods

Preparation of the inoculum. Two halotolerant, biosurfacant producers, *Bacillus* strain RS-1 and *Bacillus subtilis* subsp. *spizizenii* strain NRRL B-23049 were used as the inoculum. Both strains grow in medium with 5% NaCl. *Bacillus* strain RS-1 and *Bacillus subtilis* subsp. *spizizenii* strain NRRL B-23049 were each grown in 200 ml of medium E (48, 49). When the culture reached late exponential phase of growth, it was used to inoculate a 10-liter carboy of the same medium, which was incubated at 37ºC for 48 h. Agitation and aeration were maintained by using glass gas dispersion tubes with fritted cylinders (Fisher scientific, Inc.). The cells were concentrated by using a tangential membrane flow system (0.45 μ pore size filter) (Millipore, Bedford, MA, USA) to yield on average 2 liters of cell concentrate from each 10-liter carboy. The concentrated cells were stored at 4ºC. One-liter of cell concentrate was used to inoculate a 132-liter tank with the following components [(grams per liter of tap water): dibasic potassium phosphate (1.2), monobasic potassium phosphate (0.23), sucrose (8.6), sodium chloride (8.6), sodium nitrate (0.86), and yeast extract (0.86)]. The medium was prepared septically due to the lack of facilities on site and tanks were incubated for approximately 48 hours at ambient temperature.

Field experiment. Five oil production wells in the Bebee field (Section 19, T5N, R5E, Pontotoc City, OK) that produce from the same formation (a Viola limestone) were used for this study. Two wells received an inoculum of the *Bacillus* strains and nutrients, two wells received just nutrients, and one well received an equivalent volume of formation water and served as the negative control. Each well that was inoculated received 396 liters of *Bacillus* strain RS-1 and 264 liters of *B. subtilis*

subsp. *spizizenii* NRRL B-23049. The nutrient package consisted of 79.5 kg of glucose, 7.9 kg of sodium nitrate, 19.9 g of magnesium sulfate, 2.0 g each of manganese sulfate, zinc sulfate, and iron sulfate, 0.2 g each of copper sulfate, aluminum/potassium sulfate, boric acid, and sodium molybdate, 0.1 g of sodium selenate, and 0.6 g of nickel chloride per well. Each well that received nutrients also received fluorescein (125 g), and sodium bromide (2 kg); the former was used to detect visually (green fluorescence) when the slug was produced, and the latter served as the conservative tracer to account for dilution in the reservoir. The nutrients, inoculum, and tracers were mixed with formation water (~8000 liters) by circulation supplied by the pump truck. The formation water was obtained from a storage tank located near the production wells. Each well received an initial injection (pre-flush) of ~1600 liters (10 barrels) of formation water, an injection of ~8000 liters (50 barrels) of the treatment (nutrients and cells, nutrients only, or formation water) followed by ~8000 liters (50 barrels) of formation water as post-flush to make a total of ~17500 liters (110 barrels) of fluids injected per well. The post-flush was used to move the nutrient package 1.2 to 2.4 m into the formation. After injection of the treatment package, production from each well was stopped for 108 hours to allow time for growth and metabolism to occur in the formation. After this incubation period, production was started.

Flow meters were attached to the tubing of three of the five wells to measure the volume of fluids produced. The total volume produced was recorded when each sample was collected. Since all the wells were set to pump at almost the same rate, the volume of fluid produced during a given time interval in the two wells that did not

have flow meters was estimated from the average volume produced by the 3 wells that had the flow meters attached.

Sampling. Samples for chemical and microbiological analyses were collected on several occasions prior to treatment and for a 32-hour period after production recommenced after the 108-hour incubation period. Each sample was colleted in 2-liter glass bottle that was allowed to overflow to minimize contact with air. The temperature of the sample was immediately recorded with a hand-held probe. An aliquot for chemical analyses was filtered through a 0.45 μm membrane filter to remove particulate material and oil. The remainder of the unfiltered sample was used to measure oil-spreading activity and for microbiological enumerations. All samples were stored on ice until analyzed. The analyses for pH, conductivity, nitrate, nitrite, ammonium, alkalinity, oil-spreading, and surface tension were completed on site within two hours after sample collection. For the other analyses, the samples were transported back to the laboratory and stored at 4^{o}C until analyzed. All measurements on each sample were done in duplicate unless otherwise indicated.

Detection of biosurfactant production. Biosurfactant activity of unfiltered samples was measured by using the oil-spreading technique (33, 49). The diameter of the clear zone on the oil surface was measured in triplicate for each sample. Biosurfactant activity, defined as diameter of clearing on the oil surface in centimeters, ranged from 0 to 2 cm. Surface tension of filtered samples was measured with a Du Nuoy ring tensiometer (Fisher Scientific Inc., Hampton, NH) calibrated with water as the high surface tension standard and isopropanol as the low surface tension standard (25, 30). The lipopeptide biosurfactant was quantified by using high performance liquid

chromatography (HPLC) with a reversed phase C18 column (250 mm length x 1.5 mm ID) and 60% acetonitrile in water as the mobile phase (48). Twenty microliters of 1:4 and 1:2 dilutions of filtered samples were injected onto the column. Retention times for biosurfactant were 2, 2.3 and 3.1 minutes corresponding to 3 different fatty acid tails of the lipopeptide (50). The peak areas of the three peaks were added together and the concentration was calculated from standard curves prepared in a similar manner with surfactin (Sigma Chemical Co., St. Louis, MO) and the highly purified lipopeptides (48) produced by each of the two microorganisms, *Bacillus* strain RS-1 and *B. subtilis* subsp. *spizizenii* NRRL B-23049. The standard curve was linear up to 500 mg/l of the lipopetide. The lipopeptides detected in the samples had the same retention times as the highly purified biosurfactant obtained from cultures of each inoculum strain.

