Development of an *in situ* biosurfactant production technology for enhanced oil recovery

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I. Executive Summary

The long-term economic potential for enhanced oil recovery (EOR) is large with more than 300 billion barrels of oil remaining in domestic reservoirs after conventional technologies reach their economic limit. Actual EOR production in the United States has never been very large, less than 10% of the total U. S. production even though a number of economic incentives have been used to stimulate the development and application of EOR processes. The U.S. DOE Reservoir Data Base contains more than 600 reservoirs with over 12 billion barrels of unrecoverable oil that are potential targets for microbially enhanced oil recovery (MEOR). If MEOR could be successfully applied to reduce the residual oil saturation by 10% in a quarter of these reservoirs, more than 300 million barrels of oil could be added to the U.S. oil reserve. This would stimulate oil production from domestic reservoirs and reduce our nation's dependence on foreign imports. Laboratory studies have shown that detergent-like molecules called biosurfactants, which are produced by microorganisms, are very effective in mobilizing entrapped oil from model test systems. The biosurfactants are effective at very low concentrations. Given the promising laboratory results, it is important to determine the efficacy of using biosurfactants in actual field applications.

The goal of this project is to move biosurfactant-mediated oil recovery from laboratory investigations to actual field applications. In order to meet this goal, several important questions must be answered. First, it is critical to know whether biosurfactant-producing microbes are present in oil formations. If they are present, then it will be important to know whether a nutrient regime can be devised to stimulate their growth and activity in the reservoir. If biosurfactant producers are not present, then a suitable strain must be obtained that can be injected into oil reservoirs. We were successful in answering all three questions. The specific objectives of the project were (1) to determine the prevalence of biosurfactant producers in oil reservoirs, and (2) to develop a nutrient regime that would stimulate biosurfactant production in the oil reservoir.

The prevalence of biosurfactant producers in three carbonate and four sandstone oil-bearing formations in Oklahoma was determined by cultivation-dependent and cultivation-independent approaches. Wells that had production from the formation of interest without inter-mingling of fluids with other formations were selected for analysis. Brine analysis showed that the formations had salinities ranging from 3.2% to 15.6% and calcium concentrations ranging from 1.6 to 13.4 g/l. Sulfate levels were low in all of the brines except in brines from two wells. The sulfide levels were low in all brines sampled. Viable microorganisms were not detected in brine samples collected from any of the formations by agar plating methods whether or not the brine was heat-treated to stimulate germination of *Bacillus* spores. Liquid counting procedures conducted either in the presence or absence of oxygen using medium that matched the salinity of the formation only detected low numbers of heterotrophic bacteria (less that 10, 000 per milliliter). These findings are consistent with those of others investigators that detect only low numbers of microorganisms when traditional cultivation approaches are used. None of the enumeration approaches detected biosurfactant-producing microorganisms. However, when conditions were established to enrich for salt tolerant, heterotrophic microorganisms, biosurfactant activity was detected in these enrichments from all of the

formations. These data suggest that biosurfactant producers were present in the produced fluids from the reservoir, but were either not very numerous or not very active.

We developed several tools to detect biosurfactant-producing microorganisms in produced fluids from oil reservoirs without the need for cultivation. We developed primer DNA sequences to amplify and detect genes required for biosurfactant production by polymerase chain reaction (PCR). The genes targeted were those involved in the synthesis of lipopeptide and rhamnolipid biosurfactants, both of which have been shown to generate low interfacial tensions between oil and aqueous phases needed to recover substantial quantities of entrapped oil. The degenerative primers were highly effective at distinguishing known biosurfactant-producing strains form non-producing strains. The degenerative primers for the lipopeptide biosurfactants were able to detect the presence of two lipopeptide-producing strains in produced fluids from an oil reservoir that was inoculated with these two strains. This is the first time that the efficacy of using an inoculum for MEOR has been conclusively demonstrated. With the above methods, we were able to show conclusively that it is possible to inoculate an oil reservoir with the microorganisms needed for a microbial oil recovery process and to retrieve the same strains from the oil reservoir.

