Development of Microorganisms with Improved Transport and Biosurfactant Activity for Enhanced Oil Recovery

Annual Report

Report Start Date: June 1, 2002

Report End Date: May 31, 2004

M. J. McInerney, N. Youssef, T. Fincher, S. K. Maudgalya, M. J. Folmsbee, R. Knapp and
D. Nagle

Date of Report: May 31, 2004

DE-FC-02NT15321 R 02

Department of Botany and Microbiology and Department of Petroleum Engineering
University of Oklahoma
770 Van Vleet Oval
Norman, OK 73019-0245

Disclaimer

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, expressed or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

Table of Contents

<i>Development of Improved Anaerobic Biosurfactant Production for the Purpose of Enhanced Oil Recovery</i>	<i>1</i>
<i>Disclaimer.....</i>	<i>2</i>
<i>Table of Contents</i>	<i>3</i>
<i>List of Figures</i>	<i>6</i>
<i>List of Tables</i>	<i>8</i>
<i>Abstract.....</i>	<i>9</i>
<i>Abstract.....</i>	<i>9</i>
<i>Executive Summary</i>	<i>11</i>
<i>Chapter 1. Screening Diverse Microorganisms for Anaerobic Growth and Biosurfactant Production and the Relationship of Structure to Function for Lipopeptide Biosurfactants</i>	<i>18</i>
Abstract	18
Introduction	19
Materials and Methods	21
Bacterial Strains	21
Media	21
Screening for Biosurfactant Production	22
Biosurfactant Extraction and Purification	22
Amino acid Analysis	23
Fatty acid Analysis	24
Results.....	25
Screening Diverse Microorganisms for Anaerobic Growth and Biosurfactant Production	25
Evaluation of a New Method of Biosurfactant Extraction and Purification.....	25
Biosurfactant Yield and its Relation to Activity	26
Structure-activity Relationship Study	28
Enhancing Biosurfactant Activity	29
Discussion	31

Screening Strains for MEOR	32
Purification of Biosurfactant from Superior Strains	33
Structure-function Variation in Biosurfactants	33
Altering Growth Conditions to Favor Highly Active Structural Variants.....	34
References	35
<i>Chapter 2. Efficacy of Biosurfactant Mixtures.....</i>	38
Abstract	38
Introduction	39
Materials and methods.....	40
Bacterial Strains	40
Media	41
Measurement of Surface Tension and Determining CMD^{-1}	41
Preparing Mixtures of Different Cultures	42
Surface Tension and CMD^{-1} of Biosurfactant Mixtures.....	43
Purification of Biosurfactants	43
Interfacial Tension Measurement.....	43
Results.....	44
Surface Tension and CMD^{-1} Values of Individual Strains	44
Surface Tension and CMD^{-1} Values of Biosurfactant Mixtures.....	46
Surface and Interfacial Tensions of Partially Purified Biosurfactant	52
Discussion	55
References	56
<i>Chapter 3. Microbial Strains with Improved Transport Ability through Model Porous Systems.</i>	57
Abstract	57
Introduction	57
Materials and Methods.	60
Preparation of Spores	60
Preparation of Sand-Packed Columns.....	60
Transport Experiments	61
Plate Count Methods	63
Rep-PCR	63

Results.....	63
Rep-PCR	70
Discussion	72
References	75
 <i>Chapter 4. Tertiary oil recovery by the JF-2 biosurfactant from Berea sandstone cores and the development of a mathematical model relating oil-water interfacial tension to the concentration of the JF-2 biosurfactant.</i>	
Abstract	79
Introduction	80
Materials and Methods	82
Medium and Cultivation	82
Core Flooding	82
Establishment of a Capillary Desaturation Curve for Berea Sandstone Cores.....	83
Interfacial Tension (IFT) Measurements.....	84
Results.....	86
Core Flood Experiments	86
Capillary Desaturation	86
Analysis of Relationship Between Fractional Oil Recovery and Surfactant Concentration	89
Mathematical Model Relating Oil-water Interfacial Tension to JF-2 Biosurfactant Concentration.....	91
Mathematical Model	94
Model Prediction.....	96
Discussion	99
References	100

List of Figures

Figure 1.1 Biosurfactant extraction and purification scheme.	27
Figure 1.2. Schematic representation of the developed TLC plate.....	28
Figure 1.3. Correlation between biosurfactant yield (mg biosurfactant / g dry weight) and biosurfactant activity (expressed as the diameter of clear zones obtained when 10 µl of the surface active fraction eluted from the TLC plate and dissolved in 250µl distilled water, was tested using the oil spreading technique).	28
Figure 1.4. Relation between the specific biosurfactant activity (cm of the clear zone/ 10µg of purified biosurfactant) to the ratio of the peak areas of the two C14 isomers of the fatty acid portion of the biosurfactant.	32
Figure 2.1a. Surface tension values of different dilutions of group A strains (<i>Bacillus mojavensis</i>).	45
Figure 2.1b. Surface tension values of different dilutions of group B strains (<i>B. subtilis</i> subsp. <i>subtilis</i>).	45
Figure 2.1c. Surface tension values of different dilutions of group D strains (<i>Bacillus subtilis</i> subsp. <i>spizizensis</i>).	46
Figure 2.2a. Surface tension values of a mixture of biosurfactants from Group A strains ROB-2, ROG-4, and JF-2 compared to each individual culture.	48
Figure 2.2b. Surface tension values of a mixed biosurfactants from Group A strains ROB-2, ROQQ-2, and JF-2 compared to each individual culture.	49
Figure 2.3. Surface tension values of the mixture of biosurfactants from strains T89-42 and ROGG-2 of group B compared to each individual culture.	49
Figure 2.4a. Surface tension values of a mixture of biosurfactants from Group D strains T89-3 and CL1-14 with and without the JF-2 biosurfactant compared to the individual cultures.	51
Figure 2.4b. Surface tension values of a mixture of biosurfactants from Group D strains T89-3 and CL1-21 with JF -2 compared to each individual culture.	51
Figure 2.5a. Surface tension values of various concentrations of the partially purified biosurfactant of <i>Bacillus mojavensis</i> strain ROB-2. CMC is 17.4 g/l.	53
Figure 2.5b. Surface tension values of various concentrations of the partially purified biosurfactant of <i>Bacillus mojavensis</i> strain ROG-4. CMC is 7.74 g/l.	54

Figure 2.5c. Surface tension values of various concentrations of the partially purified biosurfactant of <i>Bacillus mojavensis</i> strain T89-14. CMC is 17.4 g/l.	54
Figure 3.1. Elution pattern of JF-2 from sand columns. Open and closed circles are duplicate columns showing EPD values for each effluent fraction. Pore volumes based on Blue Dextran ellutant was 12mls.	66
Figure 3.2. Cumulative elution pattern of JF-2 from sand columns. Pore volumes are based on Blue Dextran ellutant in 12mls.	66
Figure 3.3. Effluent pattern of JF-2(3) (early breakthrough fraction of JF-2) from sand packed columns. Fraction 3 from Figure 3.1 was sporulated and passed through duplicate columns. Pore volume of the columns ranged from 11-15ml.	67
Figure 3.4. Elution pattern of ROB-2 through sand packed columns. Open and closed circles are duplicate columns showing EPD values from each fraction.....	68
Figure 3.5. Elution pattern of early breakthrough fraction, ROB-2 (4).....	69
Figure 3.6. Cumulative elution pattern of ROB-2 from sand columns. Pore volumes were based on dry versus wet weights.....	70
Figure 3.7. Elution pattern of C-9 through sand columns.....	71
Pore volumes range from 10-14mls.....	71
Figure 3.8. Elution profile of the early breakthrough fraction of C-9, C-9 (5). Pore volume was not measured for these columns.	71
Figure 4.1. Berea sandstone capillary desaturation curve.....	87
Figure 4.2. Comparison of core data with the capillary desaturation curve.	88
Figure 4.3. Plot of tertiary oil recovery from cores (before and after removing the polymer contribution) and sand-packed columns as a function of biosurfactant concentration. .	90
Figure 4.4. The relationship between interfacial tension and biosurfactant concentration. ...	94
Figure 4.5. Model parameters used to predict IFT reductions from biosurfactant concentration.....	95
Figure 4.6. Comparison between model prediction (line) and laboratory measurements (squares).....	98

List of Tables

Table 1.1. List of references and strains used in this study.	21
Table 1.2. Growth and biosurfactant production by different strains tested in this study.	25
Table 1.3. Relative activities of different strains compared to JF-2 biosurfactant activity ...	26
Table 1.4. Comparison of biosurfactant activity and amino acid and fatty acid composition of different biosurfactants	30
Table 1.5. Comparison of the fatty acid composition of <i>Bacillus subtilis</i> subsp. <i>subtilis</i> strain T89-42 biosurfactant in presence and absence of valine in the medium.	31
Table 2.1. Names, taxonomic status, and surface activity of the strains used in this study. .	40
Table 2.2. Dilutions prepared for CMD ⁻¹ measurement.....	41
Table 2.3. Surface tension and CMD ⁻¹ values of the 15 strains used in this study.	47
Table 2.4. Comparison of surface tension obtained with mixtures to the average value of the individual strains. CMD ⁻¹ is also shown for the mixtures.	50
Table 2.5. Interfacial tensions measured for different purified biosurfactants.	52
Table 3.1. Summary of plate counts and percent recoveries for each strain applied to a column.....	64
Table 4.1. Summary of oil recovery data at biosurfactant concentrations above and below the critical micelle concentration. Corrected percent residual oil recovery is corrected for the amount of residual oil recovered by polymer alone.....	84
Table 4.2. Petrophysical properties of the Berea sandstone cores.....	85
Table 4.3. Residual oil saturation and capillary Number with increase flow rates	87
Table 4.4. Residual oil recoveries from sandstone and sand-packed model systems with different biosurfactant concentrations.....	91
Table 4.5. Interfacial tension values at different biosurfactant concentrations.	92
Table 4.6. Parameters used for the model that relates IFT to biosurfactant concentration.....	95
Table 4.7. Parameter values for biosurfactant concentrations between 0.0 and 41.0 mg/l.....	97
Table 4.8. Parameter values for concentrations between 41.0 and 58.0 ppm.....	97

Abstract

Diverse microorganisms were screened for biosurfactant production and anaerobic growth at elevated salt concentrations to obtain candidates most suitable for microbial oil recovery. Seventy percent of the 205 strains tested, mostly strains of *Bacillus mojavensis*, *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus sonorensis*, produced biosurfactants aerobically and 41% of the strains had biosurfactant activity greater than *Bacillus mojavensis* JF-2, the current candidate for oil recovery. Biosurfactant activity varied with the percentage of the 3-hydroxy-tetradecanoate isomers in the fatty acid portion of the biosurfactant. Changing the medium composition by incorporation of different precursors of 3-hydroxy tetradecanoate increased the activity of biosurfactant. The surface tension and critical micelle concentration of 15 different, biosurfactant-producing *Bacillus* strains was determined individually and in combination with other biosurfactants. Some biosurfactant mixtures were found to have synergistic effect on surface tension (e.g. surface tension was lowered from 41 to 31 mN/m in some cases) while others had a synergistic effect on CMD-1 values. We compared the transport abilities of spores from three *Bacillus* strains using a model porous system to study spore recovery and transport. Sand-packed columns were used to select for spores or cells with the best transport abilities through brine-saturated sand. Spores of *Bacillus mojavensis* strains JF-2 and ROB-2 and a natural recombinant, strain C-9, transported through sand at very high efficiencies. The earliest cells/spores that emerged from the column were re-grown, allowed to sporulate, and applied to a second column. This procedure greatly enhanced the transport of strain C-9. Spores with enhanced transport abilities can be easily obtained and that the preparation of inocula for use in MEOR is feasible.

Tertiary oil recovery experiments showed that 10 to 40 mg/l of JF-2 biosurfactant in the presence of 0.1 mM 2,3-butanediol and 1 g/l of partially hydrolyzed polyacrylamide (PHPA) recovered 10-40% of residual oil from Berea sandstone cores. When PHPA was used alone, about 10% of the residual oil was recovered. Interfacial tension (IFT) decreased in a stepwise manner as biosurfactant concentration increased with marked reductions in IFT occurring at biosurfactant concentrations of 10 and 40 mg/l. When the biosurfactant concentration was greater than 10 mg/l, residual oil recovery linearly increased with biosurfactant concentration. A mathematical model that relates oil recovery to biosurfactant concentration was modified to include the stepwise changes in IFT as biosurfactant concentrations changes. This model adequately predicted the experimentally observed changes in IFT as a function of biosurfactant concentration. Our work shows that 1) diverse microorganisms produce biosurfactants, 2) nutrient manipulation may provide a mechanism to increase biosurfactant activity, 3) biosurfactant concentrations in excess of the critical micelle concentration recover substantial amounts of residual oil, and 4) equations that describe the effect of the biosurfactant on IFT adequately predict residual oil recovery in sandstone cores.

Executive Summary

The elevated salinities and lack of oxygen in most mid-continent oil reservoirs are critical environmental factors that govern the type of microorganism used for biosurfactant-mediated oil recovery. We screened diverse microorganisms for biosurfactant production and anaerobic growth at elevated salt concentrations to obtain candidates most suitable for microbial oil recovery. We tested 205 strains, mostly strains of *Bacillus mojavensis*, *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus sonorensis*, for aerobic and anaerobic growth in 5% salt medium and biosurfactant production. All strains grew aerobically with 5% salt and 145 of these strains (70%) produced a biosurfactant. Eighty-seven strains, 40% of those tested, mostly belonging to *B. subtilis* subsp. *subtilis* and *B. subtilis* subsp. *spizizenii*, had biosurfactant activity greater than *Bacillus mojavensis* JF-2, the current candidate for oil recovery. Some strains maintained biosurfactant activity after 14 days of incubation compared to JF-2, which lost 50% of its biosurfactant activity after 7 days. Thirty-three strains grew anaerobically in the 5% salt medium. The fact that we found that biosurfactant-producing microbes can be readily isolated from uncontaminated, undisturbed arid soils as well as oil field brines argues that many oil field are likely to contain microorganisms that produce biosurfactants.

In order to optimize the activity of the biosurfactants, it is important to understand what portions of the biosurfactant molecule are most critical for its activity. We studied the relationship between biosurfactant structure and activity with a number of lipopeptide biosurfactants produced by the above *Bacillus* species. A new method of extraction and purification for lipopeptide biosurfactants was developed, which involved ammonium sulfate precipitation, solvent extraction, and thin layer chromatography. Not surprisingly, we found

that biosurfactant activity increased with increasing concentration of the lipopeptide biosurfactant. In addition, we also found that the molecular structure of the biosurfactant (e.g., amino acid and fatty acid composition) affected activity. When biosurfactants from different bacilli were tested at the same concentration, biosurfactant activity varied with the percentage of the 3-hydroxy-tetradecanoate isomers in the fatty acid portion of the biosurfactant. Changing the medium composition by incorporation of different precursors of 3-hydroxy tetradecanoate increased the activity of biosurfactant. Thus, by understanding how the structure of the biosurfactant affects activity, we were able to develop simple approach to enhance biosurfactant activity by nutrient manipulation. Our work shows that diverse microorganisms produce biosurfactants and that nutrient manipulation may provide a mechanism to increase biosurfactant activity for more efficient oil recovery.

MEOR (microbially enhanced oil recovery) depends on the use of biosurfactants to mobilize residual oil in low production or depleted reservoirs. In order to optimize the surface activity of biosurfactants, we hypothesized that mixtures of biosurfactants with diverse structures will generate lower surface tensions compared to individual biosurfactants. In this study, the surface tension for 15 different *Bacillus* strains that are known to be surface active was measured both individually and in combination with other biosurfactants. Surface tension and CMD-1 values (critical micelle dilution defined as the reciprocal of the biosurfactant dilution at which a sharp increase in surface tension is observed) were compared to assess synergistic effects of the mixtures. Some biosurfactant mixtures were found to have a synergistic effect on surface tension (e.g. surface tension was lowered from 41 to 31 mN/m in some cases) while others had a synergistic effect on CMD-1 values. Since most oil reservoirs contain diverse microorganisms, the stimulation of biosurfactant production in the reservoir

will likely result in the production of several different kinds of biosurfactants. While the presence of numerous microorganisms that could potentially compete for the injected nutrient has been viewed as a detriment to the development of MEOR, it may in fact be a benefit by allowing the production of diverse biosurfactants that may act synergistically.

We know that oil reservoirs contain active microbial communities. However, we do not know the prevalence of biosurfactant-producing microorganisms in oil fields. Thus, it is likely that the injection of biosurfactant-producing microorganisms will be needed for some reservoirs. Cells of the injected microorganisms must be able to move through the porous matrix at high efficiency. *Bacillus* species have been widely used as model organisms during MEOR research. An important characteristic of *Bacillus* species is their ability to produce spores. Spores are essential for MEOR research because of their small size compared to vegetative cells, their ability to withstand harsh environmental conditions and their increased transport ability. The objective of our study was to obtain biosurfactant-producing strains with improved transport abilities through porous materials. We compared the transport abilities of spores from three *Bacillus* strains using a model porous system to study spore recovery and transport. Sand-packed columns were used to select for spores or cells with the best transport abilities through brine-saturated sand. Spores of *Bacillus mojavensis* strains JF-2 and ROB-2 and a natural recombinant strain C-9 transported through sand at very high efficiencies (almost complete recovery of the injected spores within one to two pore volumes). The earliest cells/spores that emerged from the column were re-grown, allowed to sporulate, and applied to a second column to determine whether spores or vegetative cells had enhanced transport properties. This procedure greatly enhanced the transport of strain C-9. Our data show that

spores with enhanced transport abilities can be early obtained and that the preparation of inocula for use in MEOR is practical.