Fermentation analyses. A modified orcinol/ H_2SO_4 method was used to determine the amount of glucose in each sample (45). Acetate, ethanol, and 2,3 butanediol were measured by using gas chromatography (GC) with a 80/ 120 Carbopack B-DA*/ 4% Carbowax 20M (2m length x 2mm ID) glass column (Varian, Inc., Walnut Creek, CA). Helium was used as the carrier gas at a flow rate of 24 ml/min (18, 22). The injector temperature was 200ºC; flame ionization detector was set at 180ºC. The column temperature was kept constant at 155ºC for 3.5 minutes and then increased to 180ºC at 30ºC/ min. The temperature was then held at 180ºC for 10 minutes. One microliter of the sample diluted in 30 mM oxalic acid was injected onto the column. Lactate was measured by using HPLC with an Alltech Prevail organic acid column (250 mm length x 1.5 mm ID) (Alltech associates, Deerfield, IL) and 25 mM KH_2PO_4

(pH 2.5) as the mobile phase according to manufacturer's instructions. A 50-μl aliquot of 1:10 dilution of the sample was used. The above metabolites were identified and quantified by comparison of retention times and peak areas, respectively, with those of known standards.

To test whether the two *Bacillus* strains produced the same fermentation products from glucose as those detected in the produced fluids of the inoculated wells, *Bacillus* strain RS-1 and *B. subtilis* subsp. *spizizenii* NRRL B-23049 were each grown anaerobically in duplicate serum bottles containing 75 ml formation water supplemented with glucose, sodium nitrate, and metals at the same concentration as used for the field experiment. The cultures were incubated at 37ºC without shaking for 48 h. A three-series most probable number (MPN) technique (see below) was used to enumerate the number of viable cells immediately after inoculation and after 48 hours of incubation.

Other chemical analyses. The pH was measured on filtered samples by using a hand-held pH/ conductivity meter (EXTECH Instruments, Waltham, MA). Nitrate, nitrite, ammonium, and alkalinity (in mg/l) were measured colorimetrically by using Hach kits (Hach Chemical Co., Loveland, CO) according to the manufacturer's instructions. Bromide in filtered samples was analyzed by using liquid chromatography with an analytical anion exchange column (IonPac AS4A-SC, 4 x 250mm, Dionex corporation, CA, USA) (39). The concentration of bromide in the samples was calculated from standard curve of NaBr with concentrations ranging from 0 to 500 μM.

Microbiological analysis. A three-series MPN technique was used to enumerate total heterotrophic bacteria, spore-forming bacteria, and halotolerant bacteria in unfiltered samples from both the injected and produced fluids. The procedure was modified to use 96-well plates. Three columns of wells were used for each sample, which was serially ten-fold diluted from 10^{-1} to 10^{-5}.

Physiological properties of *Bacillus* species, e.g. biofilm formation (17), sporulation (17), and halotolerance (32), allowed the use of specific media and/or manipulations for the MPN analysis as follows. *Bacillus* biofilm growth medium (BBGM) (17) was used to enumerate heterotrophic bacteria and to promote biofilm formation since biosurfactant production has been associated with biofilm formation in *Bacillus* species (5, 19, 44). Since *Bacillus* species are known to sporulate (17), a portion of each sample was heat-treated at 85ºC for 20 min and then diluted in BBGM to estimate the number of spore-forming bacteria. Finally, to estimate the number of halotolerant bacteria and to avoid underestimation of *Bacillus* species due to heat treatment, plate count broth (Difco laboratories Inc., Detroit, MI) with 5 % NaCl was used. To estimate the number of biosurfactant producers, 5 μl of sterile crude oil was added to the surface of the medium in all wells where growth was observed and the dissipation of the oil drop was noted. The MPN of biosurfactant producers in the samples was estimated from those wells where the oil dissipated on the surface. Published tables (www.fsis.usda.gov/Ophs/Microlab/ Appendix2.02.pdf) were used to calculate the MPN.

Samples (50 μL) from the injected and produced fluids of each well were patch-inoculated onto blood agar plates and incubated overnight at 37ºC. Multiple

blood agar plates were subsequently streaked from the growth patch. Resulting β-hemolytic (12) clearing zones were picked and streak-purified until pure isolates were obtained. The β-hemolytic isolates with the same colony morphology as *Bacillus* strain RS-1 or *Bacillus subtilis* subsp. *spizizenii* NRRL B-23049 were used for further culture-dependent molecular analyses (see below).

Molecular analysis. Primers used for different analyses are shown in Table 4.1. Formation water samples (2 liters) before and after treatment were vacuum-filtered on a PES membrane of 90 mm diameter and 0.2 μm pore size (VWR, West Chester, PA) after oil separation. Membranes containing the microorganisms were cut and used for culture-independent DNA extraction. Cells were lysed by rapid thawing and bead beating to shear the membrane into small pieces (typically less than 5 mm), followed by mixing with Stool Lysis Buffer (Qiagen, Valencia, CA), vortexing for 2 minutes, and incubating at 95ºC for 10 minutes in a water bath. Using a modified QIAamp DNA Stool Minikit protocol (Qiagen, Valencia, CA), DNA was extracted from lysed cells, and amplified in a Taq DNA polymerase chain reaction (PCR) using degenerate primers designed to hybridize with the *gyrA* gene sequences of a variety of *Bacillus* strains (13, 40). PCR products were extracted from the gel (Qiagen), cloned into the pGEM vector (Promega), and plasmid DNA was purified from selected transformants (Qiagen). Four clones from each *gyrA* amplicon were sequenced by the Oklahoma Medical Research Foundation (OMRF, OKC, OK). Sequences were analyzed by DNAMAN multiple sequence alignment (Lynnon Biosoft) with the sequences obtained for *Bacillus* strain RS-1 (GenBank accession number DQ995270) and

Table 4.1. Primers used for the different molecular analyses.