The PCR approaches described above were used to survey produced fluids from seven oil formations that differed in lithology and salinity. Genes for lipopeptide biosynthesis were detected in six of the seven formations. This finding was corroborated by the concomitant detection of members of the *Bacillus subtilis-Bacillus licheniformis* group, a group known to contain many biosurfactant producers, by *gyrA* gene amplification and sequencing. 16S rRNA gene sequence analysis also detected members of this group in formations that had chloride concentrations greater than 10%. We did not detect microorganisms that produce rhamnolipids in any of the formations either by cultivation-dependent or cultivation-independent methods. Both culture-independent and culture-dependent approaches support the conclusion that biosurfactant-producing microorganisms, probably related to the *Bacillus subtilis-Bacillus licheniformis,* are present in many oil formations, even in reservoirs with very high salinities.

We systematically tested a number of nutrient components and various combinations of these nutrients in produced fluids from seven different oil formations to determine the formulation that best stimulated biosurfactant production and whether bioaugmentation (e.g., the use of an inoculum) was beneficial. Glucose and/or molasses, proteose peptone, and nitrate were the critical nutrient components needed for aerobic biosurfactant production. Anaerobic conditions are probably more reflective of the actual environmental conditions that exist in the reservoir. The information obtained with the aerobic nutrient screening allowed us to narrow the number of nutrients to be tested to glucose, molasses, nitrate and a mixture of trace metals. The highest oil-spreading activities were observed when glucose and/or molasses were present and that nitrate and the trace metals mixture stimulated biosurfactant activity. We found that the addition of glucose, nitrate and trace metals stimulated *in situ* biosurfactant production by *Bacillus licheniformis* RS-1 and *Bacillus subtilis* subsp. *spizizenii* strain NRRLB-23049 in a carbonate formation. Our work also shows that this simple nutrient formulation is

effective in stimulating anaerobic biosurfactant formation by *B. licheniformis* RS-1 in several brines that vary in salinity. Maximum biosurfactant production was observed when brines were supplemented with nutrients and *B. licheniformis* strain RS-1. Indigenous biosurfactant activity was detected in many cases, but was always much lower than that observed when an inoculum was used. These data argue that effective *in situ* biosurfactant production requires bioaugmentation. The nutrient combination that gave maximal biosurfactant production by *B. licheniformis* strain RS-1 was a mixture of trace metals, nitrate and either glucose or molasses.

We found that *B. licheniformis* strain RS-1 was a robust strain in respective to its properties useful for MEOR. It grew and produced a biosurfactant in the absence of oxygen. Strain RS-1 was able to grow aerobically in medium with 15% NaCl added and biosurfactant production occurred in medium with up to 10% NaCl added. It also grew over a wide range of pH values from 2 to 10. *B. licheniformis* strain RS- produces an biosurfactant anaerobically that generates ultra-low interfacial tensions needed for significant oil recovery and grows over a wide range of salt and temperature regimes found in many mid-Continent oil reservoirs. These properties make it an ideal strain for use in MEOR.

We injected a glucose-nitrate-mineral nutrient mixture and two biosurfactant-producing, *Bacillus* strains into two wells to correlate *in-situ* metabolism and growth with oil recovery. Two wells producing from the same Viola formation were each inoculated with 500 bbl of tank battery brine mixed with nutrients (glucose, sodium nitrate and trace metals) as well as *Bacillus licheniformis* RS-1 and *Bacillus subtilis* subsp. *subtilis spizizenii* NRRL B-23049. Analysis of production water indicated *in-situ* metabolism of the nutrients, growth of the injected strains and other heterotrophic fermenting bacteria, and the production of bacterial products including the biosurfactant. Both wells had a peak lipopeptide biosurfactant concentration of 20 and 28 mg/L, respectively, and an average carbon balance of glucose used and metabolic products and cells made of 91%. The increase in biosurfactant, acids, alcohols and carbon dioxide during the first 5 days after commencement of production corresponded directly with increasing oil recovery. Furthermore, wellhead measurements of total produced water, the water/oil ratio (WOR) and the percent oil cut as well as separation tank battery production data indicated that a corresponding net increase of at least 183 bbl in oil recovery occurred in during the first 100 days of sampling.