A critical piece of information that must be obtained for MEOR to be effective is the relationship between oil recovery and biosurfactant concentration. How much residual oil can be recovered per unit amount of biosurfactant? Will residual oil recovery increase linearly with increasing biosurfactant concentrations or is this relationship more complex? Tertiary oil recovery experiments showed that biosurfactant solutions with concentrations ranging from 10 to 40 mg/l in the presence of 0.1 mM 2,3-butanediol and 1 g/l of partially hydrolyzed polyacrylamide (PHPA) recovered 10-40% of residual oil from Berea sandstone cores. When PHPA was used alone, about 10% of the residual oil was recovered. Thus, about 10% of the residual oil recovered in these experiments was due to the increase in viscosity of the displacing fluid. The remainder of the recovered oil was due to the effect of the JF-2 biosurfactant on interfacial tension between oil and the displacing aqueous phase. The relationship between interfacial tension (IFT) reduction and biosurfactant concentration was defined. Little or no oil was recovered at biosurfactant concentrations below the critical micelle concentration (CMC) (about 10 mg/l). At concentrations lower than the CMC, IFT values were high. At biosurfactant concentrations from 10 to 40 mg/l, the IFT was 1 mN/m. As the biosurfactant concentration increased beyond 40 mg/l, IFT decreased to around 0.1 mN/m. At biosurfactant concentrations in excess of 10 mg/l, residual oil recovery was linearly related to biosurfactant concentration. A mathematical model that relates oil recovery to biosurfactant concentration was modified to include the stepwise changes in IFT as biosurfactant concentrations changes. This model adequately predicted the experimentally observed changes in IFT as a function of biosurfactant concentration.

We have met the milestones established for this period of the grant. Last year, we reported on the component present in Proteose Peptone that was required for anaerobic growth of *Bacillus mojavensis* strain JF-2. This component was deoxyribonucleic acid or its precursors, deoxyribonucleosides. We have a paper in press on this aspect of the project and will submit a topical report under a separate cover in the next two weeks. We have three strains of *Bacillus mojavensis* that exhibit enhanced transport abilities through porous materials. Additional work is needed to understand why these strains have high transport abilities and will submit a topic report on this aspect soon. We have studied the relationship of biosurfactant concentration to interfacial tension reductions, which reported on last year. We have used this information and additional data on residual oil recoveries from sandstone as a function of biosurfactant concentration to develop a mathematical relationship for interfacial tension reduction by biosurfactants. This is discussed in this report and topic report that integrates the work on biosurfactant concentration, interfacial tension, residual oil recovery and model development will be submitted shortly. The code for the model and instruction manual is submitted separately. We have begun our work on using biosurfactant mixtures (reported herein) and natural recombination to obtain improved strains (reported last year). In this report, we show that the properties of a number of strains with improved biosurfactant activity.

We have published three papers and have one paper in press on our work this year:

S. Maudgalya, M. J. McInerney, R. M. Knapp, D. P. Nagle and M. J. Folmsbee. 2004, Development of bio-surfactant based microbial enhanced oil recovery procedure. SPE 89473. Society for Petroleum Engineers, Richardson, TX.

N. H. Youssef, K. E. Duncan, D. P. Nagle, K. N. Savage, R. M. Knapp, and M. J. McInerney. 2004. Comparison of methods to detect biosurfactant production by diverse microorganisms. *J. Microbiol. Meth.* 56: 339-347.

S. Maudgalya, M. M. Folmsbee, R. M. Knapp, D. P. Nagle, and M. J. McInerney. 2003. Significant mobilization of entrapped hydrocarbon using biosurfactants with viscosity control and low molecular weight alcohol. Second International Conference of Petroleum Biotechnology, November 5-7, Instituto Mexicano del Petróleo, Mexico City, Mexico, 7 pages. (Proceedings on CD; ISBN 968-489-018-4).

M. Folmsbee, M. J. McInerney, and D. P. Nagle. 2004. Anaerobic growth of *Bacillus mojavensis* JF-2 and three other *Bacillus* strains requires deoxyribonucleosides or DNA. *Appl. Environ. Microbiol.* in press.

We presented the six papers at national and international meetings:

S. Maudgalya, M. M. Folmsbee, R. M. Knapp, D. P. Nagle, and M. J. McInerney. 2003. Significant mobilization of entrapped hydrocarbon using biosurfactants with viscosity control and low molecular weight alcohol. Second International Conference of Petroleum Biotechnology, November 5-7, Instituto Mexicano del Petróleo, Mexico City, Mexico, 7 pages. (Proceedings on CD; ISBN 968-489-018-4).

S. Maudgalya, M. J. McInerney, R. M. Knapp, D. P. Nagle, and M. M. Folmsbee. 2004. Development of bio-surfactant based microbial enhanced oil recovery procedure. SPE/DOE Fourteenth Symposium on Improved Oil Recovery, Tulsa, OK, April, 17-21, SPE 89473.

N. H. Youssef, K. E. Duncan, D. P. Nagle, and M. J. McInerney. 2004. Screening diverse microorganisms for biosurfactant production. Presented at the General Meeting of the American Society for Microbiology, New Orleans, LA, May 23-27, 2004.

S. Maudgalya, M. M. Folmsbee, R. M. Knapp, D. P. Nagle, and M. J. McInerney. 2004. Significant mobilization of entrapped hydrocarbons using the biosurfactant produced by *Bacillus mojavensis* strain JF-2. Presented at the General Meeting of the American Society for Microbiology, New Orleans, LA, May 23-27, 2004.

M. J. Folmsbee, M. J. McInerney, D. P. Nagle, and K. E. Duncan. *Bacillus subtilis* strain 168 requires deoxyribonucleosides or DNA for growth in defined medium under strict anaerobic conditions. Presented at the General Meeting of the American Society for Microbiology, New Orleans, LA, May 23-27, 2004.

One student completed her Ph. D. degree this year:

M. J. Folmsbee. 2004. *Bacillus mojavensis* and *Bacillus subtilis* require DNA or deoxyribonucleosides for anaerobic growth. Ph. D. Dissertation, University of Oklahoma, Norman, OK.

Chapter 1. Screening Diverse Microorganisms for Anaerobic Growth and Biosurfactant Production and the Relationship of Structure to Function for Lipopeptide Biosurfactants

ABSTRACT

Diverse microorganisms were screened for biosurfactant production and anaerobic growth at elevated salt concentrations to obtain candidates most suitable for microbial oil recovery. We tested 205 strains, mostly strains of *Bacillus mojavensis*, *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus sonorensis*, for aerobic and anaerobic growth in 5% salt medium and biosurfactant production. Biosurfactant production was tested by using the drop collapse and oil spreading techniques. All strains grew aerobically with 5% salt and 145 of these strains produced a biosurfactant. Eighty-seven strains, mostly belonging to *B. subtilis* subsp. *subtilis* and *B. subtilis* subsp. *spizizenii*, showed biosurfactant activity 1.25 to 2.5 times greater than *Bacillus mojavensis* JF-2, the current candidate for oil recovery. Some strains maintained biosurfactant activity after 14 days of incubation compared to JF-2, which lost 50% of its biosurfactant activity after 7 days. Thirty-three strains grew anaerobically in the 5% salt medium. In order to optimize the activity of the biosurfactants, the relationship between biosurfactant structure and activity was studied with a number of lipopeptide biosurfactants produced by the above *Bacillus* species. A new method of extraction and purification for lipopeptide biosurfactants was developed, which involved ammonium sulfate precipitation, solvent extraction, and thin layer chromatography. Biosurfactant activity increased with increasing concentration of the lipopeptide biosurfactant. The molecular structure of the

biosurfactant (e.g., amino acid and fatty acid composition) also affected activity. When biosurfactants from different *Bacilli* were tested at the same concentration, biosurfactant activity varied with the percentage of the 3-hydroxy-tetradecanoate isomers in the fatty acid portion of the biosurfactant. Changing the medium composition by incorporation of different precursors of 3-hydroxy tetradecanoate increased the activity of biosurfactant. Our work shows that diverse microorganisms produce biosurfactants and that nutrient manipulation may provide a mechanism to increase biosurfactant activity for more efficient oil recovery.

INTRODUCTION

Biosurfactants are a diverse group of surface active, chemical compounds that are produced by a wide variety of microorganisms (4). Having both polar and non-polar domains, biosurfactants are able to partition at the water/ air or water/oil interfaces and thus lower the interfacial or surface tension (4, 5, 11). Such properties make them good candidates for enhanced oil recovery (EOR) (1, 23). The microbial production of biosurfactants is a promising approach to recover residual oil in low production or depleted oil reservoirs.

Candidate microorganisms for microbially enhanced oil recovery should grow and produce their biosurfactant under harsh conditions encountered in oil reservoirs (8). Key conditions that must be considered are high temperature, high salinity and low oxygen tension. *Bacillus mojavensis* strain JF-2 is capable of producing an effective biosurfactant under these conditions (14, 15), but most of the activity was lost after extended incubations. In addition, complex nutrients are required for its anaerobic growth (10).

We hypothesize that diverse microorganisms exist that may be better candidates for microbial oil recovery. A statistical study was conducted to decide on the best method to screen

for biosurfactant production (26). Comparison of the oil spreading technique, the drop collapse method and blood agar lysis to surface tension measurements showed that the oil spreading technique was the most reliable method to screen for biosurfactant production (26). In an attempt to find a better candidate for microbial enhanced oil recovery, 205 strains, mostly *Bacillus* strains, were screened for anaerobic growth and stable biosurfactant production by using the oil spreading technique (20, 26). One hundred and forty five strains, mostly belonging to *Bacillus subtilis* subsp. *subtilis* and *B. subtilis* subsp. *spizizenii*, were capable of biosurfactant production under aerobic conditions (19). In addition, biosurfactant-producing bacteria have been detected in a number of undisturbed and contaminated soils indicating that such organisms are widely distributed in nature (7).

Most lipopeptide biosurfactants have been shown to have a structure similar to that of surfactin, the biosurfactant produced by *Bacillus subtilis* (2, 12, 16). Surfactin, is a cyclic lipopeptide with β -hydroxy fatty acids (with 13 and 15 carbons) linked to a heptapeptide (L-Glu->L-Leu->-D-leu->L-Val->L-Asp->D-Leu->L-Leu) (2, 12). Bodouret *al.* (7) stated that if two biosurfactants were produced under the same growth conditions and had different surface activity, then they have different structures. The structures might be markedly different if the biosurfactant-producing microorganisms belong to different genera or species, or the differences may be minor if the organisms belong to the same species (7).

An interesting observation from the present study was that biosurfactant activity varied markedly among the strains tested. We hypothesized that high biosurfactant activity could be due to either a high biosurfactant concentration, or a difference in the structure of the biosurfactant. One challenge was to develop a method of biosurfactant extraction and purification that recovered most of the biosurfactant activity produced by the microorganisms.

In this study, a new method of biosurfactant extraction and purification was developed which allows us to study the relationship between structure and activity for a number of lipopeptide biosurfactants produced by *Bacillus* species.

MATERIALS AND METHODS

Bacterial Strains

Table 1.1 shows the taxonomic classification and number of strains used in this study.

Table 1.1. List of references and strains used in this study.

Species	Number of strains	Reference
<i>Bacillus mojavenensis</i>	22	(22)
<i>Bacillus subtilis</i> subsp. <i>subtilis</i>	47	(9, 13)
<i>Bacillus subtilis</i> subsp. <i>spizizenii</i>	43	(9, 13)
<i>Bacillus megaterium</i>	15	(13)
<i>Bacillus licheniformis</i>	11	(9)
<i>Bacillus sonorensis</i>	9	(21)
<i>Bacillus species</i>	14	(13)
Oil well isolates of unknown taxonomic affiliation	44	OU culture collection
Total number of strains	205	

Media

All cultures were grown aerobically in liquid medium E which contained the following components: KH_2PO_4 , 2.7 g/l; K_2HPO_4 , 13.9 g/l; sucrose, 10g/l; NaCl, 50g/l; yeast extract, 0.5g/l; NaNO_3 , 1g/l; pH 6.86. The medium was autoclaved and after cooling 10 ml/l of each of the following solutions was added: 2.5 g% MgSO_4 ; 10 g% of $(\text{NH}_4)_2\text{SO}_4$; and Wolin's trace metals solution. Wolin's trace metals solution contained the following: ethylene diamine tetraacetate (EDTA), 0.5 g/l; $\text{MnSO}_4 \cdot 7 \text{H}_2\text{O}$, 3g/l; NaCl, 1 g/l; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1g/l; $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.1 g/l; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g/l; $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$, 0.01 g/l; $\text{AlK}(\text{SO}_4)_2$, 0.01 g/l; $\text{Na}_2\text{MoO}_4 \cdot 2 \text{H}_2\text{O}$, 0.01 g/l; boric acid, 0.01 g/l; Na_2SeO_4 , 0.005g/l; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.003 g/l).

For anaerobic growth, medium E with 0.1 mg/l of resazurin as a redox indicator was used. The medium was boiled and allowed to cool under a stream of 100% N₂, and then dispensed in serum tubes (10 ml/tube) with a 100% N₂ headspace. The tubes were then sealed and 0.2 ml of a 25 g/l solution of cysteine•HCl was added to each tube (3). All media and solutions were sterilized by autoclaving at 121°C for 15 min.

Screening for Biosurfactant Production

Oil spreading technique was used to screen for biosurfactant production (20, 26). 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 medium E at 37°C was added to the surface of oil. The diameter of the clear zones on the oil surface was measured and activities were expressed as diameter of clearing on the oil surface in centimeters. The activity relative to *Bacillus mojavensis* strain JF-2 biosurfactant was obtained by dividing the activity obtained by a given strain to that found with *B. mojavensis* JF-2, which had a diameter of spreading of 1.2 cm.

Biosurfactant Extraction and Purification

The method used for biosurfactant extraction and purification was modified from that previously published (18). One-liter cultures grown for 48 hours in medium E were checked for biosurfactant production by using the oil spreading technique. Cells were then removed by centrifugation at 6000 Xg for 20 minutes. The pellet was dried at 110°C overnight and then the dry weight was determined. Biosurfactant in the supernatant was precipitated with 40% ammonium sulfate and incubated overnight at room temperature. The precipitate containing the biosurfactant along with other proteins was then collected by centrifugation at 6000 Xg for 30

minutes. Extraction of the precipitate with chilled acetone (250 microliters) followed to remove most of the proteins. Further purification was achieved by running a preparative thin layer chromatogram of the acetone extract. The acetone extract (250 μ l) was spotted on the TLC plates (Whatman, Clifton, NJ) with a solvent system of isopropanol: water: 28% ammonium hydroxide (80:11:9). The TLC plates were developed with iodine vapor. The different fractions were scrapped off the plate, dissolved in 250 μ l of deionized water, and tested for surface activity by using the oil spreading technique. Surface-active fractions were lyophilized overnight. The weight of the lyophilized biosurfactant was determined and used to calculate the biosurfactant yield (biosurfactant weight/ dry weight of cells). To compare biosurfactant activity, 1 μ g/ μ l solutions of purified biosurfactants were prepared and tested by oil spreading. Activity of purified biosurfactant was expressed as the diameter of the clear zone per 10 μ g of the purified biosurfactant.

Amino acid Analysis

Amino acid analysis for purified biosurfactants was conducted in 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 6 N HCl at 110°C for 18-24 hours. The hydrolyzed samples were vacuum dried, dissolved in 0.01 N HCl and filtered through a 0.45 μ m nylon filter before analysis. Amino acid analysis was performed by cation exchange chromatography. Amino acid elution was accomplished by a two buffer system. Samples were injected onto the column equilibrated with 0.2 N sodium citrate, pH 3.28. This buffer elutes the first 9 amino acids. Next, 1 N NaCl in 0.2N sodium acetate, pH 7.4, was used to elute the remaining amino acids. The amino acids were detected by on-line post-column reaction with ninhydrin (Tritone, Pickering Lab., Inc). The 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 on a totally automated, Beckman System Gold Model 126 HPLC Amino Acid Analyzer.

Fatty acid Analysis

A methanolysis procedure, modified from a previously described method (24), was used to analyze the fatty acids. Two hundred micrograms of purified biosurfactant were hydrolyzed under vacuum for 16 hours at 90°C with 4 ml of 25:75 HCl: CH₃OH in sealed tubes. The hydrolyzed fatty acids methyl esters (FAME) were then extracted with 7 ml of 1:1 ethyl acetate: hexane (EAH solvent). The organic layer was removed and the aqueous layer was re-extracted with EAH solvent. The organic phases were combined and concentrated under a stream of N₂ to 0.6 ml. The concentrated fractions were neutralized with 0.5 ml of 0.4 mM phosphate buffer (56.8 g Na₂HPO₄ + 16 g of NaOH /L) and incubated at room temperature for 10 minutes. The organic layer was removed and the aqueous layer was re-extracted with EAH solvent. The organic layers were combined and concentrated again under N₂ to 0.6 ml. The FAME in the organic layer were derivatized with BSTFA (Pierce, Rockford, IL) and analyzed by gas chromatography/ mass spectrometry (Agilent Technologies 6890N Network GC systems/ 5973 Network Mass Selective Detector). One microliter of the sample was used for injection. The oven temperature was set at 60°C for 5 minutes and then increased to 250°C over 15 minutes. The column was a capillary column (Agilent 19091S-433) (0.25 mm X 30m X 0.25 µm) with a flow rate of 1.2 ml/min. The mass spectrometer was operated at 400Hz. Peak areas obtained on the GC chromatogram were used to calculate the percentage of the FAME isomers compared to the total FAME.

RESULTS

Screening Diverse Microorganisms for Anaerobic Growth and Biosurfactant Production

One hundred and forty five strains mostly belonging to *Bacillus subtilis* subsp. *subtilis* and *B. subtilis* subsp. *spizizenii* were capable of biosurfactant production under aerobic conditions (Table 1.2). Thirty-three strains mostly belonging to *Bacillus licheniformis* and *B. sonorensis* were able to grow anaerobically in minimal medium (Table 1.2).

Sixteen strains produced biosurfactants with activities 1.75 to 2.5 times that of JF-2 biosurfactant (Table 1.3). Thirty three percent of the strains produced biosurfactants with activities 0.83 to 1.7 times that of JF-2 biosurfactant (Table 1.3).

Table 1.2. Growth and biosurfactant production by different strains tested in this study.

Species	Number of strains	Aerobic growth in 5% salt	Aerobic biosurfactant production	Anaerobic growth in 5% salt	Anaerobic biosurfactant production
<i>Bacillus mojavenensis</i>	22	22 ^a	10 ^a	0 ^a	1 ^a
<i>B. licheniformis</i> and <i>B. sonorensis</i>	20	20	11	20	0
<i>B. subtilis</i> subsp. <i>subtilis</i>	47	47	39	0	0
<i>B. subtilis</i> subsp. <i>spizizenii</i>	43	43	38	0	0
<i>B. megaterium</i>	15	15	7	13	0
<i>Bacillus</i> species	14	14	5	0	0
Oil well isolates	44	44	35	0	0

^a: Number of strains showing growth or biosurfactant production under the conditions indicated in the column heading.