Analysis	Primers used	References
16S rDNA	907R: 5’-CCGTCAATTCCTTTRAGTTT-3’ 305F: 5'-CTCCTACGGGAGGCAGCAG-3'	(34, 35)
gyrA	gyrAF: 5’-CAGTCAGGAAATGCGTACGTCCTT-3’ gyrAR: 5’-CAAGGTAATGCTCCAGGCATTGCT-3’	(13, 40)
Rep-PCR	BOXA1R: 5’-CTACGGCAAGGCGACGCTGACG-3’	(21, 46)

Bacillus subtilis subsp. *spizizenii* strain NRRL B-23049 (GenBank accession numbers DQ995271 and AF272020).

Cell templates from isolates obtained on blood agar plates (see above) were utilized as a source of culture-dependent DNA for 16S rRNA gene and *gyrA* analyses as well as rep-PCR (21, 46). Three clones of each gene from each isolate were prepared and analyzed as described above. Consensus sequences were obtained by multiple sequence alignment and compared with those from other isolates and the strains used as inoculum.

The GenBank accession numbers for the 16S rRNA gene (551 bp) of *Bacillus* strain RS-1 and *Bacillus subtilis* subsp. *spizizenii* strain NRRL B-23049 are DQ995269 and AF074970, respectively. Rep-PCR reactions were first resolved on a 1% agarose gel and subsequently on a 5% polyacrylamide gel with 0% denaturant at 75V for 3.5 hours at 60ºC.

Calculation of total recoveries. The total amount of glucose utilized (C_{ST}) in a well was calculated from the equation, $C_{ST} = N - \Sigma C_n \bullet V_n$, where N is the number of moles of glucose injected in the well, C_n is the molar concentration of glucose in the produced fluid of n^{th} sample from the well, and V_n is the volume of fluid produced during the time interval between the n-1 and n samples from the well. The total amount of each metabolite produced (C_{PT}) was calculated from the equation, $C_{PT} = \Sigma C_n \bullet V_n$, where C_n is the molar concentration of the metabolite in the produced fluid of n^{th} sample, and V_n is the volume of fluid produced during the time interval between the n-1 and n samples. The total MPN of cells (C_{XT}) was calculated from the equation, $C_{XT} = \Sigma C_n \bullet V_n$, where C_n is the MPN per milliliter of cells in the produced fluid of n^{th}

sample, and V_n is the volume of fluid produced during the time interval between the n-1 and n samples.

Bromide was used as a conservative tracer to estimate the amount of dilution of the nutrient package by dispersion in the formation (4, 39). The bromide recovery factor was calculated for each of the three wells that had a total volumetric flow meter attached. The average bromide recovery factor from the 3 wells was 1.09 (24% variation), which indicated that little dispersion or adsorption of the tracer occurred. Values obtained from the various chemical analyses (C_{ST} or C_{PT}) from these three wells were divided by the corresponding bromide recovery factor obtained for that well. For the other two wells, the average bromide recovery factor was used. Corrected number of moles of glucose utilized and number of moles of end products were used for percentage carbon recovery calculations.

Results

Preinjection data. Samples were collected from all the wells 1 and 2 weeks prior to the treatment. The biosurfactant and common bacterial fermentation end products such as acids and alcohols were not detected in any of the wells prior to treatment (Table 4.2). In addition, we did not detect any viable bacteria at any dilution used for the MPN enumerations (Table 4.2).

Evidence that the injected strains were maintained in the inoculated wells. MPN analysis using the *Bacillus* biofilm growth medium (BBGM) showed biosurfactant producers in the injected fluids of both the inoculated wells and the nutrients only treated wells (Table 4.3). The total number of biosurfactant producers in the injected fluids was one order of magnitude higher for the inoculated wells compared to the nutrients only treated wells (4.2×10^{10} compared to 3.7×10^{9}) (Table 4.3). No spore-forming microorganisms were detected in either the injected or the produced fluids from nutrients only treated wells or the negative control. Since *Bacillus* species are known to sporulate (17), the absence of spore-forming microorganisms in the nutrients only treated wells indicates that the biosurfactant producers that were introduced into these wells were probably not *Bacillus* species, but microorganisms present in the storage tank. On the other hand, in the inoculated wells, spore-forming, biosurfactant-producing microorganisms were both introduced in and retrieved from the injected and produced fluids, respectively (Table 4.3). MPN of biosurfactant producers in the injected and produced fluids of inoculated wells were not significantly different (Table 4.3). These data indicate that biosurfactant producers in the inoculum maintained viability, but did not grow during the incubation period.

Table 4.2. Preinjection chemical and microbial analyses for all the wells.

Analysis	Inoculated wells[a]	Nutrients only wells[a]	Negative control well[b]
Temperature (ºC)	27.9 ±1.2	27.8 ± 3	28.8 ±0.3
pH	7.54 ±0.55	7.4 ±0.38	7.6 ±0.43
Glucose (μg/l)	4.22 ±5.7	4.2 ±4.3	1.97 ±1.34
Nitrate (mg/l)	11.25 ±2.4	11.75 ±2.4	9.5 ±3
Ammonium (mg/l)	8.12 ±3.75	6.25 ±1.4	7.5 ±5
Nitrite (mg/l)	ND[c]	ND	ND
Bromide (μM)	1.62 ±0.015	1.64 ±0.03	1.62 ±0.01
Fluorescein (μM)	0.21 ±0.25	0.14 ±0.16	0.18 ±0.36
Alkalinity (mg/l)	745 ±34	710 ±66	720 ±280
Acetate (mM)	ND	ND	ND
Lactate (mM)	ND	ND	ND
Ethanol (mM)	ND	ND	ND
Butanediol (mM)	ND	ND	ND
Biosurfactant (mg/l)	ND	ND	ND
Total heterotrophic bacteria (MPN)	ND	ND	ND
Biosurfactant producers (MPN)	ND	ND	ND

[a]Numbers are averages ± standard deviation of 4 replicates (2 samples collected for each of two wells within a one-week interval).