2. Introduction

The long-term economic potential for enhanced oil recovery (EOR) is large with more than 300 billion barrels of oil remaining in domestic reservoirs after conventional technologies reach their economic limit. However, cost-effective technologies must be developed to recover this entrapped oil. Actual EOR production in the United States has never been very large, less than 10% of the total U. S. production even though a number of economic incentives have been used to stimulate the development and application of EOR processes. Chemical flooding technologies such as micellar or alkaline-surfactant-polymer flooding displace tertiary oil efficiently [12], but these approaches have several significant problems. The processes are technically complex and have generally been marginally economic. The chemical solutions that contain surfactant, cosurfactant and sometimes polymer are expensive. Chemical losses due to adsorption, phase partitioning, trapping and by-passing when mobility control is not maintained can be severe [12-14]. The only way to compensate for these losses is by increasing the volume of the surfactant solutions [12]. This complexity is further complicated by reservoir heterogeneity and the necessary large capital investment. All of these factors make chemical flooding a high-risk process. The development of more cost-effective technologies to recover entrapped oil is clearly needed.

Microbially enhanced oil recovery (MEOR) has several unique advantages. MEOR processes do not consume large amounts of energy as do thermal processes, nor do MEOR processes depend on the price of crude oil as do many chemical processes. Because microbial growth occurs at exponential rates, it should be possible to produce large amounts of useful products rapidly from inexpensive and renewable resources. The results of several field projects show that MEOR can be economical. Injection of nutrients to stimulate microbial biomass production to improve sweep efficiency and oil drainage produced incremental oil for as little as \$15 per m^3 [15, 16]. The in situ production of acids, gases and solvents (end products of microbial energy metabolism) produced incremental oil for as little as three dollars per barrel [17-19]. Some microorganisms produce biosurfactants that generate very low interfacial tensions between oil and aqueous phases, comparable to that obtained with synthetic surfactants [20, 21]. In particular, the lipopeptide biosurfactant produced by *Bacillus mojavensis* strain JF-2 reduces the interfacial tension between oleic and aqueous mixtures to very low levels (<0.01 mN/m) [22, 23]. The low interfacial tensions generated by biosurfactants indicate that residual oil could be recovered if one can selectively stimulate biosurfactant production in the reservoir.

The target resource base for biosurfactant flooding is the same as the resource base considered for chemical methods in the National Petroleum Council (NPC) in-depth analysis of enhanced oil recovery potential [24]. The estimated potential of chemical methods ranged from 2.5 to 13.5 billion barrels. That target still exists since synthetic surfactants have not been broadly applied. A significant part of that potential could be produced by microbial methods. As an example, the fluvial-dominated deltaic group of reservoirs is estimated to contain more than 5 billion barrels of oil [25] and half of these reservoirs are considered to be at risk for abandonment by 2010. The Delaware-Childers Bartlesville Sandstone Reservoir in northeastern Oklahoma is a typical member of this

group and initially contained about 120 million stock tank barrels of oil. The reservoir has been extensively gas and water flooded since its discovery in 1908. Currently, it is at close to residual oil saturation (estimated at 25%) [26]. Further reducing the oil saturation to 15% by microbial surfactants could yield 12 million barrels (2 million meters^3). Bryant [27] screened the U.S. DOE Reservoir Data Base for candidate MEOR reservoirs that originally contained more than 20 million barrels of oil. She found more than 600 reservoirs containing over 12 billion barrels of oil in 10 major producing states. If MEOR could be successfully applied to reduce residual oil saturation by 10% in a quarter of these reservoirs more than 300 million barrels of oil could be added to the U.S. reserve base.