Evaluation of a New Method of Biosurfactant Extraction and Purification

Bacillus mojavenensis strain ROB-2 was found to be a promising strain for biosurfactant production due to its high biosurfactant activity. However, eighty percent of the activity of the biosurfactant produced by strain ROB-2 was lost during acid precipitation. The low recovery of activity necessitated the development of a different purification method. Ammonium sulfate

precipitation followed by acetone extraction, recovered more than 80% of the biosurfactant activity (Figure 1.1). The new method was then the method of choice for extraction and purification of biosurfactants.

Table 1.3. Relative activities of different strains compared to JF-2 biosurfactant activity

	Number of strains with biosurfactant activity relative to			
	JF-2 ranging from*			
<i>Bacillus</i> species	No activity	0.42-0.75	0.83-1.7	1.75-2.5
<i>B. mojavensis</i>	12	4	4	2
<i>B. subtilis</i> subsp. <i>subtilis</i>	8	14	20	5
<i>B. subtilis</i> subsp. <i>spizizenii</i>	5	10	20	8
<i>B. megaterium</i>	8	4	3	0
<i>B. licheniformis</i> and <i>sonorensis</i>	9	11	0	0
<i>B. species</i>	9	4	1	0
Oil well isolates	9	15	19	1
Total	60	61	68	16

* Relative activity was measured by dividing diameter of clearing of different strains by that of JF-2 (diameter of clearing =1.2 cm).

Biosurfactant Yield and its Relation to Activity

The biosurfactants of twelve different *Bacillus* strains that had different surface activities were extracted and purified. Biosurfactant yield (mg biosurfactant per gram dry weight of cells) was calculated and correlated to the activity of the surface-active fraction obtained from the TLC plates (Figure 1.2). There was a very weak correlation between biosurfactant yield and surface activity (Pearson correlation coefficient =0.33). The biosurfactant activity did not increase with the increase in biosurfactant yield, i.e. some biosurfactants were produced in high yield and had relatively low biosurfactant activity. *Bacillus mojavensis* strain ROB-2 had a biosurfactant yield of 4.9 mg/ g dry weight and the biosurfactant activity corresponded to 2.25 cm of clearing on oil/ water surface using the oil

spreading technique. *Bacillus subtilis* subsp. *subtilis* strain T89-10 had a low biosurfactant yield of 1.9 mg/ g dry weight, but the biosurfactant activity corresponded to 5.45 cm of clearing. Figure 1.3 shows the weak correlation between biosurfactant yield and activity.

Figure 1.1 Biosurfactant extraction and purification scheme.

*: 1 U is defined as 1 cm of clearing by the oil spreading technique.

Figure 1.2. Schematic representation of the developed TLC plate

Figure 1.3. Correlation between biosurfactant yield (mg biosurfactant / g dry weight) and biosurfactant activity (expressed as the diameter of clear zones obtained when 10 μ l of the surface active fraction eluted from the TLC plate and dissolved in 250 μ l distilled water, was tested using the oil spreading technique).

Structure-activity Relationship Study

Purified biosurfactants were hydrolyzed and both the amino acid and fatty acid portions were analyzed. The specific activity of purified biosurfactants is expressed as the diameter of clear zones obtained when 10 μ g of purified biosurfactant (10 μ l of a 1 μ g/ μ l solution of purified

biosurfactant) was tested with the oil spreading technique. The concentration of each biosurfactant tested was the same to rule out any effects that concentration has on activity.

All the biosurfactants tested had the same amino acids in their peptide portion: Glx: Asx: Val: Leu (1:1:1:4) (Table 1.4). 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 the biosurfactants tested may differ in the regions indicated by Glx and Asx.

When the fatty acid portions of the biosurfactants were analyzed, it was found that the biosurfactants had 3-hydroxy tridecanoate (C13), tetradecanoate (C14), pentadecanoate (C15), and hexadecanoate (C16). The C13, C14, and C15 fatty acids were present as 2 isomers each. The C16 fatty acid had one saturated isomer and two unsaturated isomers. The percentage of each of these isomers was calculated using the peak areas of the GC chromatogram. When the percentage of the different isomers and their ratios to one another were correlated to the biosurfactant activity, we found that there was a correlation ($r=0.75$, Pearson correlation coefficient) between the ratio of C14 isomers to each other and to the biosurfactant activity (Table 1.4 and Figure 1.4).

Enhancing Biosurfactant Activity

The finding that the biosurfactant activity increases with the ratio of C14 fatty acid isomers to each other (iso C14/ n-C14), led to the hypothesis that adding precursors of branched C14 fatty acids to the medium will increase the percentage of the branched C14 isomer in the fatty acid portion of the biosurfactant and hence in the biosurfactant activity. Branched amino acids (valine, leucine, and isoleucine) are known to act as precursors for branched fatty acids (17). Valine is known to act as a precursor of branched chain fatty acids

with an even number of carbons (in this case C14, and C16) (6, 24). *Bacillus subtilis* subsp. *subtilis* strain T89-42 was grown in presence and absence of valine (1 g/L) added to medium E. The biosurfactants were extracted from both cultures and purified. Biosurfactant yields (mg of purified biosurfactant/ g dry weight), activities (diameter of clear zone of 10 µg purified biosurfactant), and the ratio of C14 fatty acid isomers of the lipid portion of the biosurfactant were calculated and compared to determine the effect of adding valine on biosurfactant structure and activity (Table 1.5).

Table 1.4. Comparison of biosurfactant activity and amino acid and fatty acid composition of different biosurfactants

Strain	Activity (cm/ 10µg)	Amino acid composition				Fatty acid composition				
		lx	sx	al	eu	13	14	16	17	18
T89-10	3.25 ^a									
		.98 ^b	.95	.07	.52	.2 ^c	2.8	8.1	.2	
ROQQ-2	2.25									
		.99	.01	.99	.76	.9	5.4	7.3	.7	.6
ROB-2	2.25									
		.08	.02	.06	.84	.94	4.6	2.8	5.8	7.9
TG6-19	2									
		.02	.97		.84	.1	3.2	7.7	.4	.7
T89-15	1.95									
		.99	.99	.02	.56	.8	8.7	8.2	.2	.2
T89-42	1.75									
		.97	.99	.03	.5	.4	8.6	7.6	.4	.9
T88-8	1.55									
		.01		.99	.76	.8	4.9	3.7	0.7	.9
CL1-14	1.5									
		.01			.74	.4	8.8	8	.8	.7
T89-14	1.05									
		.04	.01	.947	.58	2.8	2.2	0.6	.2	
T89-3	0.7									
		.99	.99	.02	.57	3.6	7.5	0	.8	.9

a: Activity of purified biosurfactant expressed as diameter of spreading per 10 µg of the biosurfactant purified according to Figure 1.

b: Numbers refer to the ratio of different amino acids in the peptide portion of the biosurfactant.

c: Numbers refer to the percentage of different fatty acids in the lipid portion of the biosurfactant. The percentage was calculated by dividing individual peak areas by the total peak area.

Adding valine to medium increased the ratio of C14 branched chain fatty acid to the total fatty acids from 25% to 59%. Ratio of C14 fatty acid isomers to one another (iso C14 to

n-C14) also increased from 3.2 to 21 with valine addition. When the activities of biosurfactants in both cases were detected, we found that the specific biosurfactant activity increased from 2.9 cm/ 10µg (without valine) to 4.75 cm/ 10µg (with valine). The increase in both biosurfactant activity and the ratio of C14 isomers to each other when valine was added to the medium supports the hypothesis that the biosurfactant activity is correlated to the ratio of C14 isomers to each other.

Table 1.5. Comparison of the fatty acid composition of *Bacillus subtilis* subsp. *subtilis* strain T89-42 biosurfactant in presence and absence of valine in the medium.

Retention time (min)	Fatty acid	T89-42 - valine		T89-42 + valine	
		FA ratio to the total		FA ratio to the total	
34.9	C13	7.33		2.79	
35.1	C13	6.28		1.67	
37.4	C14	25.13	C14/C14 =	58.58	C14/ C14 =
38.3	C14	7.85	3.2	2.79	21
39.7	C15	13.09		13.95	
39.9	C15	20.94		13.25	
41.8	C16	13.09		0	
42	C16	6.28		6.97	

DISCUSSION

This chapter reports success in four major fronts in support of the goal of developing microbial strains with better biosurfactant production for use in MEOR:

- 1.) Identification of strains with potentially better qualities for MEOR than JF-2,
- 2.) Development of methods to purify biosurfactants from superior strains,
- 3.) Preliminary identification of features of structural variation in the biosurfactant molecule that affect surface activity, and
- 4.) Manipulation of growth conditions to favor the production of structural variants with higher levels of surface activity.

Figure 1.4. Relation between the specific biosurfactant activity (cm of the clear zone/ 10 μ g of purified biosurfactant) to the ratio of the peak areas of the two C14 isomers of the fatty acid portion of the biosurfactant.

Screening Strains for MEOR

The number of strains screened (205), the variety of MEOR-relevant tests performed (see also (26)), and the yield of strains with characteristics applicable for MEOR was noteworthy. The majority of strains examined 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 (9, 13, 22) and therefore not selected to be biosurfactant producers, 68% produced biosurfactant, as compared to 80% of isolates from oil wells (Table 1.2). Approximately 28% of these strains that were examined with an oil-spreading technique had relative activities 1.75 to 2.5 times higher than that of JF-2 (15 out of 54; Table 1.3), while 1 in 9 strains isolated from oil wells 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.

Purification of Biosurfactant from Superior Strains

Although purification and testing of biosurfactants focused on strains of *B. subtilis* and the closely related *B. mojavensis*, it proved necessary to devise a different method (e.g. ammonium sulfate precipitation followed by acetone extraction) to extract and purify sufficient biosurfactant from these strains for further study. Developing this method was instrumental for partitioning strain variation in production of surface-active compounds into quantity produced versus specific activity of the biosurfactant, and for examining the structural variation of the biosurfactants. 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 (Fig. 1.3), suggesting variation among strains in biosurfactant structure as well as control of the production of biosurfactant (Fig. 1.3)

Structure-function Variation in Biosurfactants

Structural variation was found in the fatty acid portion, not the peptide portion, of biosurfactants purified from ten strains (Table 1.4). The strains were chosen from among three different taxonomic groups; the relative activity of their purified biosurfactant ranged from 0.7 times to 3.25 times that of JF-2. Each strain produced biosurfactant with a variety of fatty acid

tails, but it was noted that the ratio of branched-chain fatty acids with fourteen carbons to straight-chain fatty acids of the same length (isoC14/n-C14) a strain produced showed a positive correlation with the activity of the biosurfactant (Fig. 1.4). Although attention has been previously focused on structure-function relationships with respect to the amino acid residues (12, 25)), it seems clear that variation in fatty acids is a fruitful area of investigation.

Altering Growth Conditions to Favor Highly Active Structural Variants

The addition of a precursor (valine) of branched C14 fatty acids to the growth medium was successful in increasing the ratio of isoC14/n-C14 fatty acids in the biosurfactant produced by the strain (Table 1.5). In support of the correlation between higher isoC14/n-C14 fatty acid ratios and higher activity previously observed, growth in a medium containing valine produced biosurfactant with an increased ratio of the branched-chain fatty acids and greatly increased activity. The ability to experimentally manipulate the ratios of the fatty acid tails will provide a way to increase our knowledge of structure-function relationships of biosurfactants and related molecules, as well as providing a scientifically sound method for increasing biosurfactant activity.

REFERENCES

1. **Ahimou, F., P. Jacques, and M. Deleu.** 2001. Surfactin and iturin A effects on *Bacillus subtilis* surface hydrophobicity. *Enz. Micro. Tech.* **27**:749-754.
2. **Arima, K., A. Kakinuma, and G. Tamura.** 1968. Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: isolation , characterization, and its inhibition of fibrin clot formation. *Biochem. Biophys. Res. Comm.* **31**:488-497.
3. **Balch, W. E., and R. S. Wolfe.** 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. *Appl. Environ. Microbiol.* **32**:781- 791.
4. **Banat, I. M.** 1995. Charaterization of biosurfactants and their use in pollution removal-state of the art. *Acta Biotechnol.* **15**:251-267.
5. **Banat, I. M.** 2002. Potential commercial applications of microbial surfactants. *Appl Microbiol. Biotechnol.* **53**:495 -508.
6. **Besson, F., I. Tenoux, M. Hourdou, and G. Michel.** 1992. Synthesis of b-hydroxy fatty acids and b-amino acid fatty acids by strains of *Bacillllus subtilis* producing iturinic antibiotics. *Biochem. Biophys. Acta* **1123**:51-58.
7. **Bodour, A. A., M. Drees, and R. M. Maier.** 2003. Distribution of biosurfactant-producing bacteria in undisturbed and contaminated arid southwestern soils. *Appl. Environ. Microbiol.* **69**:3280 -3287.
8. **Clark, J. B., D. M. Munnecke, and G. E. Jenneman.** 1981. In situ microbial enhancement of oil production. *Dev. Ind. Micro.* **22**:695-701.
9. **Duncan, K. E., N. Ferguson, K. Kimura, X. Zhou, and C. A. Istock.** 1994. Fine-scale genetic and phenotypic structures in natural populations of *Bacillus subtilis* and *Bacillus licheniformis*: Important implications for bacterial evolution and speciation. *Evolution* **48**:2002- 2025.
10. **Folmsbee, M., Michael McInerney, David Nagle, Michelle Staudt, Warren Frey.** 2001. Presented at the American society of Microbiology, Orlando, Fl.
11. **Georgiou, G., S. C. Lin, and M. M. Sharma.** 1992. Surface-active compounds from microorganisms. *Biotech.* **10**:60- 65.

12. **Grangemard, I., J.-M. Bonmatin, J. Bernillon, B. C. Das, and F. Peypoux.** 1999. Lichenysins G, a novel family of lipopeptide biosurfactants from *Bacillus licheniformis* IM 1307: production, isolation and structural evaluation by NMR and mass spectrometry. *Journal of Antibiotics* **52**:363-373.
13. **Istock, C. A., N. Ferguson, N. L. Istock, and K. E. Duncan.** 2001. Geographical diversity of genomic lineages in *Bacillus subtilis* (Ehrenberg) Cohn sensu lato. *Org. Divers. Evol.* **1**:179-191.
14. **Javaherj M. , G. E. Jenneman, M. J. McInerney, and R. M. Knapp.** 1985. Anaerobic production of a biosurfactant by *Bacillus licheniformis* JF-2. *Appl. Environ. Microbiol.* **50**:698-700.
15. **Jenneman, G. E., R. M. Knapp, D. E. Menzie, M. J. McInerney, D. E. Revus, J. B. Clark, and D. M. Munnecke.** 1983. Transport phenomena and plugging in Berea sandstone using microorganisms. *Proc. Int. Conf. Microb. Enhancement Oil Recovery*:71-5.
16. **Jenny, K., O. Kaeppli, and A. Fiechter.** 1991. Biosurfactants from *Bacillus licheniformis*: structural analysis and characterization. *Appl Microbiol. Biotechnol.* **36**:5-13.
17. **Kaneda, T.** 1991. Iso-and anteiso-fatty acids in bacteria: biosynthesis, function and taxonomic significance. *Microbiol. Rev.* **55**:288-302.
18. **Kim, H.-S., S.-B. Kim, S.-H. Park, H.-M. Oh, Y.-I. Park, C.-K. Kim, T. Katsuragi, Y. Tani, and B.-D. Yoon.** 2000. Expression of *sfp* gene and hydrocarbon degradation by *Bacillus subtilis*. *Biotech. Lett.* **22**:1431-1436.
19. **McInerney, M. J., R. M. Knapp, D. P. Nagle, K. E. Duncan, N. Youssef, M. J. Folmsbee, and S. Maudgalya.** 2003. Development of microorganisms with improved transport and biosurfactant activity for enhanced oil recovery DE-FE-02NT15321. DOE.
20. **Morikawa, M., Y. Hirata, and T. Imanaka.** 2000. A study on the structure-function relationship of lipopeptide biosurfactants. *Biochim. Biophys. Acta* **1488**:211-218.
21. **Palmisano, M. N., L. K. Nakamura, K. E. Duncan, C. A. Istock, and F. M. Cohen.** 2001. *Bacillus sonorensis* species nov., a close relative of *Bacillus*

- licheniformis*, isolated from the soil in the Sonoran desert, Arizona. Inter. J. System. Evol. Microbiol. **51**:1671- 1679.
22. **Roberts, M. S., L. K. Nakamura, and F. M. Cohen.** 1994. *Bacillus mojavensis* sp. nov., distinguished from *Bacillus subtilis* by sexual isolation, divergence in DNA sequence, and differences in fatty acid composition. Inter. J. Syst. Bacteriol. **44**:256-264.
 23. **Van Dyke, M. I., H. Lee, and J. T. Trevors.** 1991. Applications of microbial surfactants. Biotech. Adv. **9**:241-252.
 24. **Yakimov, M. M., H. L. Fredrickson, and K. N. Timmis.** 1996. Effect of heterogeneity of hydrophobic moieties on surface activity of lichenysin A, a lipopeptide biosurfactant from *Bacillus licheniformis* BAS50. Biotechnol. Appl. Biochem. **23**:13- 18.
 25. **Yakimov, M. M., L. Giuliano, K. N. Timmis, and P. N. Golyshin.** 2000. Recombinant acylheptapeptide lichenysin: high level of production by *Bacillus subtilis* cells. J. Mol. Microbiol. Biotechnol. **2**:217-224.
 26. **Youssef, N., K. E. Duncan, D. Nagle, K. N. Savage, R. Knapp, and M. J. McInerney.** 2004. Comparison of methods to detect biosurfactant production by diverse microorganisms. J. Microbiol. Methods **56**:339-347.