[b]Numbers are averages ± range of 2 samples collected within a one-week interval.

[c]ND, not detected

Table 4.3. MPN analysis of total and spore-forming heterotrophic bacteria and biosurfactant producers in the injected and the produced fluids[a].

Wells		MPN in the injected fluid[b]		MPN in produced fluid[b]	
		Heterotrophic bacteria	Biosurfactant producers	Heterotrophic bacteria	Biosurfactant producers
Inoculated	Total	1×10^{15} (15±0.01)	4.2×10^{10} (10.3 ±0.6)	4.3×10^{14} (14.6 ±0.4)	--[c]
	Spore formers	3.1×10^{11} (11.2 ±0.6)	3.1×10^{11} (11.2 ±0.6)	2.5×10^{11} (10.3 ±0.2)	2.5×10^{11} (10.3 ±0.2)
Nutrients only treated	Total	2.5×10^{11} (11.5 ±2.3)	3.7×10^{9} (9.6 ±0.6)	2.7×10^{12} (12.1 ±0.8)	--
	Spore formers	--	--	--	--
Negative control	Total	1.4×10^{9} (9.15 ±2.3)	--	5.3×10^{9} (9.7 ±0.11)	--
	Spore formers	--	--	--	--

[a] *Bacillus* biofilm growth medium (BBGM) was used for the MPN analysis.

[b] Numbers in parentheses are average $\log_{10}$total MPN ± standard deviation of 4 independent determinations except for the negative control, where numbers are averages ± range of 2 independent determinations.

[c] --; no growth was detected in the MPN medium.

Although data in Table 4.3 suggest that biosurfactant-producing *Bacillus* species were only present in the produced fluids from inoculated wells but not from nutrients only treated wells, it was essential to eliminate any underestimation of *Bacillus* MPN due to the heat treatment. Plate count broth (PCB) medium modified to contain 5% salt was used for the MPN analysis to select for the halotolerant *Bacillus* species (Table 4.4). Although halotolerant, biosurfactant producers were detected in the produced fluids from nutrients only treated wells, the total MPN of these microorganisms was 53-fold lower than that in the injected fluids from the same wells. On the other hand, MPN data showed that the total MPN of halotolerant, biosurfactant producers in the produced fluids of inoculated wells was about 1.5-times that present in the injected fluids of the same wells.

The MPN data suggest the absence of *Bacillus* species (no spore-forming, biosurfactant-producing microorganisms) and the presence of non-spore-forming, halotolerant, biosurfactant producers in the nutrients only treated wells. The latter were probably introduced into the wells with the treatment (e.g., from the storage tank formation water), but were not maintained. On the other hand, MPN data from inoculated wells indicate the survival of *Bacillus* species (spore-forming, halotolerant, biosurfactant-producing microorganisms) in these wells.

Colonies with the same morphologies as those of the two strains used as the inoculum, *Bacillus* strain RS-1 and *B. subtilis* subsp. *spizizenii* NRRL B-23049, were retrieved on blood agar plates from both the injected and produced fluids of the inoculated wells, but not from the injected and produced fluids of the wells that received only nutrients or the negative control well. The 16S rRNA and *gyrA* gene sequences of

Table 4.4. MPN analysis of halotolerant heterotrophic bacteria and biosurfactant producers in the injected and the produced fluids [a]

Wells	MPN in the injected fluids		MPN in produced fluids	
	Heterotrophic bacteria	Biosurfactant producers	Heterotrophic bacteria	Biosurfactant producers
Inoculated	1.25×10^{12} (12 ±0.3) [b]	1×10^{12} (11.9 ±0.3) [b]	1.8×10^{15} (15 ±0.5) [b]	1.56×10^{12} (12.2 ±0.05) [b]
Nutrients only	1.9×10^{12} (12 ±0.45)	3.3×10^{11} (10.6 ±1.4)	6.5×10^{13} (13.7 ±0.3)	6.2×10^{9} (9.8 ±0.3)
Negative control	3.1×10^{12} (12.5 ±0.4)	2.5×10^{9} (9.4 ±0.4)	1.2×10^{11} (11.1 ±0.04)	-- [c]

[a] Plate count broth supplemented with 5% NaCl was used for the MPN analysis.

[b] Numbers in parentheses are average $\log_{10}$MPN ± standard deviation of 4 independent determinations except for the negative control, where numbers are averages ± range of 2 independent determinations.

[c] --; no growth was detected in the MPN medium

these were 100% similar to *Bacillus* strain RS-1 and *B. subtilis* subsp. *spizizenii* NRRL B-23049.

Culture-independent *gyrA* amplicons were obtained with DNA extracted from produced fluids of the inoculated wells and not from DNA extracted from produced fluids from the wells that received only nutrients or the control well that received brine. The *gyrA* amplicons were not detected with DNA extracted from produced fluids of the inoculated wells collected prior to treatment. The resulting sequences of *gyrA* clones obtained were 99.72% similar to *Bacillus* strain RS-1 and *B. subtilis* subsp. *spizizenii* NRRL B-23049.

Repetitive extragenic palindromic PCR (rep-PCR) reactions (21, 46) on blood agar plates isolates from the injected and produced fluids of the inoculated wells showed patterns similar to those of the inoculum strains, *Bacillus* strain RS-1 and *B. subtilis* subsp. *spizizenii* NRRL B-23049 (Figure 4.1).