To be successful, a microbial surfactant flooding processes must address the problems associated with chemical flooding technologies. In contrast to synthetic surfactants, the use of biosurfactant offers no long-term risk to the environment since biosurfactants and the materials from which they are made are readily degraded [28-31]. The microbial processes used in field pilots have not been technically complex. The nutrients have been uncomplicated and inexpensive. It is not known if adsorption or trapping losses are significant, but since the surfactants are produced in situ they are not expected to move far to reach the oil-water interface to mobilize the oil. Clearly, more work is needed to quantify the interactions between biosurfactants and the rock matrix at the pore scale. In laboratory tests, microbial cells and products other than the surfactant appear to serve as mobility control agents so that little bypassing has been observed [15, 32, 33]. This would help deal with reservoir heterogeneity. Finally, capital requirements for pilot studies have been modest. Only slight modifications to normal water flood injection equipment to mix the nutrients with the injection water have been needed. The most successful MEOR process by Brown and his coworkers (2000) resulted in oil recovery for as little as \$15 per m^3.

With such a promising outlook for biosurfactant flooding, one must ask the question why the technology has not been implemented more extensively. The results of several laboratory studies show that residual oil recoveries by biosurfactant-producing microorganisms are low and inconsistent [34-39]. There are several potential reasons for this including the inconsistent production of the biosurfactant, loss of biosurfactant activity with extended incubation, and nutrient limitations that delay the growth of the requisite microorganisms. The inconsistent performance of MEOR has led to criticisms whether sufficient quantities of microbial products can be produced in oil reservoirs at rates sufficient to result in economic oil recovery [40]. In our previous DOE project (DE-FC26-02NT1531), we addressed many of these concerns. We have obtained a number of microbial strains that have high biosurfactant activity that is not lost after extended incubation [41]. We have identified the nutrients needed to support luxurious anaerobic growth of biosurfactant-producing bacteria, i. e., deoxyribonucleotides often found in commercially available meat digests [42]. Finally, we have found that substantial mobilization of residual oil occurs at low biosurfactant concentrations (20 to 50 mg/l) if a polymer and 2,3-butanediol, a product of the biosurfactant-producing bacteria, are present [43].

Thus, we feel that we have answered a number of the criticisms that have been leveled at MEOR processes. However, additional information is needed to move biosurfactant-mediated oil recovery from laboratory-based studies to actual field applications. First, we must know if oil fields contain biosurfactant-producing microorganisms or whether these cells must be injected. Cell injection adds increased cost, which a company would want to avoid if possible. Secondly, we must know what nutrients to inject to stimulate and maintain in situ biosurfactant production. This is a much more difficult challenge than other MEOR processes faced since we are trying to produce a metabolite that is not a direct result of the energy metabolism of the cell as are acids, gases, solvents or the microbial cell itself. Our preliminary data indicates that a mixture of glucose, proteose peptone (a commercially available meat digest) and 27 mM nitrate selectively stimulates the growth of biosurfactant-producing bacteria in groundwater samples [44]. We need to demonstrate that this approach is effective in the presence of microbes indigenous to oil reservoirs and that it will also lead to in situ biosurfactant production.

Factors affecting oil recovery.

A large amount of oil remains in small pores or dead-end pores after waterflooding. The forces that entrap this oil control the ultimate oil recovery factor in most reservoirs, especially in reservoirs near their economic limit of production. The viscous and capillary forces that hold this oil in place are expressed as a ratio called the capillary number (N_{ca}) [45]:

$$N_{ca} = (\mu_w v_w)/(\square_{ow})$$

where μ_w is the viscosity, v_w is the flux of fluid, and $\square_{ow}$ is the oil-water interfacial tension (IFT). Large changes in the capillary number (about a factor of 1000) are needed for substantial oil recovery [46]. The Chun-Huh relationship demonstrates that as the IFT decreases, the mass of oil solubilized per mass of surfactant increases [47]. Capillary (trapping) number curves illustrate that there is a threshold IFT below which significant mobilization occurs. Since large changes in viscous forces are only possible for the recovery of heavy oil, the reduction in interfacial tension by surfactants is the only way to achieve large changes in capillary number. Chemical flooding techniques have very high microscopic displacement efficiencies in laboratory studies [48-50], but economics and other concerns have prevented widespread use of these technologies. Several biosurfactants generate ultra-low interfacial tensions [22, 23] and engineering analysis indicates that this may result in significant oil recovery factor [51].