Chapter 2. Efficacy of Biosurfactant Mixtures

ABSTRACT

MEOR (microbially enhanced oil recovery) depends on the use of biosurfactants to mobilize residual oil in low production or depleted reservoirs. In order to optimize the surface activity of biosurfactants, we hypothesized that mixtures of biosurfactants with diverse structures will generate lower surface and interfacial tensions compared to individual biosurfactants. In this study, the surface tension for 15 different *Bacillus* strains that are known to be surface active was measured both individually and in combination with other biosurfactants. Surface tension as well as CMD^{-1} values (critical micelle dilution defined as the reciprocal of the biosurfactant dilution at which a sharp increase in surface tension is observed) were compared to assess synergistic effects of the mixtures. The lowest surface tension was obtained with *Bacillus subtilis* subsp. *spizizensis* strain TG3-43 (34 mN/m). The lowest CMD^{-1} value was obtained with *Bacillus subtilis* subsp. *spizizensis* strain CL1-14 (10). Some biosurfactant mixtures were found to have synergistic effect on surface tension (e.g. surface tension was lowered from 41 to 31 mN/m in some cases); others had a synergistic effect on CMD^{-1} values. Moreover, surface and interfacial tensions were measured for partially purified biosurfactants to determine their CMC (critical micelle concentration defined as the concentration of the biosurfactant at which a sharp increase in surface tension is observed). CMC values in the range of 7 – 17 $\mu\text{g/ml}$ and interfacial tensions as low as 5.7 mN/m were obtained with some biosurfactants. Further work will involve measuring the surface and interfacial tensions for mixtures of purified biosurfactants to determine the best mixture that

can significantly lower surface and/ or interfacial tension or that will have the lowest CMC value.

INTRODUCTION

Biosurfactants are surface-active compounds produced by a wide variety of microorganisms (1). The chemical structure of biosurfactants allows them to partition at the oil/ water interfaces and hence lower the interfacial tension. Biosurfactants belong to several groups including lipopeptides synthesized by many bacilli and other species, 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 (2, 6). Despite their different structures, all biosurfactants possess both polar and non-polar domains that allow their partitioning at interfaces and thus lowering surface or interfacial tensions.

Surfactin and iturin are two different lipopeptides produced by *Bacillus* species. Surfactin has the ability to lower interfacial tension and also has some antibacterial activity. Iturins on the other hand show antifungal activity. Surfactin was found to increase the antifungal activity of iturin by forming mixed micelles (4).

One hundred and forty five diverse strains (mostly belonging to the genus *Bacillus*) were found to produce biosurfactants using several screening methods (7). Upon studying the structure of some of these biosurfactants (Chapter 1), we found that the biosurfactants produced by *Bacillus* species have very similar structures (i.e. they are all lipopeptide in structure). However, they do have minor differences in their amino acid (e.g. some will have glutamate instead of glutamine and/or aspartate instead of asparagine) and in their fatty acid compositions (e.g. differences in the carbon number and branching). We hypothesized that

mixtures of biosurfactants with different structures will generate lower surface and interfacial tensions than the individual biosurfactants do.

In this study, we measured surface tension for several strains which were found to be biosurfactant producers by the oil spreading technique, either alone or in combination with other biosurfactant producers. The effect on surface tension and/ or critical micelle dilution (CMD^{-1} is defined as the reciprocal of the biosurfactant dilution at which a sharp increase in surface tension is observed) was compared for single cultures and mixtures of more than one biosurfactant producer in order to quantify any synergistic effect that the mixture might have on surface activity.

MATERIALS AND METHODS

Bacterial Strains

Table 2.1 shows the names, taxonomic status, and surface activity (expressed as the diameter of clear zones on the oil surface in centimeters by the oil spreading technique (7) of the strains used in this study.

Table 2.1. Names, taxonomic status, and surface activity of the strains used in this study.

Strain	Species (Group)	Oil spreading diameter (cm)
ROB-2	<i>Bacillus mojavensis</i> (A)	2.7
ROG-4	<i>Bacillus mojavensis</i> (A)	2
ROQQ-2	<i>Bacillus mojavensis</i> (A)	2.1
T89-14	<i>Bacillus mojavensis</i> (A)	1.2
JF-2	<i>Bacillus mojavensis</i> (A)	1.2
T89-42	<i>Bacillus subtilis</i> subsp. <i>subtilis</i> (B)	3
T89-1	<i>Bacillus subtilis</i> subsp. <i>subtilis</i> (B)	2.4
ROGG-2	<i>Bacillus subtilis</i> subsp. <i>subtilis</i> (B)	2.1
T89-15	<i>Bacillus subtilis</i> subsp. <i>subtilis</i> (B)	1.8
T89-46	<i>Bacillus subtilis</i> subsp. <i>subtilis</i> (B)	1.5
T89-3	<i>Bacillus subtilis</i> subsp. <i>spizizensis</i> (D)	3
T89-9	<i>Bacillus subtilis</i> subsp. <i>spizizensis</i> (D)	2.7
CL1-14	<i>Bacillus subtilis</i> subsp. <i>spizizensis</i> (D)	2.4
TG3-43	<i>Bacillus subtilis</i> subsp. <i>spizizensis</i> (D)	2.1
CL1-21	<i>Bacillus subtilis</i> subsp. <i>spizizensis</i> (D)	1.8

Media

All cultures were grown aerobically for 24 hours at 37°C in liquid medium E (KH_2PO_4 , 2.7 g/l; K_2HPO_4 , 13.9 g/l; sucrose, 10g/l; NaCl, 50g/l; yeast extract, 0.5g/l; NaNO_3 , 1g/l; pH 6.86). The medium was autoclaved and, after cooling, 10 ml/l of each of the following solutions was added: 2. 5% MgSO_4 ; 10% $(\text{NH}_4)_2\text{SO}_4$; and Wolin's trace metals solution. Wolin's trace metals solution contained the following: EDTA, 0.5 g/l; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 3g/l; NaCl, 1 g/l; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1g/l; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g/l; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g/l; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.01 g/l; $\text{AlK}(\text{SO}_4)_2$, 0.01 g/l; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.01 g/l; boric acid, 0.01 g/l; Na_2SeO_4 , 0.005g/l; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.003 g/l).

Measurement of Surface Tension and Determining CMD^{-1}

Biosurfactant activity was determined by the oil spreading technique (7) and by estimating the units of activity from surface tension measurements in samples diluted to below the CMD^{-1} . The critical micelle dilution (CMD^{-1}) is defined as the reciprocal of the biosurfactant dilution at which a sharp increase in surface tension is observed. To determine the CMD^{-1} different dilutions of the culture or biosurfactant solution were made in uninoculated medium E according to Table 2.2.

Table 2.2. Dilutions prepared for CMD^{-1} measurement.

Volume of culture (ml)	Volume of medium E (diluent) (ml)	Dilution
4	0	0
3	1	0.75x
2	2	0.5x
2	4	0.33x
1	3	0.25x
0.65	3.35	0.165x
0.5	3.5	0.125x
0.5	4.5	0.1x

Surface tension was measured for each dilution using a Du Nouy ring tensiometer (3). Two milliliters of each dilution was used for the measurement. The critical micelle dilution was estimated graphically as the dilution where a sharp increase in surface tension occurred.

The number of units of biosurfactant activity present in each sample was quantified from the surface tension of dilutions below the CMD^{-1} . At a dilution below CMD^{-1} , surface tension values are linearly proportional to biosurfactant activity. At this dilution, surface tension value was subtracted from surface tension of uninoculated medium E (72 mN/m) which was used as the diluent and the resulting number was divided by 10 mN/m to give an estimate of the number of units of biosurfactant activity in the sample. One unit of activity is defined as the amount of surfactant that lowers the surface tension of water by 10 mN/m. Water and isopropanol were used to calibrate the instrument before measurement.

Preparing Mixtures of Different Cultures

Mixtures of different biosurfactants were prepared by growing individual strains separately in medium E for 24 hours. The next day, biosurfactant production was checked with the oil spreading technique, the surface tension of different dilutions of the culture was measured, and the CMD^{-1} was determined. Mixtures of biosurfactants were prepared such that each biosurfactant in the mixture was present at its CMD^{-1} . The volume of each culture added to the mixture was adjusted such that the final dilution for each individual biosurfactant equaled its CMD^{-1} . Mixtures of strains within the same taxonomic group (e.g. group A is *B. mojavensis* strains, group B is *B. subtilis* subsp. *subtilis* strains, and group D is *B. subtilis* subsp. *spizizensis* strains) were mixed together. All mixtures were made with and without the addition of *Bacillus mojavensis* strain JF-2.

Surface Tension and CMD^{-1} of Biosurfactant Mixtures

After a mixture of several biosurfactants was prepared, the mixture was diluted in medium E to give final dilutions of 1x, 0.5x, and 0.25x. The surface tension of each dilution was measured and the CMD^{-1} was determined as described above for individual biosurfactants. To detect any synergistic effects, the surface tension of the undiluted mixture of biosurfactants was compared to the average of surface tension values of each individual biosurfactant that comprised the mixture. The latter were measured at the dilution used to create the mixture. A positive synergistic effect is defined as a surface tension of the mixture that is lower than the average surface tension of individual biosurfactants that comprise the mixture or a CMD^{-1} value that is lower than the CMD^{-1} values of the individual biosurfactants that comprise the mixture.

Purification of Biosurfactants

Biosurfactants from *Bacillus mojavensis* strains ROB-2, ROG-4, and T89-14 were extracted and purified by using the method outlined in Chapter 1. The purified biosurfactants was diluted and the surface tension of each dilution was measured. This information was used to graphically determine the critical micelle concentration (CMC). The critical micelle concentration (CMC) is defined as the concentration of the biosurfactant at which a sharp increase in surface tension is observed. Purification of additional biosurfactants is underway to determine if they have lower CMC's than previously characterized biosurfactants and if they act synergistically with mixtures of other biosurfactants.

Interfacial Tension Measurement

A model 500 Spinning Drop Tensiometer was employed for all interfacial tension measurements. Samples were prepared from the biosurfactant such that the denser phase (the aqueous surfactant) is continuous and the less dense phase (oil phase) forms the drop. One milliliter of a 1 mg/l solution of purified biosurfactant and three microliters of toluene were used. Each biosurfactant solution was measured in triplicate.

RESULTS

Surface Tension and CMD^{-1} Values of Individual Strains

Fifteen strains belonging to three different taxonomic groups of *Bacillus* that had high oil spreading activity (Table 2.1) were chosen to test for synergistic effects of biosurfactant mixtures(7) . Figure 2.1 shows the surface tension of different dilutions of each strain. *Bacillus subtilis* subsp. *spizizensis* strain TG3-43 had a surface tension of 34 mN/m (group D, Figure 2.1c; Table 2.3), which was the lowest surface tension value of all the strains tested. Low surface tension values have been encountered before with other *Bacillus* strains (e.g. *Bacillus mojavensis* strain JF-2 showed surface tension of 27 mN/m, (3), although in this set of experiments the value for JF-2 was 27 mN/m². The surface tension value often depends on the stage of growth at which the surface tension is measured. *Bacillus* species are known to produce their biosurfactant at the end of the log phase and the beginning of the stationary phase (5). Since we did not follow surface tension during the course of growth, the surface tension values shown in Figure 2.1 may not correspond to the lowest surface tension that can be attained by these strains.

Figure 2.1a. Surface tension values of different dilutions of group A strains (*Bacillus mojavensis*).

Figure 2.1b. Surface tension values of different dilutions of group B strains (*B. subtilis* subsp. *subtilis*).

Figure 2.1c. Surface tension values of different dilutions of group D strains (*Bacillus subtilis* subsp. *spizizensis*).

Surface Tension and CMD^{-1} Values of Biosurfactant Mixtures

CMD^{-1} is defined as the reciprocal of the biosurfactant dilution at which a sharp increase in surface tension is observed. The higher the CMD^{-1} value, the more effective the biosurfactant is since the surface tension remains low even as the biosurfactant concentration is lowered. The highest CMD^{-1} value was obtained with *Bacillus subtilis* subsp. *spizizensis* strain CL1-14 (a CMD^{-1} value of 10) (Table 2.3).

Biosurfactant mixtures were prepared by mixing cultures of individual strains according to the CMD^{-1} . Culture medium of each individual strain was added to the mixture in an amount corresponding to its CMD^{-1} such that any synergistic effect on either surface tension or CMD^{-1} can be easily interpreted. Surface tension values of the biosurfactant mixtures were compared to the average surface tension value of each individual strain measured at its CMD^{-1} . Any decrease in the surface tension of the mixture would then be considered as a synergistic effect and would suggest mixed micelles were being formed at the surface. Since each biosurfactant

in the mixture was added at its CMD^{-1} , then a lower CMD^{-1} of the mixture compared to the CMD^{-1} of each individual biosurfactant indicates a positive synergistic.

Table 2.3. Surface tension and CMD^{-1} values of the 15 strains used in this study.

Strain (group)	Surface tension of 1x (mN/m)	CMD^{-1}
ROB-2 (A)	41.5	2
ROG-4 (A)	43	2
ROQQ-2 (A)	38	3
T89-14 (A)	38	3
JF-2 (A)	38	3
T89-42 (B)	38.5	8
T89-1 (B)	37	4
ROGG-2 (B)	37	4
T89-15 (B)	41.5	2
T89-46 (B)	39.5	2
T89-3 (D)	36.5	4
T89-9 (D)	42.5	2
CL1-14 (D)	42	10
TG3-43 (D)	34	6
CL1-21 (D)	42	4

Figure 2.2a shows the effect of mixing cultures of *Bacillus mojavensis* strains ROB-2, ROG-4, and JF-2 on surface tension. The surface tension of the mixture was 33.5 mN/m. This mixture contained 40% ROB-2, 40% ROG-4, and 20% JF-2 culture fluid (each present at its CMD^{-1}). Surface tension values for these cultures at the indicated dilutions were 40, 45, and 44, respectively (Figure 2.1a). The average of the three surface tension values is 43 mN/m. This clearly shows the positive synergistic effect of biosurfactant mixtures on surface tension. The CMD^{-1} of the mixture was 2, which is not better than the CMD^{-1} 's of the individual strains.

Figure 2.2b shows the effect of mixing biosurfactants from a different combination of Group A strains. The mixture contained 50% ROB-2, 25% ROQQ-2, and 25% JF-2 culture fluid and had a surface tension of 41 mN/m, which is not different from average surface tension of the individual biosurfactants (Table 2.4). However, the CMD^{-1} of the mixture was

greater than 4 (Figure 2.2b; Table 2.4), which is better than the CMD^{-1} values of individual cultures of strains ROB-2, ROQQ-2, and JF-2 (2, 2, and 3, respectively; Table 2.3). At a CMD^{-1} of 4, the mixture contained only 12.5% of ROB-2 and 6.25% of each of ROQQ-2 and JF-2 cultures, but still generated a much lower surface tension than if 100% of each culture was used. We could not determine the actual CMD^{-1} of this mixture since dilutions greater than those used are required. However, it is clear that mixing ROB-2, ROQQ-2, and JF-2 biosurfactants had a synergistic effect on the CMD^{-1} .

Figure 2.2a. Surface tension values of a mixture of biosurfactants from Group A strains ROB-2, ROG-4, and JF-2 compared to each individual culture.

When biosurfactants from Group B strains T89-42 and ROGG-2 were mixed together, effects on surface tension but not CMD^{-1} were observed (Figure 2.3). The mixture contained 12.5% T89-42 and 25% ROGG-2 culture fluid. The surface tension of the mixture was 31 mN/m. The surface tension values for these strains at the above concentrations can be deduced from Figure 2.1c to be 41 and 41 mN/m, respectively. The average surface tension of the individual cultures is 41 mN/m, which is much higher than that of the mixture (Table 2.4).

Thus, the mixture had much better surface tension lowering ability than either of the two biosurfactants alone.

Figure 2.2b. Surface tension values of a mixed biosurfactants from Group A strains ROB-2, ROQQ-2, and JF-2 compared to each individual culture.

Figure 2.3. Surface tension values of the mixture of biosurfactants from strains T89-42 and ROGG-2 of group B compared to each individual culture.

Table 2.4. Comparison of surface tension obtained with mixtures to the average value of the individual strains. CMD⁻¹ is also shown for the mixtures.

Group	Strains mixed	Surface tension of mixture (mN/m)	Surface tension of individual strains (avg)	CMD ⁻¹ of the mixture
A	ROB-2+ROG-4	40	39.75	2
	ROB-2+ROQQ-2	40	42	2
	ROB-2+T89-14	41	41	2
	ROB-2+ROG-4+JF-2	33.5	43	2
	ROB-2+ROQQ-2+JF-2	41	42.7	>4
	ROB-2+T89-14+JF-2	35	42	Between 2 and 4
B	T89-42+T89-1	34	39	2
	T89-42+ROGG-2	31	41	2
	T89-42+T89-15	43.5	40.5	2
	T89-42+T89-46	35	40.5	Between 2 and 4
B and JF-2	T89-42+T89-1+JF-2	36.5	40.6	2
	T89-42+ROGG-2+JF-2	40	42	Between 2 and 4
	T89-42+T89-15+JF-2	35	41.6	Between 2 and 4
	T89-42+T89-46+JF-2	42	41.6	2
	T89-42+JF-2	37	42.5	Between 2 and 4
	T89-3+T89-9	32	39.25	Between 2 and 4
D	T89-3+CL1-14	33	39.5	Between 2 and 4
	T89-3+TG3-43	36	38	Between 2 and 4
	T89-3+CL1-21	34	39	Between 2 and 4
	T89-3+JF-2	34	40	Between 2 and 4
D and JF-2	T89-3+CL1-14+JF-2	31	41	Between 2 and 4
	T89-3+TG3-43+JF-2	36	40	Between 2 and 4
	T89-3+CL1-21+JF-2	43	40.6	>4

Mixing biosurfactants from Group D strains showed effects on surface tension and possibly on the CMD⁻¹. Figure 2.4a shows the effect on surface tension when cultures of strains T89-3, CL1-14, and JF-2 were mixed together. A binary mixture of T89-3 and CL1-14 biosurfactants had a surface tension of 33 mN/m. This mixture contained 25% T89-3 and 12.5% CL1-14 culture fluid. Surface tension values for these strains at these concentrations were deduced from Figure 2.1c to be 36 and 43, respectively, giving an average of 39.5 mN/m (Table 2.4). Again, this is an example where the surface tension of the mixture is much lower than the average surface tension of the individual biosurfactants. A positive synergistic effect was also observed when JF-2 was added to the above binary mixture. The surface tension of

the mixture was 31 mN/m. This mixture contained 25% T89-3, 12.5% CL1-14, and 25% JF-2. Surface tension values for culture of these strains at the above concentrations were deduced from Figure 2.1c to be 36, 43, and 44, respectively, giving an average of 41 mN/m. For these combinations of strains, no positive synergistic effect was observed on the CMD⁻¹.