Collectively, microbiological and molecular data show that the microorganisms injected into the formation were retrieved in the produced fluids of the inoculated wells.

Lipopeptide biosurfactant production. The presence of the biosurfactant in produced fluids was followed over the time of sampling by using three different methods. Oil spreading technique (33, 49) and surface tension measurement (25) were used to detect the presence of surface-active compounds in the produced fluids from all the wells. Surface activity was observed only in production fluids from the inoculated wells as evidenced by an increase in oil spreading activity and a decrease in surface tension (Figure 4.2A, and 4.2B). No evidence for surface activity was detected

Figure 4.1: Polyacrylamide gel of the Rep-PCR reaction for identification of *Bacillus* strains in the injected and produced fluids from the inoculated wells. DNA was extracted from isolates that had colonies with the same morphology as the two strains used as the inoculum (*Bacillus* strain RS-1 and *Bacillus subtilis* subsp. *spizizenii* NRRL B-23049) and used for the PCR reactions. Lanes 2 and 9 are DNA from *B. licheniformis* and *B. subtilis* subsp. *spizizenii* type strains, respectively. Lanes 3 and 10 are DNA from laboratory-grown *Bacillus* strain RS-1 and *Bacillus subtilis* subsp. *spizizenii* NRRL B-23049, respectively. Lanes 4 and 11 are DNA from *Bacillus* strain RS-1- and *Bacillus subtilis* subsp. *spizizenii*-like isolates, respectively, both obtained from the tanks used for the inoculation. Lanes 5 and 6 are DNA from *Bacillus* strain RS-1-like isolates obtained from the injected fluids for the two inoculated wells, respectively. Lanes 7 and 8 are DNA from *Bacillus* strain RS-1-like isolates obtained from the produced fluids from the two inoculated wells, respectively. Lanes 12 and 13 are DNA from *Bacillus subtilis* subsp. *spizizenii* -like isolates obtained from the injected fluids for the two inoculated wells, respectively. Lanes 14 and 15 are DNA from *Bacillus subtilis* subsp. *spizizenii* -like isolates obtained from the produced fluids from the two inoculated wells, respectively. Lanes 1 and 16 are DNA ladder.

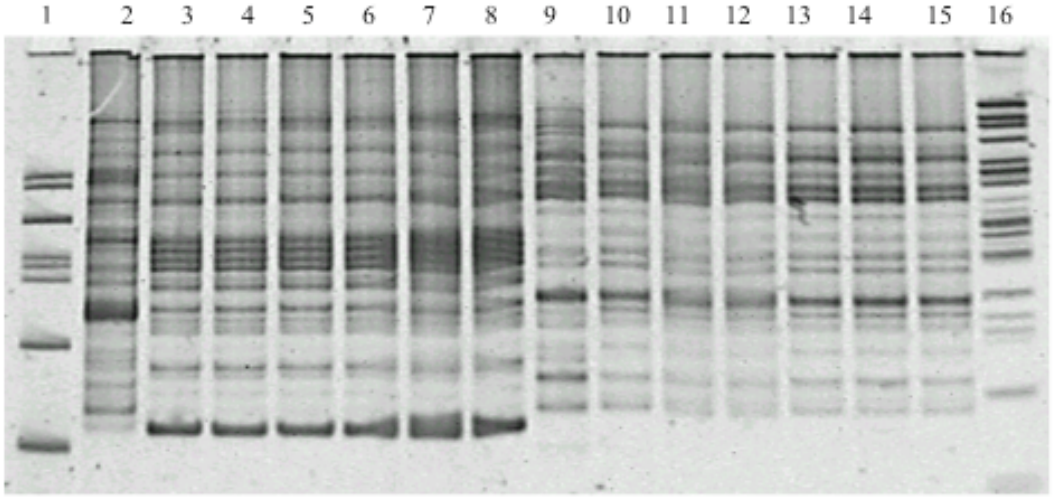
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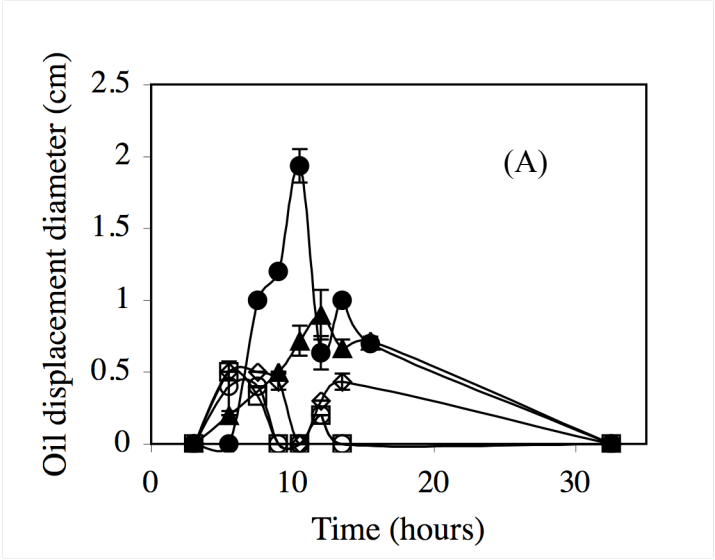
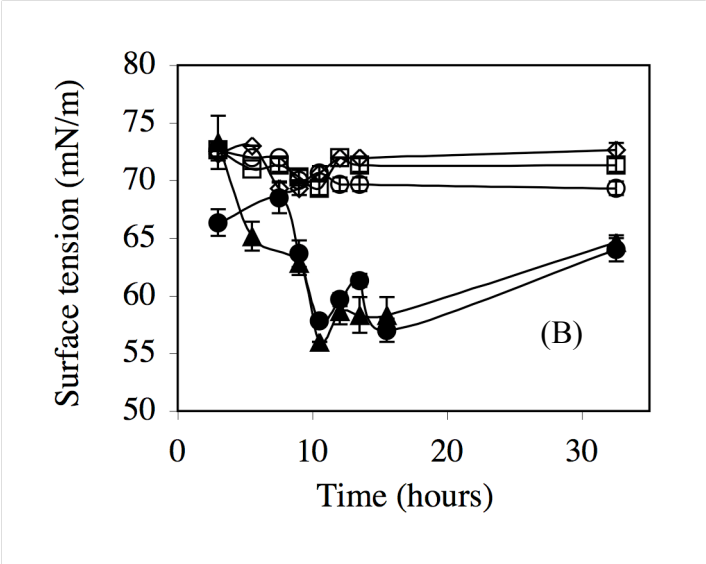
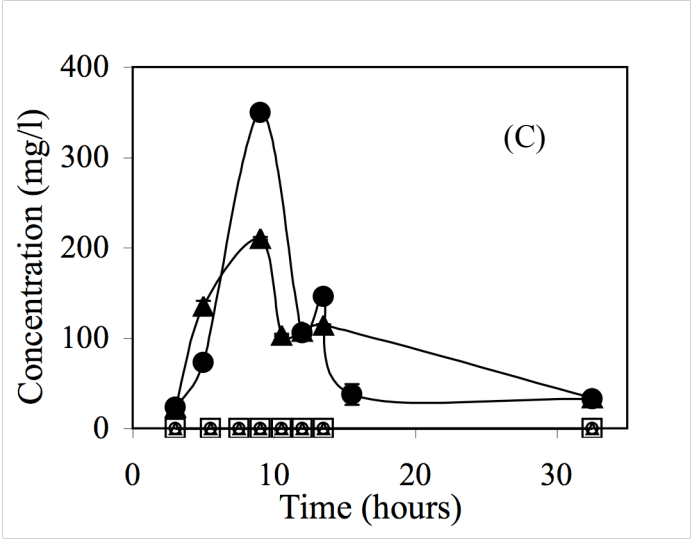
Figure 4.2. Evidence for *in situ* biosurfactant production. X-axis represents the time in hours after the production started from the wells. Closed circles and triangles represent data from the 2 inoculated wells, open squares and open diamonds represent data from the 2 nutrient-treated wells, and open circles represent data from the negative control well. (A) By the oil spreading technique. Error bars represent standard deviations of 3 measurements. (B) By surface tension measurement. Error bars represent standard deviation of 3 measurements. (C) By determination of biosurfactant concentration by high performance liquid chromatography. Error bars represent ranges of duplicate measurements.