Biosurfactants

Biosurfactant production has traditionally been viewed as a mechanism to enhance hydrocarbon biodegradation by increasing the apparent aqueous solubility of the hydrocarbon [52-61] or by enhancing the interaction of the microbial cell with the hydrocarbon [60, 62, 63]. By dispersing or increasing the apparent solubility of poorly soluble hydrocarbons, especially polynuclear aromatic compounds, these compounds become more bioavailable and, thus, more amenable to biodegradation [57, 61, 64-66]. However, there are several biosurfactants that generate the low interfacial tensions between the hydrocarbon and the aqueous phases required to mobilize entrapped

hydrocarbon [21, 67-69]. In particular, the lipopeptide biosurfactant produced by *Bacillus* species [21, 34, 36, 38, 39, 70, 71] and the rhamnolipid produced by various *Pseudomonas* species [59] reduce the interfacial tension between the hydrocarbon and aqueous phases to very low levels (<0.01 mN/m) [22, 67, 69]. In addition, the critical micelle concentrations are low (20-50 mg/l), indicating that the biosurfactants are effective at very low concentrations [67]. Figure 2.1 shows the structures of lipopeptide and rhamnolipid biosurfactants. Often, microorganisms produce a series of these compounds that differ in the length and branching of the fatty acid side chains. However, there may also be differences in the number of sugars or amino acids as well as the type of amino acids present in these molecules. How these variations in structure affect the interfacial activity of the compounds is not well understood, but slight