Figure 2.4a. Surface tension values of a mixture of biosurfactants from Group D strains T89-3 and CL1-14 with and without the JF-2 biosurfactant compared to the individual cultures.

Figure 2.4b. Surface tension values of a mixture of biosurfactants from Group D strains T89-3 and CL1-21 with JF-2 compared to each individual culture.

Mixing biosurfactants from a different combination of Group D strains gave a positive synergistic effect on the CMD^{-1} , but not the surface tension (Figure 2.4b). When T89-3, CL1-21, and JF-2 were mixed together in ratios of 25% of each, the CMD^{-1} of the mixture was greater than 4, which corresponds to a concentration of each biosurfactant of <6.25% each. T89-3, CL1-21, and JF-2 cultures had CMD^{-1} values 4, 4, and 3, respectively (Table 2.3). However, the mixture had a CMD^{-1} greater than this when only 6.25% each culture fluid was present showing that mixing these 3 strains did have a synergistic effect. Table 2.4 summarizes the effect of mixing different biosurfactants on surface tension and CMD^{-1} .

Surface and Interfacial Tensions of Partially Purified Biosurfactant

Biosurfactants produced by some *Bacillus* strains were partially purified using a method that combines ammonium sulfate precipitation, acetone extraction and thin layer chromatography (Chapter 1). The partially purified biosurfactants were then used for both surface and interfacial tension measurements. The critical micelle concentration (CMC) of each partially purified biosurfactant was also determined. Figure 2.5 shows the surface tension of different concentrations of partially purified biosurfactants of *Bacillus mojavensis* strains ROB-2, ROG-4 and T89-14. The CMC values obtained for the partially purified biosurfactants from each strain ranged between 7 and 17 g/l. These concentrations are very low compared to synthetic surfactants.

Table 2.5. Interfacial tensions measured for different purified biosurfactants.

Biosurfactant purified from strain (group)	Interfacial tension mN/m
T89-3 (D)	11.6 ± 0.3
CL1-14 (D)	5.7 ± 0.1
CL1-21 (D)	11.9 ± 0.3
ROGG-2 (B)	8.8 ± 0.4
T89-46 (B)	12 ± 0.3
T89-1 (B)	13.8 ± 0.2
T89-42 (B)	5.9 ± 0.2

Interfacial tensions were measured for some purified biosurfactants by using Spinning drop tensiometer. The samples were prepared in 1 mg/l concentration. Interfacial tensions obtained are reported in Table 2.5. The lowest interfacial tension was obtained with biosurfactants from *Bacillus subtilis* subsp. *spizizensis* strain CL1-14 and *Bacillus subtilis* subsp. *subtilis* strain T89-42. Meanwhile, larger amounts of biosurfactants are being prepared to determine interfacial tensions over a range of concentrations for each biosurfactant.

Figure 2.5a. Surface tension values of various concentrations of the partially purified biosurfactant of *Bacillus mojavensis* strain ROB-2. CMC is 17.4 g/l.

Figure 2.5b. Surface tension values of various concentrations of the partially purified biosurfactant of *Bacillus mojavensis* strain ROG-4. CMC is 7.74 g/l.

Figure 2.5c. Surface tension values of various concentrations of the partially purified biosurfactant of *Bacillus mojavensis* strain T89-14. CMC is 17.4 g/l.

DISCUSSION

In these experiments, we compared the surface activity of mixtures of biosurfactants produced by two or three strains of *Bacillus* to the activity of the biosurfactants assayed singly. A synergistic effect on surface tension and/or on the CMD^{-1} value (reciprocal of the critical micelle dilution) was often found. Although only 15 strains and a fraction of the possible combinations were tested, in 14 instances out of 23, the surface tension of the mixture of biosurfactants was 5-10 mN/m lower than that of the weighted average of the individual strains. These findings are significant for MEOR for two reasons. First, it suggests a new approach for MEOR, e.g., the use of mixtures of biosurfactant than individual biosurfactants. Such an approach has not been considered for enhanced oil recovery previously. Secondly, since most oil field microbial communities contain diverse microorganisms, it is likely that these communities will produce diverse types of biosurfactants. Thus, the stimulation of populations indigenous to oil reservoirs may allow the production of self-generating biosurfactant mixtures that may be very effective for oil recovery.

Our work also raises the question why combinations of biosurfactants are more effective than individual biosurfactants in lowering surface tension. With single biosurfactants, repulsion due to charge or similar hydrophobic/hydrophilic properties may not generate micelles structures most effective for oil mobilization. When different biosurfactants with different structures are present, there is the possibility that the molecules align to reduce charge repulsion or detrimental hydrophobic interactions. Thus, the micelles may be more compact or form a structure that is more conducive for oil recovery.

REFERENCES

1. **Banat, I. M.** 1995. Charaterization of biosurfactants and their use in pollution removal-state of the art. *Acta Biotechnol.* **15**:251-267.
2. **Bodour, A. A., and R. M. Miller-Maier.** 2002. Biosurfactants: types, screening methods, and applications., p. 750-770, *Encyclopedia of environmental microbiology*. Wiley and Sons, N.Y.
3. **McInerney, M. J., M. Javaheri, and D. P. Nagle.** 1990. Properties of the biosurfactant produced by *Bacillus licheniformis* strain JF-2. *J. Indust. Microbiol.* **5**:95-102.
4. **Peypoux, F., J. M. Bonmatin, and J. Wallach.** 1999. Recent trends in the biochemistry of surfactin. *Appl Microbiol. Biotechnol.* **51**:553-563.
5. **Sullivan, E. R.** 1998. Molecular genetics of biosurfactant production. *Curr. Opin. Biotech.* **9**:263-269.
6. **Van Dyke, M. I., H. Lee, and J. T. Trevors.** 1991. Applications of microbial surfactants. *Biotech. Adv.* **9**:241-252.
7. **Youssef, N., K. E. Duncan, D. Nagle, K. N. Savage, R. Knapp, and M. J. McInerney.** 2004. Comparison of methods to dectect biosurfactant production by diverse microorganisms. *J. Microbiol. Methods* **56**:339-347.

Chapter 3. Microbial Strains with Improved Transport Ability through Model Porous Systems.

ABSTRACT

Bacillus species have been widely used as model organisms during MEOR research. An important characteristic of *Bacillus* species is their ability to produce spores. Spores are essential for MEOR research because of their small size compared to vegetative cells, their ability to withstand harsh environmental conditions and their increased transport ability. The objective of this study was to obtain biosurfactant producing strains with improved transport abilities through sandstone. We compared the transport abilities of spores from three *Bacillus* strains using a model porous system to study spore recovery and transport. Sand-packed columns were used to select for spores or cells with the best transport abilities through brine-saturated sand. Spores of *Bacillus mojavensis* strains JF-2 and ROB-2 and a natural recombinant strain C-9 transported through sand at very high efficiencies. The earliest cells/spores that emerged from the column were re-grown, allowed to sporulate, and applied to a second column to determine whether spores or vegetative cells had enhanced transport properties. This procedure greatly enhanced the transport of strain C-9. Our data show that spores with enhanced transport abilities are obtained early in effluent and that the preparation of inocula is a practical and useful tool to study microbial enhanced oil recovery.

INTRODUCTION

Bacillus species capable of producing biosurfactants are commonly isolated from oil reservoirs (1, 28, 34). However, indigenous strains may not produce biosurfactants that generate the ultra low interfacial tensions. Thus, the injection of one or more biosurfactant-producing strains into the reservoir may be required. For this reason, an understanding of the

factors that affect microbial transport in porous media is needed. The physical, chemical and biological properties that affect microbial transport in porous media are grouped into two probabilities described by filtration theory: the probability of bacterial cell collision with sediment grain collectors upon approach (collector efficiency) and the probability of bacterial cell attachment to the collector upon collision (collision efficiency) (11, 12). The collector efficiency accounts for the physical factors that control the frequency of bacterial cell collisions with the grain surfaces such as interception, diffusion and gravitational settling. Due to the small pore size, straining will be an important factor controlling the penetration of bacteria in reservoirs (14, 20, 32). Jang, 1983, found that, because of their small size, spores were more effective at transport through Berea sandstone cores than vegetative cells. Spore formation is a key characteristic of the genus *Bacillus* and with their ability to make effective biosurfactants these are the organisms of choice for MEOR.

The small size of spores should allow them to penetrate greater distances within the reservoir than vegetative cells. However, less than 0.01% of the injected concentration of spores of *Bacillus licheniformis* were recovered in the effluent of short sandstone cores (10). Thus, collision efficiency is important. Collision efficiency describes the total interactive forces between bacterial and mineral surfaces as the sum of the double layer, London-van der Waals and acid-base potential energies over the distance separating the cell from the surface (11). In addition to cell size, bacterial surface charge (usually negative), cell hydrophobicity and cell motility are important biological factors controlling collision efficiency (3, 13, 24, 25, 29, 30). In aquifers, positively charged ferric iron oxides promote adhesion of the negatively charged bacterial cell (25). Most petroleum reservoirs are anaerobic so iron will be in its reduced form. However, it is not clear how reduced iron minerals affect microbial transport. Oil reservoirs also have sandstones partially coated with crude oil so hydrophobic

interactions will be important. Cell hydrophobicity controls adhesion of cells to surfaces that are not strongly hydrophilic (29, 30, 32). Wiencek *et al.* (1990) found that spores of several species of *Bacillus* and *Clostridium* were more hydrophobic and thus adhered to surfaces more effectively than vegetative cells (33).

Populations of bacterial cells exhibit a wide range of heterogeneities with respect to their ability to adhere to grain surfaces (2, 27). Subsets of the bacterial population often have much reduced collision efficiencies, which may explain the migration of bacterial cells over large distances within aquifers (17). If low collision efficiency is a stable trait, we should be able to select for bacteria with improved transport properties by selecting for these cells or spores that do not adhere to crushed sandstone (4-6). This approach led to the development of a strain that showed to migrate over large distances in a fine-sand aquifer (17).

Bacillus species have been used in the research for microbial enhanced oil recovery because of their ability to produce spores and the production of biosurfactant. Spores are dormant cells, which are produced from stresses in the environment such as starvation. Spores are able to withstand many extreme environmental changes such as starvation, marked temperature and pH changes, desiccation, and many other factors. Biosurfactants produced by bacteria consist of a wide variety of surface and interfacially active compounds (9). These compounds are able to reduce surface and interfacial tensions between oil and water or oil and sand, making them excellent candidates for microbial enhanced oil recovery. An understanding of how biosurfactant-producing microorganisms transport and migrate through porous media will aid in the improvement of MEOR processes.

Jang *et al.* (1983) found that spores are better candidates for enhanced transport than vegetative cells (14). They also found that a large inocula of cells injected into a bench-top model increases the potential for plugging (14). The use of spores *in situ* could decrease the

potential for plugging since spores are smaller than vegetative cells and have a decreased probability of blocking pore channels. Also, since spores are in a dormant state, nutrient injection to maintain viability is not required during injection and thus, spores should be able to transport further than vegetative cells. Several studies have shown penetration rates and permeability factors were key components when studying cell movement through porous media (15, 16, 18, 23, 26).

In this study, the migration of spores and vegetative cells of three different strains of *Bacillus mojavensis*, JF-2, ROB-2 and C-9, through sand packed columns was studied to select for a strain with enhanced transport abilities. Thus far, this is the first study on the potential of enhanced spore transport through unconsolidated porous media.

MATERIALS AND METHODS

Preparation of Spores

Bacillus mojavensis strains JF-2 and ROB-2 and the natural recombinant strain C-9 were grown and sporulated in Difco sporulation broth (22) with the following modifications: Difco nutrient broth with 0.6 ml of 1 M NaOH, 10 ml of 1.2% (w/v) MgSO_4 , and 10 ml of 10% (w/v) KCl per liter. After autoclaving, the sporulation broth was supplemented with 1 ml of a mineral solution containing 0.01 M MnCl_2 , 1M $\text{Ca}(\text{NO}_3)_2$, and 0.001M $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ per liter. Each culture was grown for 7 to 11 days at 37 °C. The spores were harvested by centrifugation (10,000 x g; 20 min; 4°C) and washed twice by suspending of the pellet in 35 ml of 0.85% NaCl solution and centrifugation as above. The spore preparation was stored at 4 °C until used as the inoculum for packed-sand columns.

Preparation of Sand-Packed Columns

Borosilicate glass columns with polypropylene luer-lock fittings, approximately 2.5 cm in diameter by 10 cm in length, were used for the transport studies. Each column was

packed with 95-100 g of quartz sand while sonicating the column in a bench-top sonicator. Silica gel was applied to the inside rim of the column cap to prevent leakage. After sonication, the columns were saturated with 0.85% NaCl and autoclaved (121 °C; 15 min.). The sonication step and autoclave sterilization was then repeated. The columns were allowed to equilibrate overnight before applying the spore preparation. An empty, sterile column fitted with a cotton-filled syringe on the cap was attached to the top of the sand column by a three-way, polypropylene, luer-lock fitting. The empty column served as the reservoir for the brine to flow into the sand column by natural flow.

Pore volume of the column was determined by weighing the column before and after saturation of the sand column with sterile brine (0.85% NaCl solution). Pore volumes were calculated as the difference between the wet-weight and dry weight of the sand-packed column. The dry weight of the sand-packed column was obtained after overnight incubation under constant airflow. In addition, Blue Dextran, a high molecular weight, inert material, was used to determine the pore volume of the sand column. One 1 ml of concentrated Blue Dextran was injected into the sand column and fractions were collected until the blue color began to appear in the effluent. The volume of the fractions collected prior to the appearance of the blue color was determined volumetrically. Pore volume for sand-packed columns containing 95-100 g of sand ranged from 11 to 16 ml, respectively.

Transport Experiments

After overnight equilibration with 0.85 % NaCl solution, approximately 10 pore volumes of sterile 0.85% NaCl was injected into the column. Next, 1 ml of the spore preparation was injected into the sand column through the three-way valve by using a sterile, 1-ml syringe. The reservoir column was filled completely with 0.85% NaCl and allowed to flow through the sand-packed column. Effluent fractions were collected as follows: fractions

1-5, 3 ml; fractions 6-23, 1 ml; fractions 24-29, 3 ml; and fractions 30-36, 5 ml. The total effluent volume collected in each case was 86 ml. As a control, 1 ml of sterile brine was injected into the column to ensure sterility of the column preparation procedure.

The concentration of both vegetative cells and spores in each effluent sample was estimated by end point dilution (EPD). Each fraction was serially diluted ten-fold by adding a 25 μ l aliquot of the fraction to 225 μ l of Medium E to the first well of a column of a 96-well, sterile microtiter plate. After mixing, 25 μ l was withdrawn and used to inoculate the next well until a dilution of $1:10^8$ was achieved. Microtiter plates were incubated for 24 hours at 37 °C. Positive wells were those that had increased turbidity compared to uninoculated, control wells. The EPD is the reciprocal of the most dilute well that showed growth. Composition of Medium E is given in Chapter 1. No growth was observed in any well inoculated with fractions collected when sterile brine was injected into the column.

The total number of viable microorganisms (colony-forming units, CFU·ml⁻¹), which includes both vegetative cells and spores and spores alone were determined by pooling the total volume of each fractions together and serially diluting (ten fold) the pooled fraction in 0.85% NaCl, and inoculating agar medium. Viable plate counts were done to obtain a viable cell count (vegetative cells and spores) and spore count of inoculum used for each column. To determine the spore concentration, the fraction was heated for 20-25 min. at 85 °C to kill vegetative cells and to assist in germination of spores.

To obtain transport-enhanced strains, aliquots of the first effluent fractions (fraction 3, 4, or 5, depending on the strain) were grown and a new spore preparation was prepared as above. The transport properties of the original strain and the effluent fraction were compared.

Plate Count Methods

Plate counts were done to determine CFU·ml⁻¹ for both total viable cells (vegetative cells and spores) and spores alone for each preparation. Serial dilutions from 10⁻¹ to 10⁻⁸ were achieved by adding 100 µl of preparation to 900 µl of 0.85% of NaCl in sterile glass tubes until a dilution of 10⁻⁸ was obtained. Then, 100µl from three dilutions were separately plated onto standard plate count agar in triplicate and incubated for 16 hours at 37 °C. Counts were only considered accurate for plates containing 30-300 colonies.

Rep-PCR

Isolated genomic DNA from strains JF-2, ROB-2 and C-9 was used as templates for repetitive sequence-based, polymerase chain reaction (rep-PCR) (19, 31). DNA was isolated according to Dneasy Tissue Kit for Animal Tissue (Qiagen 2003). BoxA1R repetitive sequence-based oligonucleotide primers (5'-ctacggcaaggcgacgctgacg-3') were used to identify any differences between the spores injected into the column and the spores recovered in the effluent (31). Each 30 µl PCR reaction contained 8.75 µl water, 8 µl of 25 mM MgCl₂ and 10x PCR buffer mix, 1 µl 10 mM dNTP, 2 µl boxA1R primer (0.3 µg/µl), 0.25 µl Taq Polymerase (10nmol), and 10 µl of DNA template. PCR amplifications were carried out using a thermocycler with an initial denaturation at 94 °C for 4min., followed by 30 cycles of denaturation at 94 °C for 1 min. and annealing at 50 °C for 1 min. and an extension of 72 °C for 8 min. A final extension was run for one cycle at 72 °C for 8 min. The products of the PCR reactions were run on an 0.8% agarose gel.