Oil displacement diameter (cm)
2.5
2
1.5
1
0.5
0
0
10
20
30
Time (hours)
(A)


Surface tension (mN/m)
80
75
70
65
60
55
50
0
10
20
30
Time (hours)
(B)


Concentration (mg/l)
400
300
200
100
0
0
10
20
30
Time (hours)
(C)

in production fluids from the nutrients only treated wells or the negative control well (Figure 4.2A, and 4.2B). To determine the nature and the concentration of the biosurfactant produced in the inoculated wells, high-pressure liquid chromatography (HPLC) was used (48). The HPLC profile of the samples from the produced fluids of the inoculated wells matched those of the lipopeptide biosurfactants purified from laboratory cultures of the two strains used as inoculum, *Bacillus* strain RS-1 and *B. subtilis* subsp. *spizizenii* NRRL B-23049. The average concentration of the lipopeptide biosurfactant in the produced fluids of inoculated wells was about 90 mg/l (a total amount of 7 moles in ~ 80,000 L of produced water) (Table 4.5, and Figure 4.2C). The maximum concentration was as high as 350 mg/l in the produced fluids from the inoculated wells (Figure 4.2C). These maximum concentrations are more than 20-times higher than the reported critical micelle concentration for lipopeptide biosurfactants (ranging from 10-20 mg/l) (33). HPLC analysis of the produced fluids of the nutrients only treated wells and the negative control wells showed no lipopeptide biosurfactant production.

Glucose utilization and product formation. Glucose was the carbon and energy source in our treatments. While complete glucose utilization did not occur, it was clear that large amounts of glucose were used since only 20-40% of the glucose added to the nutrients-treated wells (both inoculated and un-inoculated) was recovered in the produced fluids and products of microbial metabolism were detected in the produced fluids. Produced fluids from all of the nutrients-treated wells (both inoculated and un-inoculated) showed an increase in alkalinity and carbon dioxide concentration compared to pre-treatment levels, most likely resulting from microbial growth and

Table 4.5. *In situ* mass balance and product formation rates and yields compared to those obtained in laboratory culture.

Products	Inoculated wells[a]		Nutrients only-treated wells[a]		Laboratory culture[a]	
	Moles[b]	Rate (h^{-1})[c]	Moles[b]	Rate (h^{-1})[c]	Moles[b]	Rate (h^{-1})[c]
Acetate	16.6±7	0.045 ±0.004	ND[d]	NA[e]	0.57±0.03	0.04±0.001
Butanediol	67.7±44	0.06±0.007	ND	NA	0.54±0.014	0.04±0.0005
Lactate	39.9±0.9	0.06±0.0002	ND	NA	2.06±0.19	0.06±0.001
Ethanol	902.6±50	0.08±0.001	130±48	0.07 ±0.003	0.39±0.12	0.03±0.007
CO_2	644±113	0.017±0.001	202±76	0.006 ±0.003	11.4±0.56	0.07±0.001
Biosurfactant	6.8±0.67	0.02±0.001	ND	NA	0.11±0.05	0.05±0.009
Cells	7.3 ± 8	0.06 ±0.01	0.26 ±0.15	0.18±0.006	0.12 ±0.17	0.18±0.06

[a] The number of moles of glucose used were 506 ± 118 moles, 360 ± 34 moles, and 4.88 ± 0.0004 moles for the inoculated wells, nutrients only-treated wells, and the laboratory cultures, respectively. Percent carbon recoveries were 106.7 ± 34, 20.7 ±9, and 94.5 ±12.8 for the inoculated wells, nutrients only-treated wells, and the laboratory cultures, respectively.