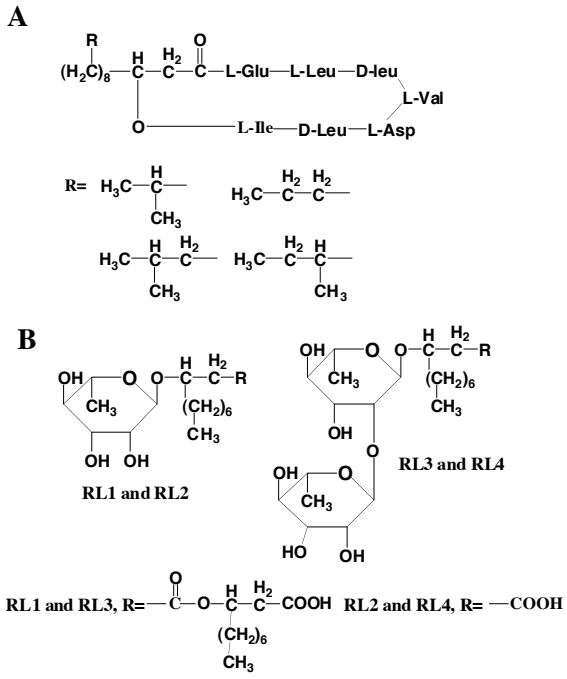


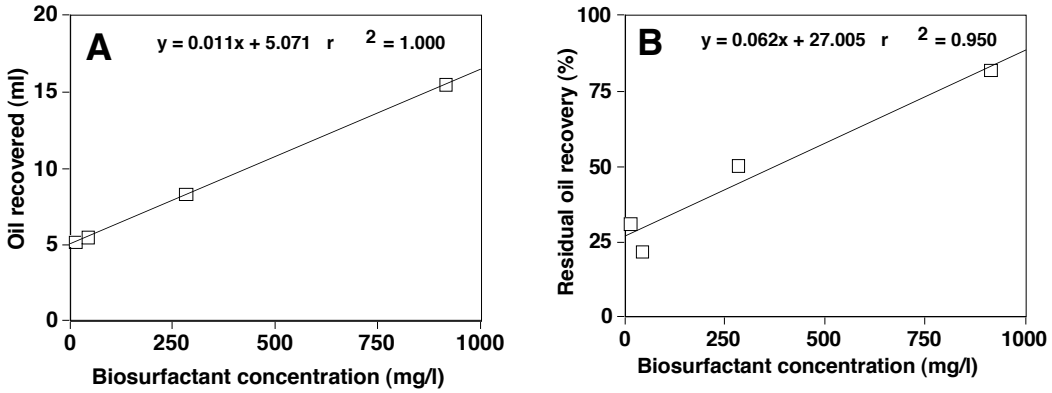
Figure 2.1. Biosurfactant structures [72]. A. The lipopeptide biosurfactants made by *Bacillus licheniformis* and, B. The rhamnolipids made by *Pseudomonas* species.

variations in structure do affect activity [73]. Since mixtures of synthetic surfactants are known to be more effective than pure surfactants in mobilizing complex hydrocarbons such as crude oil [47], biosurfactants have a natural advantage. The use of biosurfactants to mobilize residual hydrocarbon has met with mixed results. From 20 to 90% of hydrocarbons present in contaminated soils or oil shale cuttings were removed in the presence of biosurfactants [56, 74]. The rhamnolipid biosurfactant produced by certain strains of *Pseudomonas* was 20 times more effective in solubilizing hexadecane than some synthetic surfactants [55] and mobilized up to 75% of the residual hexadecane from sand-packed columns [75, 76]. However, the number of pore volumes required (40 to 70) was large. Residual oil is recovered when a biosurfactant-producing bacterium and the nutrients needed to support growth are introduced into sandstone cores [38, 77, 78], but residual hydrocarbon recoveries were often low (5 to 20%) and required multiple pore volumes of recovery fluid.

We found that the lipopeptide biosurfactant produced by *B. mojavensis* strain JF-2 mobilized large amounts of residual hydrocarbon from sand-packed columns (Figure 2.2) [43] at concentrations about 10 to 100-fold lower than typically used for synthetic surfactant-enhanced recovery process [79-82]. Approximately 82% of the residual hydrocarbon in sand-packed columns was recovered when less than one pore volume of culture fluid containing about 900 mg/l of the biosurfactant was injected into the column.

We have recently found that about 50% of the residual oil is recovered from sandstone cores when low biosurfactant concentrations (about 50 mg/l) were used (S. Maudagalya and R. M. Knapp, unpublished data). We obtained about 5.3 milliliters of residual hydrocarbon per milligram of biosurfactant. *B. mojavensis* strain JF-2 grows well and produces biosurfactant concentrations ranging from 0.1 to 0.7 g/l with cheap renewable resources. Culture fluids that contain 56 mg/l of the biosurfactant generate low interfacial tensions (about 0.2 mN/M) consistent with the high residual oil recoveries obtained when using these culture fluids. Other laboratories have reported residual oil recovery by in situ biosurfactant production [36, 38, 39] and, in one case, residual oil recoveries up to 39% were reported [34].

Figure 2.2. Effect of increasing concentrations of the JF-2 biosurfactant on oil recovery from sand-packed columns flooded to residual oil recovery. A. Volume of oil recovered. B. Percent residual oil recovery.



In addition to the biosurfactant, a small molecular weight alcohol (2,3-butanediol) and a polymer were required [43]. The role of the polymer was most likely to provide mobility control to prevent the oil bank from dissipating before it reached the effluent end of the sand pack. The role of 2,3-butanediol is unclear, but it may act to prevent surfactant liquid crystal formation or to increase the effective surfactant concentration as found with other alcohols. We should note that 2,3-butanediol is a common fermentation product of *Bacillus* sp. [83], produced at concentrations of around 5-10 mM by our bacterium. Thus, this chemical will not have purchased, but will be made naturally by the bacterium.