RESULTS

B. mojavensis strains were inoculated into Difco sporulation broth and incubated for 7-11 days, shaking at 150rpm at 37°C. Cultures were centrifuged, suspended in brine (0.85% NaCl) and injected into a sterile column, after which, the earliest effluent fraction was

collected, sporulated and applied to a second column. First, we quantified the recoveries of spores and cells exiting the columns to obtain an estimate of the degree of cell retention (Table 3.1). The variation in cell and spore recoveries was estimated by calculating the range since duplicate columns were run for each strain. The percent recovery of total viable colony forming units had ranges of 0.15 to 0.54 and the percent spore recovery had ranges of 0.09 to 1.55 (Table 3.1). This indicates that differences in percent recovery greater than two-fold are likely to be significant. The total viable colony forming units and spore counts of effluents were performed several weeks after the column was run. Thus, effluent percent recoveries based on plate counts may be an underestimation of the actual recoveries. However, our recovery data are several orders of magnitude higher than that cited in the literature. In addition, these recoveries were obtained using small pore volumes of displacement fluid. As shown the various figures, the elution patterns for different replicates of each strain were reproducible. The variation between replicates for a given fraction was within one order of magnitude, which is within the precision of the MPN method. Thus, elution patterns that differ by more than an order of magnitude are likely to be significant.

Table 3.1. Summary of plate counts and percent recoveries for each strain applied to a column.

Strain	CFU·ml ⁻¹ Influent		% Spores Influent	CFU·ml ⁻¹ Effluent		Total Recovery	% Spore Recovery Spore
	Viable	Spore		Viable	Spore		
JF-2	3.1e8 (9.0e7)	2.9e8 (3.0e7)	96.5 (18.6)	1.5e6 (1.0e4)	1.6e6 (5.0e4)	0.50 (0.15)	0.53 (0.14)
JF-2(3)	4.03e7 (4.5e6)	4.1e7 (1.3e7)	100.1 (19.8)	3.29e5 (3.75e4)	3.1e5 (2.5e3)	0.82 (0.19)	0.78 (0.09)
ROB-2	2.3e8 (2.0e8)	1.7e8 (9.5e6)	89.5 (74.6)	6.7e5 (4.03e5)	1.19e6 (1.83e6)	0.42 (0.54)	0.86 (1.55)
ROB-2(4)	3.8e7 (0)	3.01e7 (0)	79.2	3.9e5 (8e4)	2.9e5 (6.5e4)	1.03 (0.21)	0.77 (0.17)
C-9	NA	5.0e8	NA		3.5		NA
C-9(5)	NA	2.4e9	NA		93.7		2.23e9

*Percent spores in influent and percent spore recovery were calculated by the ratio of spore to viable cell counts for influent and effluent fractions. Parentheses show ranges between means of duplicate columns.

Table 3.1 summarizes the data collected for each transport experiments with strains JF-2, ROB-2, and C-9, and the early effluent fractions of these strains, JF-2(3), ROB-2(4) and C-9(5) that were regrown and reinjected into new columns. After passage of JF-2 through the column, the mean concentration of viable cells collected was 1.5e6 CFU·ml⁻¹, while the average number of spores collected from the column was 1.6e6 spore·ml⁻¹. The ratio of influent spores and the effluent spores shows that the percentage of JF-2 spores recovered from the column was 0.53%. We used an end point dilution to estimate the concentration of viable cells and spores (together) in each effluent fraction. Since a large number of fractions (36) were collected for each column, it was not possible to determine viable cell counts for each fraction by the agar plate technique.

Figure 3.1. Elution pattern of JF-2 from sand columns. Open and closed circles are duplicate columns showing EPD values for each effluent fraction. Pore volumes based on Blue Dextran ellutant was 12mls.

Figure 3.2. Cumulative elution pattern of JF-2 from sand columns. Pore volumes are based on Blue Dextran ellutant in 12mls.

Figure 3.1 compares the log concentrations of JF-2 cells $\cdot \text{ml}^{-1}$ versus volume of effluent that passed through the column. Comparing both columns of JF-2, the majority of the cells and spores injected into the column was collected within the first two pore volumes at a significantly high cell concentrations, 10^8 - 10^7 cells $\cdot \text{ml}^{-1}$ (Figure 3.1). This suggests that JF-2 is moving in an ordered rather than a dispersed manner. When these data are expressed

as the cumulative number of viable cells and spores collected with pore volume of effluent, one can clearly see that almost all of the viable cells and spores initially injected into the column was recovered (Figure 3.2). Initially, about 3.1×10^8 viable cells and spores of JF-2 were injected into the column. By EPD, we estimate that about 3.75×10^8 viable cells and spores were recovered in the first 1.5 pore volumes of effluent. This shows that JF-2 transported through the column at very high efficiency.

Figure 3.3. Effluent pattern of JF-2(3) (early breakthrough fraction of JF-2) from sand packed columns. Fraction 3 from Figure 3.1 was sporulated and passed through duplicate columns. Pore volume of the columns ranged from 11-15ml.

The third effluent fraction from each JF-2 column was collected, allowed to sporulate and injected into a second column. Figure 3.3 shows the log concentration of each fraction collected versus the volume in milliliters of effluent that passed through the column for the early breakthrough fraction of the original JF-2 culture, designated JF-2(3). The average percent recovery of spores of the duplicate JF-2(3) columns was 0.78% which was a 0.25% increase from the original strain. Figure 3.2 shows that nearly 100% recovery of the injected

viable cells and spores were obtained within 1 to 2 pore volumes of displacement fluid.

Again, showing that JF-2 transported through the column at high efficiency.

Figure 3.4. Elution pattern of ROB-2 through sand packed columns. Open and closed circles are duplicate columns showing EPD values from each fraction.

ROB-2 transported through the column in a manner similar to strain JF-2. After passage of ROB-2 through the column, the mean concentration of viable cells collected was 6.7×10^5 CFU·ml⁻¹, while the average number of spores collected from the column was 1.2×10^6 spore·ml⁻¹. The ratio of influent spores and the effluent spores shows that the percentage of JF-2 spores recovered from the column was 0.86%, slightly higher than the recovery of JF-2. ROB-2 had higher concentration of cells/spores being released in the early fractions than JF-2. The recoveries of the early breakthrough fraction of ROB-2, ROB-2(4), were similar to that of the original ROB-2 preparation (Table 3.1). Effluent pattern shows that the majority of ROB-2 or ROB-2(4) cells and spores were released from the column within the second pore volume (Figure 3.4 and 3.5). Figure 3.4 shows the effluent pattern of ROB-2 had more fractions with higher EPD than the original JF-2 or its early breakthrough fraction, JF-2(3).

When these data are expressed as the cumulative number of viable cells and spores collected with pore volume of effluent, it is clear that ROB-2 and its early breakthrough fraction, ROB-2(4) were transported at high efficiency (Figure 3.6). Initially, about 2.3×10^8 and 3.8×10^7 viable cells and spores of ROB-2 and ROB-2(4), respectively, were injected into the column. By EPD, we estimate that about 1×10^9 viable cells and spores for each were recovered in the first 2 pore volumes of effluent.

Figure 3.5. Elution pattern of early breakthrough fraction, ROB-2 (4).

C-9, a natural genetic recombinant strain of *Bacillus mojavensis* (21), was tested in our column system to compare its elution pattern to the other two strains. Figure 3.7 shows the effluent concentrations of each fraction collected of the C-9 original culture and Figure 3.8 provides this information for the early breakthrough fractions, C-9(5). The influent concentration of C-9 was 5.0×10^8 CFU·ml⁻¹ and 2.4×10^9 for C-9(5) (Table 3.1). Based on EPD estimates, we recovered about 1.17×10^7 viable cells and spores of C-9 from the column, for an estimated percent recovery of 3.5. Interestingly, the early breakthrough fraction, C-9(5),

transported through the column at much higher efficiencies than the original C-9. The percent spore recovery exceeded 91%, the influent spore concentration was 2.4×10^9 CFU·ml⁻¹ and the effluent concentration was 2.2×10^9 CFU·ml⁻¹. Fraction 4 in Figure 3.4 was collected, grown and spores were injected into another series of columns.

Figure 3.6. Cumulative elution pattern of ROB-2 from sand columns. Pore volumes were based on dry versus wet weights.

Rep-PCR

During transport experiments, different colony morphologies were observed when plating effluents of JF-2, ROB-2 and C-9. The colonies were similar for all strains, mixtures of smooth and rough colonies. Rep-PCR was performed in order to determine if the different colony morphologies were due to variations in the same strain injected into the column, or by an unknown contaminant possibly present in the spore preparation (31). The results of the rep-PCR experiment showed that the smooth and rough colonies were variations in colony morphology. There was no change in the band pattern from the original strain to the early effluent fractions. The band pattern for JF-2 was compared to band patterns of *Bacillus subtilis* 168; the band patterns were slightly different but this was expected since they are

different strains. The rep-PCR banding patterns were not different enough to believe that the initial spore preparations used to inject into the columns were contaminated for any of the strains.

Figure 3.7. Elution pattern of C-9 through sand columns. Pore volumes range from 10-14mls.

Figure 3.8. Elution profile of the early breakthrough fraction of C-9, C-9 (5). Pore volume was not measured for these columns.

DISCUSSION

Several important characteristics contribute to an organism's ability to transport through porous media. These characteristics include size, motility, exopolymer production, hydrophobicity, electrostatic charge, as well as others. The three *Bacillus mojavensis* strains used to select for spores with enhanced transport through saturated unconsolidated porous media were allowed to flow through the columns naturally without added pressure. We believed that we could select for spores with enhanced transport ability by collecting the earliest fractions that exited the cores and running these fractions through columns until cells or spores are released in the first pore volume. However, we found that all of the strains passed through the sand packed columns at very high efficiencies. Transport was so efficient that, in only one case, e. g., strain C-9, could we distinguish a difference in transport between the original preparation and the early breakthrough fraction (Figures 3.7 and 3.8). Collecting the early effluent fractions may not be as necessary as first thought. Testing different variables such as higher salt concentrations and shorter inoculation times should now be looked at to predict the best method for enhanced transport and biosurfactant production.

Generally, cell recoveries from transport studies are much lower, often less than 0.01% of the injected concentration (10), an order of magnitude less than our minimum estimate of cell and spore recoveries are. The recovery of *B. licheniformis* spores was very low (0.001%). It is possible that the procedure that we used to prepare our inoculum contributed to the high transport efficiency. The culture was incubated for 7 to 11 days to allow sporulation. For much of this period, the culture would have been without needed nutrients. This may have provided an environment that allowed the organism not only to

form spores, but also to produce spores with low collision efficiencies. Thus, the inoculum preparation procedure may be the most important aspect in obtaining strains with high transport efficiencies.

Figure 3.1 and 3.3 show the difference of effluent collected between JF-2 and JF-2(3). JF-2 had a spore percent recovery of 0.53% while fraction 3 had a spore percent recovery of 0.78%. This is a 0.25% increase of spores recovered with fraction 3 versus the original culture. Duplicate columns of JF-2 and JF-2(3), shows that fraction 3 travels through the column similar to its original strain. There was no significant difference in viable cell recovery compared to spore recovery in JF-2(3) or JF-2 showing that spores were moving at the same rate or faster than viable cells. Unlike JF-2, JF-2(3) passed in high concentration as a band of cells through the second pore volume, while JF-2 eluted more diffusely from the column.

Recoveries of ROB-2 cells and spores from the column were similar to that of JF-2. However, the elution of ROB-2(4) had one fraction with very high concentration, 10^9 cells/ml, which is an order of magnitude higher than found for ROB-2 or JF-2. C-9 shows similar spore recoveries to JF-2 with 3.5% released from the original column. These data were based on EPD and not plate counts. The early breakthrough fraction for C-9, C-9(5), was transported through the column at much higher efficiencies than the original preparation. This is the one case where it is clear that a transport-enhanced strain was obtained.

Many studies have focused on the movement of bacterial cells through saturated porous media, but there has been fewer studies using spores. Jang et al. (1983) studied three different bacterial species for their ability to move through porous media as spores or vegetative cells (14). They found that *Clostridia* spores easily moved through porous media more so than vegetative cells. There has been reports of two adhesion deficient strains,

Comamonas sp. and *Erwinia herbicola*, were studied based on attachment and detachment rates. They found 67% to 55% recoveries with pore volumes ranging from 530 – 754 milliliters (7). In the current study we have been able to get efficient transport with smaller pore volumes. JF-2 spores move through the column at the same rate as vegetative cells, although further analysis of ROB-2 and C-9 will be needed to determine if these spores move through at the same rate or faster than vegetative cells. By running the earliest fraction through the column again, we may be able to select for smaller more transportable spores. Additional columns will allow for the selection of a strain with small spore size as well as possible changes in cell surface characteristics and increased biosurfactant production.

The use of sand columns for seeking out biosurfactant-producing strains with enhanced transport abilities is an efficient tool. We found two strains, JF-2 and ROB-2, that naturally had high transport efficiencies through sand and were able to select for a transport enhanced variant of strain C-9. The fact that all of the *Bacillus* strains studied in this project had high transport efficiencies coupled with the high percentage of biosurfactant producers among isolates from diverse environments suggests these organism may be present in many oil fields. Previous studies have focused on the transport of vegetative through porous media. Fontes et al. reported percent recoveries ranging from 49% to 11% using nonsporeforming bacteria through high ionic strength sand columns (8). In this case the average pore volume was 88cm³. We believe spores are good candidates because of their resilience to severe environmental conditions, and the fact that spores do not need a means to replenish nutrients. Another advantage of spores over vegetative cells is their small, consistent size, which allows movement through small pore sizes in porous media. Further studies are needed using oil saturated models to test the effects of oil on the transport of spores.

REFERENCES

1. **Abdulrazag, Y., R. A. Almehaideb, and O. Chaalal.** 1999. Presented at the SPE Annual Technical Conference and Exhibition, 1991.
2. **Albinger, O., B. K. Biesemeyer, R. G. Arnold, and B. E. Logan.** 1994. Effect of bacterial heterogeneity on adhesion to uniform collectors by monoclonal populations. *FEMS Microbiol. Let.* **124**:321-6.
3. **Camesano, T. A., and B. E. Logan.** 1998. Influence of Fluid Velocity and Cell Concentration on the Transport of Motile and Nonmotile Bacteria in Porous Media. *Env. Sci. Tech.* **32**:1699-1708.
4. **DeFlaun, M. F., B. M. Marshall, E. P. Kulle, and S. B. Levy.** 1994. Tn5 insertion mutants of *Pseudomonas fluorescens* defective in adhesion to soil and seeds. *Appl. Environ. Microbiol.* **60**:2637-42.
5. **DeFlaun, M. F., S. R. Oppenheimer, S. Streger, C. W. Condee, and M. Fletcher.** 1999. Alterations in adhesion, transport, and membrane characteristics in an adhesion-deficient pseudomonad. *Appl. Environ. Microbiol.* **65**:759-765.
6. **DeFlaun, M. F., A. S. Tanzer, A. L. McAteer, B. Marshall, and S. B. Levy.** 1990. Development of an adhesion assay and characterization of an adhesion-deficient mutant of *Pseudomonas fluorescens*. *Appl. Environ. Microbiol.* **56**:112-19.
7. **Dong, H., R. Rothmel, T. Onstott, M. E. Fuller, M. F. DeFlaun, S. H. Streger, R. Dunlap, and M. Fletcher.** 2002. Simultaneous transport of two bacterial strains in intact cores from Oyster, Virginia: Biological effects and numerical modeling. *Appl. Environ. Microbiol.* **68**:2120-2132.
8. **Fontes, D. E., A. L. Mills, G. M. Horrnerberger, and J. S. Herman.** 1991. Physical and chemical factors influencing transport of microorganisms through porous media. *Appl. Environ. Microbiol.* **57**.
9. **Georgiou, G., S. C. Lin, and M. M. Sharma.** 1992. Surface-active compounds from microorganisms. *Biotech.* **10**:60-65.
10. **Gullapalli, I. L., J. H. Bae, K. Hejl, and A. Edwards.** 2000. Laboratory design and field implementation of microbial profile modification process. *SEP Reservoir Eval. Eng.* **3**:42-49.

11. **Harvey, R. W.** 1991. Parameters involved in modeling movement of bacteria in groundwater, p. 89-114. *In* C. J. Hurst (ed.), Modeling the environmental fate of microorganisms. ASM Press, Washington, D. C.
12. **Harvey, R. W., and S. P. Garabedian.** 1991. Use of colloid filtration theory in modeling movement of bacteria through a contaminated sandy aquifer. *Env. Sci. Tech.* **25**:178-85.
13. **Harvey, R. W., L. H. George, R. L. Smith, and D. R. LeBlanc.** 1989. Transport of microspheres and indigenous bacteria through a sandy aquifer: results of natural- and forced-gradient tracer experiments. *Env. Sci. Tech.* **23**:51-6.
14. **Jang, L. K., P. W. Chang, J. E. Findley, and T. F. Yen.** 1983. Selection of bacteria with favorable transport properties through porous rock for the application of microbially enhanced oil recovery. *Appl. Environ. Microbiol.* **46**:1066-1072.
15. **Jenneman, G. E.** 1985. Microbial penetration through Berea sandstone and the effect of nitrate on biogenic sulfide production: their relevance to microbial enhanced oil recovery. Univ. OK., Norman, OK.
16. **Jenneman, G. E., M. J. McInerney, and R. M. Knapp.** 1986. Effect of nitrate on biogenic sulfide production. *Appl. Environ. Microbiol.* **51**:1205-1211.
17. **Johnson, W. P., P. Zhang, M. E. Fuller, T. D. Scheibe, B. J. Mailloux, T. C. Onstott, M. F. DeFlaun, S. S. Hubbard, J. Radtke, W. P. Kovacik, and W. Holben.** 2001. Ferrographic tracking of bacterial transport in the field at the narrow channel focus area, Oyster, VA. *Env. Sci. Tech.* **35**:182-91.
18. **Johnson, W. P., P. Zhang, P. M. Gardner, M. E. Fuller, and M. F. DeFlaunn.** 2001. Evidence for detachment of indigeous bacteria from aquifer sediment in response to arrival of infected bacteria. *Appl. Environ. Microbiol.* **67**:4908-4913.
19. **Judd, A. K., M. Schneider, M. J. Sadowsky, and F. J. De Bruijn.** 1993. Use of repetitive sequences and the polymerase chain reaction technique to classify genetically related *Bradyrhizoboum japonicum* serocluster 123 strains. *Appl. Environ. Microbiol.* **59**:1702-1708.
20. **McInerney, M. J.** 1991. Use of models to predict bacterial penetration and movement within a subsurface matrix, p. 115-135. *In* C. J. Hurst (ed.), Modeling the environmental fate of microorganisms. ASM Press, Washington, D.C.