[b] The numbers are averages ± standard deviation of 4 independent determinations. Values were corrected for adsorption using bromide recovery factor.

[c] The rates were calculated from the equation, $\ln (C_t/C_0) = k \bullet t$, where C_t is the concentration at time t, C_0 is the initial concentration, k is the rate of production, and t (time) was 108 hours. The numbers are averages ± standard deviation of 4 independent determinations.

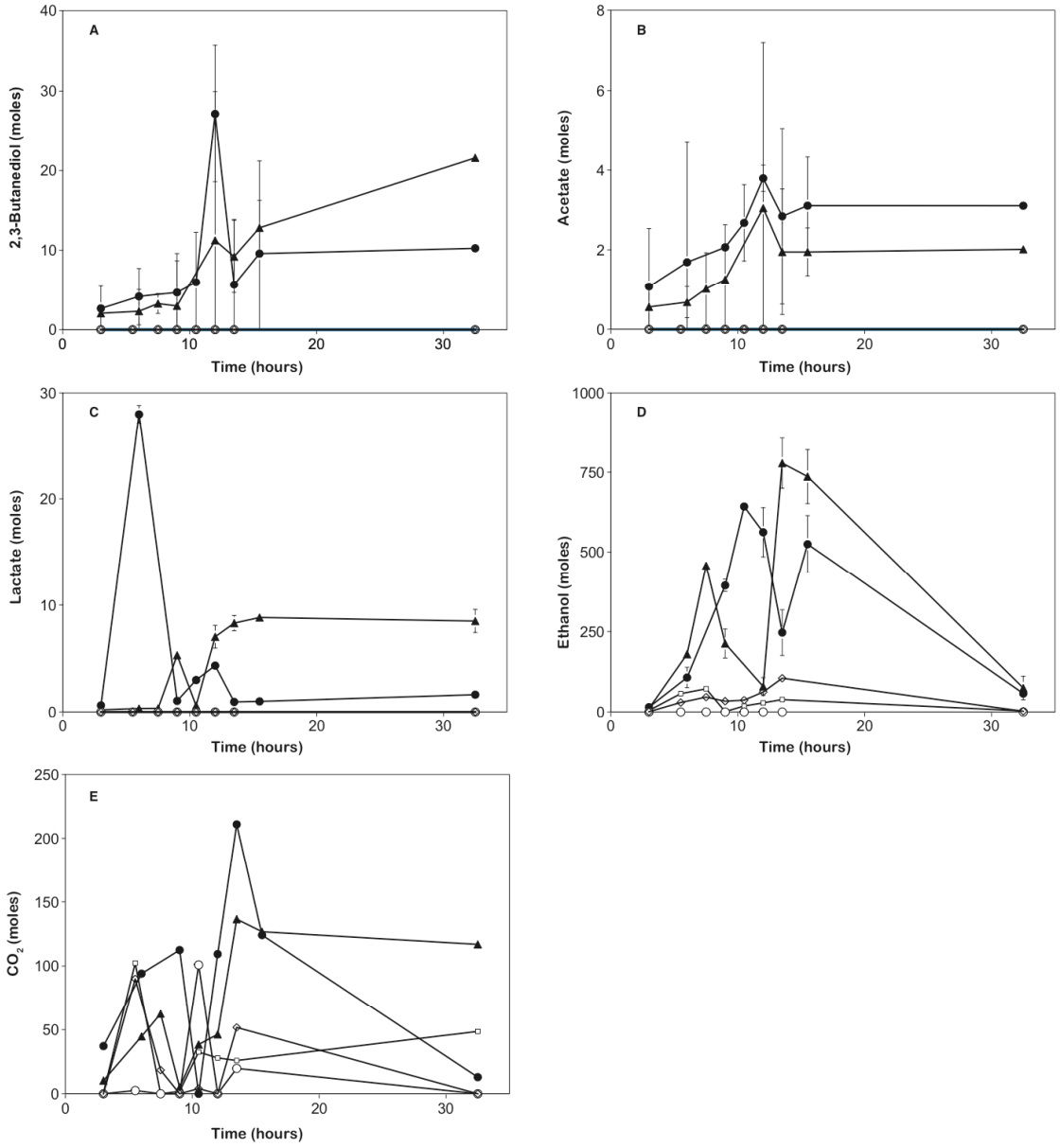
[d] ND: not detected

[e] NA: not applicable.

activity. Produced fluids from the inoculated wells had a much higher CO_2 concentration following treatment compared to that from the nutrients only-treated wells (Table 4.5, Figure 4.3E). In addition to CO_2, acetate, lactate, ethanol, and 2,3 butanediol were detected in the produced fluids of the inoculated wells (Table 4.5, Figure 4.3A-D). The percent carbon recovery was 107 ±34 % for the inoculated wells (Table 4.5). Similar fermentation products from glucose metabolism were obtained by the pure cultures of the strains used as inoculum, *Bacillus* strain RS-1 and *B. subtilis* subsp. *spizizenii* NRRL B-23049, when the strains were individually grown in formation water supplemented with the nutrients used for well treatments (Table 4.5). These data argue that the products detected in the produced fluids of the inoculated wells were products of anaerobic glucose metabolism by the inoculum. Although glucose was partially utilized in the wells that received only nutrients, none of the above fermentation products were detected with the exception of CO_2 and some ethanol (Figure 4.3). We do not know the fate of the remaining glucose carbon in these wells.