The ratio of moles of oil recovered per mole of biosurfactant present was about 100 times greater than the reported molar solubility ratios (MRS) for synthetic surfactants. The rhamnolipid biosurfactant was also shown to have a MSR 20 times greater than alkyl benzyl sulfonate surfactants [55]. Thangamani and Shreve [55] argued that the rhamnolipid structure results in a large volume, low-density micelle that accommodates more hydrocarbon than alkyl benzyl sulfonate micelles. However, in our work, an oil bank formed, which suggests that once mobilized, the oil formed a separate phase that may not have required large amounts of biosurfactants to maintain. These data are very encouraging in that they suggest that low concentrations of biosurfactants may allow

supersolubilization or mobilization of hydrocarbon contaminants at much lower concentrations than observed with synthetic surfactants.

Selective stimulation of in situ biosurfactant production.

Our laboratory studies clearly show that biosurfactants mobilize significant amounts of residual oil at biosurfactant concentrations made naturally by the cell (e. g., around 30 to 50 mg/l). The next major hurdle to overcome is to develop an approach to produce biosurfactants in the oil reservoir. Many individuals and companies naively think that all one has to do is inject cells and nutrients and the appropriate activity will occur. However, this approach ignores the microbial ecology of the oil reservoir. All oil reservoirs that have been studied contain diverse and active microbial communities [84-89]. Thus, the nutrients could be used by any number of different microorganisms and there is no guarantee that the injected bacterium will be able to compete with indigenous organisms. While it is known that oil reservoirs contain active and diverse microbial communities, we do not know whether biosurfactant-producing bacteria commonly occur in oil reservoirs. If they do, then we only need to develop a nutrient package and injection protocol to selectively stimulate them. Even if one can stimulate the growth of biosurfactant-producing bacteria, there is no guarantee that the biosurfactant will be made since this compound is not directly linked to the energy metabolism of the cell. Thus, we must first understand what triggers biosurfactant production in natural communities.

Our hypothesis is that biosurfactant-producing bacteria are common in oil reservoirs. We based this hypothesis on several lines of evidence. *Bacillus* species and phylogenetically related bacteria are commonly detected in oil production fluids [90, 91] and 85% of the 160 strains of different *Bacillus* species that we surveyed produced biosurfactants [41]. Thus, it is likely that oil reservoirs will contain biosurfactant producers. In addition, many of the biosurfactant-producing strains that have been used in MEOR have been isolated from oil field production fluids [34, 36, 92-94]. Lastly, biosurfactant-producing bacteria have been detected in a number of undisturbed and contaminated soils indicating that such organisms are widely distributed in nature [95]. We tested this hypothesis by determining the prevalence of biosurfactant-producing bacteria in produced brines and surfaces from production wells from a number of different oil reservoirs that vary in lithology and brine chemistry.
Certain strains of *Bacillus subtilis, B. mojavensis*, and *B. licheniformis* produce as the cyclic lipopeptides biosurfactants such as surfactin and lichenysin [96]. These biosurfactants share a common mode of assembly through the action of peptide synthetases and a thioesterase [97]. The genetics and control of surfactin production have been extensively studied in *B. subtilis* [98, 99] and genes homologous to the peptide synthetases and thioesterase have been described from *B. licheniformis* [97]. A gene probe and set of primers based on the sequence of the peptide synthetase *srfA* of *B. subtilis* developed in the McInerney lab detected the presence of *srfA* in *B. mojavensis* strain JF-2 (McInerney et al., 2001) and 21 additional strains of *B. subtilis* subspecies *subtilis*, *B. subtilis* subspecies *spizizenii*, and *B. mojavensis* isolated from three deserts on two different continents (unpublished data, Youssef 2003). The correspondence between detection of *srfA* by polymerase chain reaction (PCR) and the ability of the strain to produce surface-active compounds was 90%. Therefore, this gene can be used to