21. **McInerney, M. J., R. M. Knapp, D. P. Nagle, K. E. Duncan, N. Youssef, M. J. Folmsbee, and S. Maudgalya.** 2003. Development of microorganisms with improved transport and biosurfactant activity for enhanced oil recovery DE-FE-02NT15321. DOE.
22. **Nakano, M. M., M. A. Maraheil, and P. Zuber.** 1988. identification of a genetic locus required for biosynthesis of the lipopeptide antibiotic surfactin in *Bacillus subtilis*. J. Bact. **170**:5662-5668.
23. **Reynolds, P. J., P. Sharma, G. E. Jenneman, and M. J. McInerney.** 1989. Mechanisms of microbial movement in subsurface materials. Appl. Environ. Microbiol. **55**:2280-2286.
24. **Scholl, M. A., and R. W. Harvey.** 1992. Laboratory investigations on the role of sediment surface and groundwater chemistry in transport of bacteria through a contaminated sandy aquifer. Env. Sci. Tech. **26**:1410-17.
25. **Scholl, M. A., A. L. Mills, J. S. Herman, and G. M. Hornberger.** 1990. The influence of mineralogy and solution chemistry on the attachment of bacteria to representative aquifer materials. J. Cont. Hydrol. **6**:321-36.
26. **Sharma, M. M., and G. Georgiou.** 1993. Microbial enhanced oil recovery research. Final Report for Annex X. Bartlesville Project Office, U. S. Department of Energy, Bartlesville, OK, DOE/BC/14445-12.
27. **Simoni, S. F.** 1998. Population heterogeneity affects transport of bacteria through sand columns at low flow rates. Environ. Sci. Technol. **32**:2100-2105.
28. **Sugihardjo, E. H., and S. W. Pratomo.** 1999. Presented at the SPE Asia Pacific Improved Oil Recovery Conference, Kuala Lumpur, Malaysia.
29. **Van Loosdrecht, M. C. M., J. Lyklema, W. Norde, and A. J. B. Zehnder.** 1989. Bacterial adhesion: a physicochemical approach. Micro. Eco. **17**:1-15.
30. **Van Loosdrecht, M. C. M., J. Lyklema, W. Norde, and A. J. B. Zehnder.** 1990. Influence of interfaces on microbial activity. Microbiol. Rev. **54**:75-87.
31. **Versalovic, J., M. Schneider, F. J. De Bruijn, and J. R. Lupski.** 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Meth. Mol. Cell Bio. **5**:25-40.

32. **Weiss, T. H., A. L. Mills, G. M. Hornberger, and J. S. Herman.** 1995. Effect of Bacterial Cell Shape on Transport of Bacteria in Porous Media. *Environmental Science and Technology* **29**:1737-40.
33. **Wiencek, K. M., N. A. Klapes, and P. M. Foegeding.** 1990. Hydrophobicity of Bacillus and Clostridium spores. *Applied and environmental microbiology* **56**:2600-5.
34. **Yonebayashi, Y., K. Ono, H. Enomoto, T. Chida, C.-X. Hong, and K. Fujiwara.** 1997. Presented at the SPE Asia Pacific Oil and Gas Conference, Kuala Lumpur.

Chapter 4. Tertiary oil recovery by the JF-2 biosurfactant from Berea sandstone cores and the development of a mathematical model relating oil-water interfacial tension to the concentration of the JF-2 biosurfactant.

ABSTRACT

Efficacy of the JF-2 biosurfactant was tested using Berea sandstone cores and sand-packed columns which were flooded to residual oil saturation. Tertiary oil recovery experiments showed that biosurfactant solutions with concentrations ranging from 10 to 920 mg/l in the presence of 0.1 mM 2,3-butanediol and 1 g/l of partially hydrolyzed polyacrylamide (PHPA) recovered 10-40% of residual oil from Berea sandstone cores. When PHPA was used alone, about 10% of the residual oil was recovered. Thus, about 10% of the residual oil recovered in these experiments was due to the increase in viscosity of the displacing fluid. The remainder of the recovered oil was due to the effect of the JF-2 biosurfactant on interfacial tension between oil and the displacing aqueous phase. Capillary trapping may have prevented mobilized oil from being produced ahead of the biosurfactant front, but this oil was produced when a post surfactant brine solution was injected at three times the rate that the initial biosurfactant sludge was injected into the core. The relationship between interfacial tension (IFT) reduction and biosurfactant concentration was defined. Little or no oil was recovered at biosurfactant concentrations below the critical micelle concentration (CMC) (about 10 mg/l). At concentrations lower than the CMC, IFT values were high. At biosurfactant concentrations from 10 to 40 mg/l, the IFT was 1 mN/m. As the biosurfactant concentration increased beyond 40 mg/l, IFT decreased to around 0.1 mN/m. At biosurfactant concentrations in excess of 10 mg/l, residual oil recovery was linearly related to biosurfactant concentration. A mathematical

model that relates oil recovery to biosurfactant concentration was modified to include the stepwise changes in IFT as biosurfactant concentrations changes. This model adequately predicted the experimentally observed changes in IFT as a function of biosurfactant concentration.

INTRODUCTION

The widespread use of petroleum hydrocarbons has resulted in the contamination of valuable groundwater resources. Petroleum hydrocarbons may exist in the vadose and saturated zones as a free liquid or ganglia of residual hydrocarbon (3, 5). Even if the free liquid hydrocarbon can be removed, substantial amounts of residual hydrocarbon remain entrapped by capillary forces and represent a long-term source of contamination (5). Entrapment of petroleum hydrocarbons by capillary forces is also a major factor that limits oil recovery (1, 14, 16). Current technology recovers only one-third to one-half of the oil that is originally present in an oil reservoir. Since almost all regions of the world have been intensively explored for oil, the discovery of large new oil resources is unlikely and the exploitation of oil resources in existing reservoirs will be essential in the future.

Surfactants of synthetic or biological origin enhance hydrocarbon biodegradation by increasing the apparent aqueous solubility of the hydrocarbon or by enhancing the interaction of the microbial cell with the hydrocarbon. Alternately, bulk hydrocarbon displacement can occur if the capillary forces that entrap the hydrocarbon are reduced. Interfacial tension (IFT) between the hydrocarbon and aqueous phases is largely responsible for trapping the hydrocarbon in the porous matrix. Ultra-low values (several orders of magnitude reduction) of IFT are needed for hydrocarbon mobilization. To achieve these ultra-low IFT values, very high

concentrations ($> \text{g l}^{-1}$) of synthetic surfactants must be used, which makes chemical surfactant flooding expensive. Microbially-produced biosurfactants may be an economical method to recover residual hydrocarbons since they are effective at low concentrations (as indicated by their low critical micelle concentrations). However, the recovery of residual hydrocarbon by biosurfactants from model porous systems is inconsistent and often low.

Microorganisms produce a variety of biosurfactants (4), several of which generate the low interfacial tensions between the hydrocarbon and the aqueous phases required to mobilize residual hydrocarbon (4, 7, 10). In particular, the lipopeptide biosurfactant produced by *Bacillus mojavensis* strain JF-2 reduces the interfacial tension between oleic and aqueous phases to very low levels ($<0.016 \text{ mN/m}$) (9, 10). The critical micelle concentration is 20 mg/l , indicating that the biosurfactant is effective even at very low concentrations (10). Residual oil is recovered when a biosurfactant-producing bacterium and the nutrients needed to support growth are introduced into sandstone cores (11, 18, 20), but residual hydrocarbon recoveries were often low (5 to 20%) and required multiple pore volumes of recovery fluid (11, 18).

Previously, we showed that substantial mobilization of residual hydrocarbon from a model porous system occurs at biosurfactant concentrations made naturally by *B. mojavensis* strain JF-1 if a polymer and 2,3-butanediol were present (12).

In this report, we include data on oil recovery from Berea sandstone experiments along with our previous data from sand pack columns in order to relate biosurfactant concentration to the fraction of oil recovered (12). A capillary desaturation curve was obtained between water flood phase capillary numbers and residual oil saturation in Berea sandstone cores. This curve indicates the change in the magnitude of the capillary number required to lower residual oil saturation in a core.

MATERIALS AND METHODS

Medium and Cultivation

Bacillus mojavensis strain JF-2 was grown in medium E as described in Chapter 1 of this report. The culture was centrifuged to remove cells (10,000 x g; 10 min; 4 °C). The concentration of the JF-2 biosurfactant was determined by high-pressure liquid chromatography (13). The cell-free culture fluid was used immediately for injection into the core. When more dilute biosurfactant concentrations were required, the cell-free culture fluid was diluted with sterile medium E. The partially hydrolyzed polyacrylamide (PHPA) and 2,3-butanediol were added to give final concentrations of 1 g/l and 10 mM, respectively, prior to injection in the cores.

Core Flooding

Berea sandstone cores were dried in an oven at 60° C for 4 days. The dried cores were weighed and their length and diameter measured. The core was inserted into a Hassler holder and placed under vacuum for 24 hours to remove air. The core was placed at 2000 psig and then flooded with at least multiple pore volumes of deaerated 5% NaCl brine. After brine saturation, the core was flooded to connate water saturation (until no more brine exited the core) with crude oil (32° API gravity). The core was then flooded with 5% NaCl brine until near residual oil saturation, where only a trace of oil was detected in the effluent of the core.

After the core reached near residual oil saturation, cell-free culture fluid containing the indicated biosurfactant concentration and 1 g/l PHPA and 10 mM 2,3-butanediol was injected into core. Table 4.1 gives the pore volumes and flow rates used for biosurfactant injection. In most cases, the core was then treated with 5% NaCl brine after biosurfactant sludge was

injected as indicated in Table 4.1. The flow rates used for post-flush brine injection are also given in Table 4.1.

Effluent samples were collected in flasks and the amounts of oil and brine collected were determined volumetrically.

Petrophysical properties of the Berea sandstone cores are given in Table 4.2. Brine viscosity ranged from 1.03 to 1.1 cp and crude oil viscosity ranged from 2.0 to 6.0 cp.

Establishment of a Capillary Desaturation Curve for Berea Sandstone Cores

Capillary number is defined as the ratio of inertial to capillary forces. Capillary number increases with increases in the inertial forces or decreases in the interfacial forces. Increases in capillary number lower the residual oil saturation in the core and increase residual oil recovery.

Capillary number is mathematically defined as:

$$N_{CP} = \frac{v\mu}{\sigma}$$

where,

v: velocity through porous media (cm/sec), $Q/A\Phi$

μ : viscosity of displacing fluid (brine), cp

σ : Interfacial tension between oil and water, dynes/cm

and Q: the water flooding rate, A is the core's cross-sectional area and Φ is the porosity of the core.

Table 4.1. Summary of oil recovery data at biosurfactant concentrations above and below the critical micelle concentration. Corrected percent residual oil recovery is corrected for the amount of residual oil recovered by polymer alone.

Core	Biosurfactant concentration (mg/l)	Volume of recovery sludge (PV)	Biosurfactant injection rate (ml/h)	Volume of brine post flush (PV)	Post-flush rate (ml/h)	Residual oil recovered (ml)	Percent residual oil recovery	Corrected percent residual oil recovery (%)
1	11	2	3.14	0	3.14	0	0	0
2	39	2	3.14	3	3.14	2.3	39	29.3
3	38	1	2.54	1	5.14	2	47	37.3
4	38	1	5.14	1	10	3	45	35.3
5	21	1	5.4	1	30.9	2.7	26.5	16.8
6	21	1	6.4	1	30.9	3	27.8	18.1
7	10.5	1	6.4	1	30.9	2	13.4	3.7
8	10.5	1	6.4	1	30.9	1.7	16.2	6.5
9	11	2	5.14	1	20.53	2.3	20.2	10.5
10	11	1	5.14	1	20.53	1.7	13.8	4.1
11	11	1	5.14	1	20.53	1.8	15.9	6.2
12	5.5	1	5.14	1	20.53	1	8.8	0
13	5.5	1	5.14	1	20.53	1.2	9.6	0
14	2.75	1	5.14	1	20.53	1.3	10.3	0.6
15	2.75	1	5.14	1	20.53	1.7	13.3	3.6
16	0	1	6.43	1		1.2	9.7	0

A Berea sandstone cores was dried in an oven at 60⁰ C for four days, then weighed and its length and diameter measured. The core was place under vacuum for 24 h to remove trapped air inside the core, saturated with deareated 5.0 % NaCl brine, and flooded to connate water saturation using crude oil. In the water flooding phase, 5.0% NaCl brine was injected at a flow rate of 2.5 ml/h until the core reached residual oil saturation (e. g., until no more oil was recovered from the core). The rate of brine injection was doubled (5.1 ml/h) and the core was again water flooded to residual oil saturation. The doubling of the flow rate continued until the brine flow rate reached a maximum of 576.0 ml/h. The amount of oil recovered at each flow rate was measured and the residual oil saturation determined.

Interfacial Tension (IFT) Measurements

Biosurfactant was prepared aerobically in separate batches and had different biosurfactant concentrations. Some batches were diluted by one-half or one-quarter of the original biosurfactant concentration by diluting the cell-free culture fluid with sterile medium.

E. Composition and preparation of medium, growth of *B. mojavensis* strain JF-2, and preparation of cell-free culture fluids is given in Chapter 1. The concentration of the biosurfactant was determined by high-pressure liquid chromatography as described previously (13).

Table 4.2. Petrophysical properties of the Berea sandstone cores.

Core	Porosity (%)	Pore volume (ml)	Absolute permeability (md)	$K_{O, Eff}$ (md)	$K_{W, Eff}$ (md)	Oil saturation (%)	Residual oil (ml)
1	13.9	23.9	35	27.2	13.7	20.9	5
2	23.1	39	26.5	14.3	8.3	15.1	5.9
3	13.9	18.9	31.3	21.3	1.2	20.9	4.8
4	13.4	27	31	14.3	4.2	24.8	6.7
5	14.9	25	34.8	21.1	5.2	40.8	10.2
6	18	30	22.8	19.2	2.8	36	10.8
7	16.7	29		21.6	6.7	51.4	14.9
8	15.7	26.5	29.7	21.6	5.4	39.6	10.5
9	18.4	31	103	36.3	20.2	36.8	11.4
10	17.9	31.5	108	39.5	13.8	39	12.3
11	18.2	32	72	39.5	15.9	35.3	11.3
12	18.2	32	72.2	39.5	4.5	35.6	11.4
13	17.4	30	68.7	37.7	4.3	40	12
14	18.2	31.8	60.9	38	5	39.6	12.6
15	18.4	32.5	122.2	48	6	39.4	12.8
16	18.7	33	240.1	47.8	8.8	36.1	11.9

Interfacial tension measurements (IFT) were made by using a spinning drop tensiometer. Each sample was measured three times and in some cases four times for greater accuracy. The crude oil used was 32⁰ API oil with a viscosity of 6.0 cp. The tensiometer readings were taken at room temperature (26⁰ C).

RESULTS

Core Flood Experiments

Table 4.1 summarizes the results of a series of core flood experiments with different biosurfactant concentrations and flow regimes. Little or no oil was recovered at biosurfactant concentrations less than 21 mg/l. At biosurfactant concentrations ranging from 2.75 to 11 mg/l, the amount of residual oil recovered was similar to that of the control that lacked biosurfactant (Core 16, Table 4.1). Oil recovery at these low biosurfactant concentrations are most likely the result of increase in viscous forces due to polymer injection. When the biosurfactant concentration was 21 mg/l (Cores 5 and 6; Table 4.1), additional residual oil was recovered. At a biosurfactant concentration of 39 mg/l (Core 2-5; Table 4.1), the percent of residual oil that was recovered, corrected for residual oil recovered by the polymer alone, was twice that when the biosurfactant concentration was 21 mg/l (Table 4.1). These data indicate that once a threshold value of biosurfactant is reached, residual oil recovery becomes linearly proportional to the biosurfactant concentration. This linear dependence of residual oil recovery on biosurfactant concentration was observed previously in sand pack columns at higher biosurfactant concentrations (12). The threshold value is between 10 to 20 mg/l, which is the critical micelle concentration of the JF-2 biosurfactant (7).

Capillary Desaturation

To determine if the residual oil recoveries by biosurfactant injection were an artifact of the core flooding process (e. g., unusually high residual oil saturations or flow rates that may not be reflective of actual field conditions), a capillary desaturation curve was generated by

measuring the oil saturations at different flow rates. The residual saturations and the capillary numbers obtained with different flow rates are shown in Table 4.3 and this relationship is plotted in Figure 4.1.

Table 4.3. Residual oil saturation and capillary Number with increase flow rates

Q_w (cc/hr)	S_{or,WF} (%)	N_{CP}
2.5	.3667	1.51 E-5
5.0	.3458	3.02 E-5
10.5	.3396	6.34 E-5
20.53	.3292	1.24 E-4
30.86	.3104	1.86 E-4
61.0	.3021	3.68 E-4
123.4	.2688	7.45 E-4
246.9	.2479	1.49 E-3
493.1	.2250	2.98 E-3
576.0	.2250	3.48 E-3

Figure 4.1. Berea sandstone capillary desaturation curve.

Figure 4.2. Comparison of core data with the capillary desaturation curve.

Figure 4.2 shows this same curve as in given in Figure 4.1, with the residual oil saturations obtained for some of the Berea sandstone cores used in the biosurfactant flooding experiments included.

Hysteresis of the oil trapping process results in larger inertial force being required to displace oil from a pore for discontinuous systems, where capillary number is increased in a stepwise manner, compared to a system that is continuous (capillary number does not change) or where capillary number changes continually (15). Because it is expected that the biosurfactant process will be used in fields that have undergone water flooding to near residual oil saturations, the discontinuous process is more representative of the capillary number requirements to displace residual oil by biosurfactants. Comparison of the residual oil saturations of cores used for biosurfactant injection to the capillary desaturation curve shows that in some cases higher and in other cases lower residual oil saturations were obtained. Thus, our experimental model is representative of the expected field conditions. When the capillary

number at a given residual oil saturation for an individual core water flood is lower than that for the same residual oil saturation on the capillary desaturation curve, this would be advantageous from a tertiary recovery point of view since displacement will be a discontinuous process and the capillary number required to mobilize oil would be higher than that required for continuous process.