The detection of 2,3-butanediol, a product often produced by *Bacillus* species during fermentation (32, 43) and the biosurfactant indicates that we stimulated the microorganisms responsible for biosurfactant production.

Figure 4.3: Total moles of glucose fermentation products in produced fluid from the wells. X-axis represents the time in hours after the production started from the wells. Closed circles and triangles represent data from the 2 inoculated wells, open squares and open diamonds represent data from the 2 nutrient-treated wells, and open circles represent data from the negative control well. (A) 2, 3-butanediol, (B) acetate, (C) lactate, (D) ethanol. and (E) CO_2.


A
2,3-Butanediol (moles)
Time (hours)
B
Acetate (moles)
Time (hours)
C
Lactate (moles)
Time (hours)
D
Ethanol (moles)
Time (hours)
E
CO_2 (moles)
Time (hours)

Discussion

A major concern with MEOR is whether exogenous microorganisms will be metabolically active in the presence of diverse, natural populations of microorganisms that inhabit oil reservoirs (2, 8, 32). Biosurfactant-mediated oil recovery is even more problematical in that a metabolite, e.g., the biosurfactant, not related to the main energy metabolism of the cell must be produced. The data presented in Figures 4.2 and 4.3 and Table 4.5 clearly show that the appropriate metabolism was stimulated in the formation and resulted in production of the biosurfactant and products indicative of *Bacillus* fermentation (45). The MPN data indicate that microorganisms physiologically similar to those used as the inoculum (halotolerant, spore-forming biosurfactant producers) were present in high numbers in the produced fluids from the inoculated wells after the incubation period. Molecular characterization of the isolates obtained from produced fluids from the inoculated wells clearly showed that the same strains used in the inoculum were retrieved from the produced fluids of the inoculated wells. These data provide clear evidence that biosurfactant-mediated oil recovery is technically feasible.

Even though microorganisms exist in the reservoir (as evidenced by growth and glucose utilization in the wells that received only nutrients), the indigenous microorganisms did not prevent the strains used as the inoculum from establishing and metabolizing in the reservoir. The success of the inoculation procedure might be because the type and amount of nutrients used were more favorable for *Bacillus* species compared to indigenous microorganisms. Pre-treatment sampling did not detect the presence of microorganisms in produced fluids from the wells that were

capable of growing in the various media used for enumeration (Table 4.2). So, it is possible that the injection of nutrients created a niche that allowed the injected strains to be metabolically active. The MPN analysis did not indicate that the biosurfactant producers in the inoculated wells grew since there was not a significant difference in the total MPN of the spore-forming biosurfactant producers (Table 4.3) or the halotolerant biosurfactant producers (Table 4.4) present in the injected fluids compared to the produced fluids. The lack of growth may have been due to a nutrient limitation. Nitrate was used as the nitrogen source and limiting amounts of nitrate were added to the treated wells to shift the carbon flow from cell mass production to a secondary metabolite production. Previous studies show that nitrogen limitation is associated with an increase in biosurfactant production (11).

Sand-packed column studies have shown oil recoveries up to 95% occur when the columns are treated with lipopeptide biosurfactants (3, 27, 36, 38). Recent studies have shown that at least 11 mg/l of a lipopeptide biosurfactant is required to mobilize oil from sandstone cores and recoveries as high as 40% of entrapped oil were obtained with as little as 38 mg/l of the lipopeptide biosurfactant (20, 28). In the current field test, the average concentration of lipopeptide biosurfactant in the produced fluids of inoculated wells was about 90 mg/l. This concentration is ~ 9-times the minimum concentration required to mobilize entrapped oil from sandstone cores (28). These results showed that *in situ* lipopeptide biosurfactant production indeed meets this important engineering criterion.

Previous laboratory studies that used both sandstone cores and sand-packed columns suggested that 2.2 ml of oil could be recovered per mg of a lipopeptide

biosurfactant (29). Based on this information, the seven moles of lipopeptide biosurfactant recovered from the produced fluids of the two inoculated wells could recover approximately 16 m^3 (100 barrels) of oil. The total material expenses to produce seven moles of biosurfactant were about $164 ($82 per well). The cost of the *in situ* biosurfactant production process could be as low as $10 per m^3 ($1.6 per barrel). Since the main goal of the study was to test whether *in situ* biosurfactant production is possible, the volume of the reservoir that was contacted was small and thus significant amounts of additional oil were not expected. However, the company followed oil production before and after treatment and these data can be used to judge the effectiveness of the process. The lease that had the two inoculated wells, one of the nutrient only-treated wells and several other wells showed an average increase of oil production of one barrel of oil per day compared to the oil production rate before treatment. This increase in oil production was maintained for a period of 7 weeks following treatment. On the other hand, oil production rate of the other well that received only nutrients slightly decreased during the 7 weeks after treatment compared to pre-treatment oil production rates. Although the oil production data suggest that *in situ* process was not as efficient and cost effective as predicted from laboratory data, the data still argue for the cost-effectiveness of MEOR compared to the current price of oil (> $65/ barrel). However, more definitive results can be obtained if the size of the treatment is scaled up.

Our data show that *in situ* biosurfactant production is possible and occurred in amounts exceeding the engineering criterion to mobilize oil from sandstone cores. This work also provides essential data for modeling MEOR processes *in situ* (Table

4.5) including growth rates (0.06 ± 0.01 h^{-1}), carbon balance (107 ± 34 %), biosurfactant production rates (0.02 ± 0.001 h^{-1}), and biosurfactant yields (0.015 ± 0.001 mole biosurfactant/ mole glucose). These data can be used to study computationally microbial activity in subsurface environments including petroleum reservoirs. We should note that this is the first time that an *in situ* carbon/mass balance has been obtained for any MEOR process. Overall, the work emphasizes technical feasibility and cost-effectiveness of MEOR.

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