Analysis of Relationship Between Fractional Oil Recovery and Surfactant Concentration

Table 4.4 summarizes the data that relate biosurfactant concentration to residual oil recovery for Berea sandstone and sand-packed column experiments. The data on residual oil recovery from Berea sandstone are corrected for the amount of oil produced by the polymer alone are included. Injection of the polymer alone in sand-packed columns did not recovery residual oil. These data can be used to relate interfacial tension, biosurfactant concentration, and oil recovery.

Figure 4.3 shows that the fraction of oil recovered by the viscous-biosurfactant solution, either corrected or not corrected for the contribution of the polymer, is linearly dependent on biosurfactant concentration when the concentration is greater than about 10 mg/l. When biosurfactant concentration was close to 10-11 ppm, the fraction of oil recovered was close to zero. The data from sand-packed experimental systems also show a linear relationship between the fraction of oil recovered and biosurfactant concentration. However, the slope of this line differs from that obtained with Berea sandstone cores. This may reflect the differences in the petrophysical properties of the two porous systems or differences in the treatment protocols for biosurfactant injection. With sand packs, a viscous pre-flush ahead of the biosurfactant solution and post flush with different viscosities were used.

Figure 4.3. Plot of tertiary oil recovery from cores (before and after removing the polymer contribution) and sand-packed columns as a function of biosurfactant concentration. The closed symbols represent tertiary oil recoveries using the viscous-surfactant solution from sandstone cores before correcting for the polymer contribution. The open symbols are the recoveries after the 10% residual oil recovered by the polymer solution alone was subtracted from the oil recovery data. Measurements from surfactant treatment of horizontal sand packs were also included in the plot (small closed triangles). In the sand pack experiments, each pack was flooded with a pore volume of viscous surfactant solution. These points are included to provide a greater range of surfactant concentrations over which oil recovery could be analyzed. The single solid circle is the oil recovery fraction after two pore volumes of biosurfactant (11 mg/l) was flooded through a core.

The black circles on the plot (Figure 4.3) represent the fraction of oil recovered when two pore volumes of a 11 mg/l biosurfactant solution was used. After correcting for the contribution of the polymer contribution, a single pore volume treatment at 11 mg/l of the biosurfactant did not recover any oil, but the two pore volume treatment at 11 mg/l of the biosurfactant recovered 0.1 of the residual oil. The adsorption of the biosurfactant to the porous matrix or trapping of the micelles in the pores may have lowered the effective biosurfactant concentration and prevented oil recovery when only one pore volume of the biosurfactant solution was used (6) (8). Adsorption of biosurfactant in the second pore volume would have been reduced, allowing for some residual oil recovery oil. As found with synthetic surfactants,

these data indicate that adsorption and trapping of biosurfactants occur. This probably lowers the biosurfactant concentration below that needed for oil recovery. Biosurfactant-mediated water floods would require large volumes of recovery fluid or a high concentration of biosurfactant.

Table 4.4. Residual oil recoveries from sandstone and sand-packed model systems with different biosurfactant concentrations.

Surfactant concentration (ppm)	Oil recovery (Frac.)	Oil recovery after removing polymer contribution (Frac.)
41	0.39	0.29
38	0.48	0.38
38	0.45	0.35
21	0.27	0.17
21	0.28	0.18
10.5	0.13	0.04
10.5	0.16	0.07
11.0	0.14	0.04
11.0	0.16	0.06
5.5	0.09	0.0
5.5	0.10	0.0
2.75	0.10	0.06
2.75	0.13	0.04
920	0.64	-
920	0.63	-
283	0.53	-
283	0.48	-
43	0.22	-
43	0.22	-

Mathematical Model Relating Oil-water Interfacial Tension to JF-2 Biosurfactant Concentration

The construction and analysis of a mathematical relationship between oil-water interfacial tension (IFT) and biosurfactant concentration, salinity and co-surfactant 2,3-butenediol is presented here. Last year, we reported on the dependence of IFT and bio-

surfactant concentration, salinity, and co-surfactant alcohol using a two-way analysis of variance method (13). We found that, at biosurfactant concentrations made naturally by *B. mojavensis* strain JF-2, IFT between the aqueous and oil phases was lowered by two orders of magnitude in some cases. Increasing salinity from 5% NaCl to 7.5 and 10%, with or without 2,3-butanediol present, increase the interfacial tension. The lowest IFT observed was 0.1 mN/m at 5% NaCl in the presence of 2,3-butanediol.

Here the effect of all three variables on IFT between oil and water is studied and a mathematical relationship between oil-water IFT and bio-surfactant concentration is presented.

Interfacial tension values at different biosurfactant concentrations are shown below in Table 4.5. These data were obtained using biosurfactant samples from different batches of aerobically grown cultures. The method of preparation and composition was the same for each batch. A spinning drop tensiometer was used to measure the data used to calculate IFT. Each measurement was repeated three times for greater accuracy.

Table 4.5. Interfacial tension values at different biosurfactant concentrations.

Concentration of surfactant (ppm)	IFT (Dynes/cm)
58.0	0.35
29.0	0.38
11.6	1.88
54.0	0.168
27.0	0.42
10.8	0.37
57.0	0.10
28.5	1.50
11.4	2.50
11.0	2.54
41.0	1.21
38.0	1.48
21.0	1.50
10.5	2.00
11.0	0.93
5.5	3.00
2.75	4.20

Figure 4.4 shows a stepwise decrease in IFT with increasing biosurfactant concentration. The IFT between crude oil and 5% NaCl brine was measured first and its value was repeatedly found to be 29.0 mN/m. Interfacial tension first decreased from 29 mN/m to 1.0 mN/m as biosurfactant concentration increased from 0.0 to 11.0 mg/l. From 11.0 mg/l to 41.0 mg/l, the IFT stayed steady in a region close to 1.0 dyne/cm. When the bio-surfactant concentration increased beyond 41.0 ppm, IFT declined again with increasing concentration until it reached a region between 54-58 ppm. At this point, IFT was close to 0.1 dyne/cm. On the basis of conservative error at concentrations close to 58 ppm, IFT appeared to remain unchanged at concentrations beyond 58.0 ppm. But this has not been confirmed because 58.0 ppm was the highest concentration obtained in the laboratory.

Two critical concentrations were identified from inspection of Figure 4.4. The first critical biosurfactant concentration is around 11 mg/l. At this concentration, IFT decreases to 1.0 mN/m. IFT value remains unchanged until the biosurfactant concentration reaches 41.0 mg/l. The biosurfactant concentration of 11.0 mg/l may represent the critical micellar concentration (CMC) for the bio-surfactant. At the CMC, the concentration of surfactant molecules is sufficient to form micelles. The CMC of the purified JF-2 biosurfactant has been reported to be 10 mg/l (10), consistent with our findings. The second critical biosurfactant concentration is between 40 and 60 mg/l and this is where another decrease in IFT is observed. This region may be the critical microemulsion concentration (CMEC). When surfactant concentrations reach the CMEC, a third phase called a microemulsion (in addition to the oil and aqueous phases) forms. The microemulsion phase is generally associated with ultra-low IFT values. The microemulsion phase region contains oil, water, and a microemulsion that may

have oil and/or water molecules surrounded the surfactant molecules (6) (8). The two critical biosurfactant concentration regions are indicated with circles in Figure 4.4 below.

Figure 4.4. The relationship between interfacial tension and biosurfactant concentration.

Mathematical Model

The mathematical model used to represent the change in IFT with changing biosurfactant concentration had been previously derived from laboratory experiments on synthetic surfactants (2, 17). It has also been used in an earlier model for biosurfactant-based microbial enhanced oil recovery (19). Approximating IFT through this relationship is straightforward. Studies have shown that other equations may be required where IFT is a function of the equivalent alkane number of the crude oil, salinity, or temperature. The exponent, ES, is an exponent factor that describes the dependency of interfacial tension on biosurfactant concentration. The concentration exponent is reported to be less than unity at low concentrations. The model parameters are given in Table 4.6. The equation is shown below as **Equation (a)**.

$$\text{Log}_{10}(\text{IFT}_{C, \text{Surf}}) = \text{Log}_{10}(\text{IFT}_{\text{Min}}) + (\text{Log}_{10}(\text{IFT}_{\text{Max}}/\text{IFT}_{\text{Min}})) * ((C_{\text{Surf,Max}} - C_{\text{Surf}})/\text{Delsuf})^E \dots (a)$$

Based on our analyses, we will use a different system of nomenclature from **Equation (a)** to identify parameters used to predict IFT as a function of biosurfactant concentration as illustrated in Figure 4.5.

Table 4.6. Parameters used for the model that relates IFT to biosurfactant concentration.

Variable	Value
$\text{IFT}_{C_{\text{Surf}}}$	IFT at surfactant concentration between $C_{\text{Surf,Max}}$ and $C_{\text{Surf,Min}}$
IFT_{Min}	Minimum interfacial tension (dynes/cm)
IFT_{Max}	Maximum interfacial tension (dynes/cm)
$C_{\text{Surf,Max}}$	Maximum surfactant concentration (mg/L)
$C_{\text{Surf,Min}}$	Minimum surfactant concentration (mg/L)
C_{Surf}	Concentration of biosurfactant (mg/L)
Delsuf	$C_{\text{Surf,Max}} - C_{\text{Surf,Min}}$ (mg/L)
ES	Concentration exponent

Figure 4.5. Model parameters used to predict IFT reductions from biosurfactant concentration.

To model the stepwise profile that we obtained, two concentration ranges were identified. One range was between 0.0 mg/l and 41.0 mg/l. In this range, IFT reaches a minimum of 1.0 mN/m once the CMC of 11.0 mg/l is reached and appears to remain constant until 41 mg/l. The value 41.0 mg/l is called the higher critical micellar concentration (CMCH) and 11.0 mg/l is called the lower critical micellar concentration (CMCL). This region is defined by **Equation (b)**. When concentrations exceed 41 mg/l, **Equation (c)** is used to define the relationship between IFT and higher biosurfactant concentrations. The minimum concentration for this region is called the higher critical micellar concentration and the maximum concentration (CMAX) has been assumed to equal a biosurfactant concentration greater than 58.0 mg/l. The IFT reaches a minimum of 0.1 mN/m at a critical microemulsion concentration (CMEC) of 58.0 mg/l and from then IFT is assumed to remain constant with further increases in biosurfactant concentrations.

For bio-surfactant concentrations between 0.0 and 41.0 mg/l, the model is defined by **Equation b**:

$$\text{Log}_{10} (\text{IFT}_{\text{C, Surf}}) = \text{Log}_{10} (\text{IFT}_{\text{Min1}}) + (\text{Log}_{10} (\text{IFT}_{\text{Max}}/\text{IFT}_{\text{Min1}})) * ((\text{C}_{\text{Surf}}, \text{CMCH} - \text{C}_{\text{Surf}}) / \text{Delsuf1})^{\text{ES1}} \dots (\text{b})$$

For bio-surfactant concentrations between 41.0 to 58.0 mg/l and for larger biosurfactant concentrations, the model is defined by **Equation c**:

$$\text{Log}_{10} (\text{IFT}_{\text{C, Surf}}) = \text{Log}_{10} (\text{IFT}_{\text{Min2}}) + (\text{Log}_{10} (\text{IFT}_{\text{Min2}}/\text{IFT}_{\text{Min1}})) * ((\text{C}_{\text{Surf}, \text{Max}} - \text{C}_{\text{Surf}}) / \text{Delsuf2})^{\text{ES2}} \dots (\text{c})$$

Model Prediction

When the biosurfactant concentration is between 0 and 41 mg/l, the model has a specific set of values (Table 4.7). These parameters differ when the concentration exceeds 41 mg/l (Table 4.8). This way, the stepwise behavior of the IFT is modeled by using the same mathematical equation, but with different parametric values. The model prediction is shown in Figure 4.6 below. The values for the parameters were obtained from the laboratory measurements.

Table 4.7. Parameter values for biosurfactant concentrations between 0.0 and 41.0 mg/l

Variable	Value
$C_{Surf, \text{Min}}$ (mg/L)	0.0
$C_{Surf, \text{CMCH}}$ (Higher critical micellar concentration) (mg/L)	0.041
$C_{Surf, \text{CMCL}}$ (Lower critical micellar concentration) (mg/L)	0.011
IFT_{Min1} (dynes/cm)	1.0
IFT_{Max} (dynes/cm)	29
ES1	7.0
Delsuf1 (mg/L)	$C_{Surf, \text{CMCH}} - C_{Surf, \text{Min}}$

Table 4.8. Parameter values for concentrations between 41.0 and 58.0 ppm

Variable	Value
$C_{Surf, \text{CMCH}}$ (mg/L)	0.041
$C_{Surf, \text{Max}}$ (mg/L)	0.080
$C_{Surf, \text{CMEC}}$ (Critical microemulsion concentration) (mg/L)	0.058
IFT_{Min2} (dynes/cm)	0.1
IFT_{Min1} (dynes/cm)	1.0
ES2	3.0
Delsuf2 (mg/L)	$C_{Surf, \text{Max}} - C_{Surf, \text{CMCH}}$

Figure 4.6. Comparison between model prediction (line) and laboratory measurements (squares)

DISCUSSION

We have modified our previous model that related biosurfactant concentration to IFT by incorporating the stepwise behavior of IFT as a function of biosurfactant concentration (19). By using the same relationship with different input parameter values for different concentration ranges of the biosurfactant, we are able to model the changes in IFT behavior more accurately. A single set of parameter values did not model the observations accurately. A maximum biosurfactant concentration of 80.0 mg/l was used. This is an assumed value equal to the critical microemulsion concentration. The model has also been further improved by estimating the model parameters from laboratory data. The concentration exponent, 'ES' has a value greater than one for both critical biosurfactant concentrations. Though one expects that the value of ES should be less than unity for low biosurfactant concentrations (8), no specific surfactant concentration has been defined in the literature where ES would become less than unity. Since a value for ES of 7.0 for the first concentration range and 3.0 in the second concentration range provided a good fit, they were used in the model to simulate biosurfactant-based oil recovery.

REFERENCES

1. **Austad, T., and K. Taugbol.** 1995. Chemical flooding of oil reservoirs 1. Low tension polymer flood using a polymer gradient in three-phase region. Colloids and Surfaces, A: Physiochemical and Engineering aspects **101**:87-97.
2. **Bang, H. W., and B. H. Caudle.** 1984. Modeling of a micellar/polymer process. SPEJ **Dec.**:617-627.
3. **Brusseau, M. L., D. A. Sabatini, G. J.S., and M. D. Annable.** 1999. Surfactant selection criteria for enhanced subsurface remediation. Adv. Chem. Series **725**:8-23.
4. **Desai, J., and I. M. Banat.** 1997. Microbial production of surfactants and their commercial potential. Microbiol. Mol. Biol. Rev. **61**:47-64.
5. **Deshpande, S., B. J. Shiau, D. Wade, D. A. Sabatini, and J. H. Harwell.** 1999. Surfactant selection for enhancing ex situ soil washing. Wat. Res. **33**:351-360.
6. **Dominquez, J. G., and G. P. Willhite.** 1977. Retention and flow characteristics of polymer solutions in porous media. SPEJ **April**:111-121.
7. **Georgiou, G., S. C. Lin, and M. M. Sharma.** 1992. Surface-active compounds from microorganisms. Biotech. **10**:60-65.
8. **Green, D. W., and G. P. Willhite.** 1998. Enhanced oil Recovery, vol. 6. SPE.
9. **Knapp, R. M., J. L. Chisholm, and M. J. McInerney.** 1990. Presented at the Proceedings of the SPE/UH emerging technologies conference.
10. **Lin, S. C., K. S. Carswell, M. M. Sharma, and G. Georgiou.** 1994. Continuous production of the lipopeptide biosurfactant of *Bacillus licheniformis* JF-2. Applied Microbiology and Biotechnology **41**:281-5.

11. **Marsh, T. L., X. Zhang, R. M. Knapp, M. J. McInerney, P. K. Sharma, and B. E. Jackson.** 1995. Mechanisms of microbial oil recovery by *Clostridium acetobutylicum* and *Bacillus* strain JF-2., p. 593-610. In R. S. Bryant and K. L. Sublette (ed.), The Fifth International Conference on Microbial Enhanced Oil Recovery and Related Problems for Solving Environmental Problems. Office of Scientific and Technical Information, CONF-9509173.
12. **McInerney, M. J., S. O. Han, S. Maudgalya, H. Mouttaki, M. Folmsbee, R. Knapp, D. Nagle, B. Jackson, M. Staudt, and W. Frey.** 2001. Development of more effective biosurfactants for enhanced oil recovery. Final Technical Report for DOE DE-AC26-98BC15113. University of Oklahoma.
13. **McInerney, M. J., R. M. Knapp, D. P. Nagle, K. E. Duncan, N. Youssef, M. J. Folmsbee, and S. Maudgalya.** 2003. Development of microorganisms with improved transport and biosurfactant activity for enhanced oil recovery DE-FE-02NT15321. DOE.
14. **Miller, D. J., S.-P. von Halasz, M. Schmidt, and G. Pusch.** 1991. Dual surfactant systems for enhanced oil recovery at high salinities. J. Petr. Sci. Engineer. **6**:63-72.
15. **Moore, T. F., and R. C. Slobod.** 1956. The effect of viscosity and capillarity on the displacement of oil by water. Producers Monthly **Aug**:20-30.
16. **Reed, R. L., and R. Healy, N.** 1977. Some physical aspects of microemulsion flooding: a review., p. 383-437. In D. O. Shah and R. S. Scheckter (ed.), Improved Oil Recovery by Surfactant and Polymer Flooding. Academic Press, Inc., New York.
17. **Saad, N.** 1989. Field scale simulation of chemical flooding, Ph. D. Dissertation, University of Texas, Austin.

18. **Thomas, C. P., G. A. Bala, and M. L. Duvall.** 1993. Surfactant-based enhanced oil recovery mediated by naturally occurring microorganisms. Soc. Petrol. Eng. Reservoir Eng. **11**:285-291.
19. **Xhang, Z.** 1994. Development of microbial oil recovery simulator, Ph. D. Dissertation, University of Oklahoma, Norman.
20. **Yakimov, M. M., M. Amro, M. Bock, K. Boseker, H. L. Fredrickson, D. G. Kessel, and K. N. Timmis.** 1997. The potential of *Bacillus licheniformis* strains for in situ enhanced oil recovery. J. Petr. Sci. Engineer. **18**:147-160.