determine the prevalence of biosurfactant-producing bacteria from various reservoirs with a high degree of confidence. PCR was used because of its greater accuracy and sensitivity compared to other techniques (Gruntzig, et al. 2001; Harms, et al., 2003). Because of the large difference in gene sequence between *licA* and *srfA-A* (Konz et al. 1999), we had to design another set of primers to detect the homologous *licA* sequence in *B. licheniformis* strains. Similar to the situation for *Bacillus*, the *rhlR* gene was used to detect an essential gene for rhamnolipid production in *Pseudomonas aeruginosa* and related species. These molecular approaches will allow us to detect biosurfactant producers in brine and surface samples even if we have difficulty in culturing these organisms.

Whether biosurfactant-producing bacteria are present or not in the formation, the next step will be to selectively stimulate their growth and activity. We hypothesize that we can selectively stimulate the growth of these organisms by the types and concentrations of nutrients that are injected into the formation. During our previous DOE funded project, we found that the addition of glucose, 27 mM nitrate and Proteose peptone (a commercially available meat digest) to groundwater inoculated with our biosurfactant producer (*Bacillus mojavensis* strain JF-2) selectively stimulated its growth and metabolism. The presence of 2,3-butanediol was detected when Proteose peptone was added and a very high percentage of the viable microbial population was biosurfactant-producing bacteria when 27 mM nitrate was used. We need to replicate this experiment to ensure that this approach will be effective in oil reservoirs and that this approach will also lead to in situ biosurfactant production.

Project Objectives

The ultimate goal of the project was to move biosurfactant-mediated oil recovery from laboratory investigations to actual field applications. In order to achieve this objective, it was first necessary to determine if oil reservoirs contain biosurfactant-producing microorganisms. If biosurfactant producers are not present, then inoculation f the oil reservoirs biosurfactant-producing microorganisms would be necessary. Secondly, it is also necessary to know the types of nutrients needed to stimulate biosurfactant production in the reservoir.

The specific objective of this project were:

(1) to determine the prevalence of biosurfactant producers in oil reservoirs and thus determine the need for cell injection, and

(2) to test the efficacy of nutritional supplements to stimulate growth and biosurfactant production in oil reservoir brines.

Scope of Work

The prevalence of indigenous bacteria that produce rhamnolipid or lipopeptide biosurfactants was determined by cultivation-dependent and cultivation-independent methods. We developed polymerase chain reaction (PCR) methods to detected genes involved in the production of lipopeptide and rhamnolipid biosurfactants in DNA extracted from production fluids obtained from reservoirs with different salinities. We also surveyed these brines for the presence of biosurfactant producers by using a number of different cultivation approaches. We systematically tested different nutrients to

determine the optimal formulation that stimulates biosurfactant production in these brines. Our work shows that biosurfactant producers are prevalent but the bioaugmentation (e.g., the addition of an inoculum) may be the recommended strategy to stimulate biosurfactant production in oil reservoirs.

3. Detection and stimulation of biosurfactant activity in oil field brines

Abstract

The stimulation of biosurfactant production *in situ* may be an effective method to recover crude oil that remains entrapped in oil reservoirs as current recovery methods reach their economic limit. Knowledge of the prevalence of biosurfactant producers in oil reservoirs is needed to determine whether stimulation of indigenous microorganisms is possible or whether inoculation will be required. The prevalence of biosurfactant producers in two carbonate and four sandstone oil-bearing formations in Oklahoma was determined by cultivation-dependent methods. Wells that had production from the formation of interest without inter-mingling of fluids with other formations were selected for analysis. Brine analysis showed that the formations had salinities ranging from 3.2% to 15.6% and calcium concentrations ranging from 1.6 to 13.4 g/l. Sulfate levels were low in all of the brines except in brines from two wells. The sulfide levels were low in all brines sampled. Viable microorganisms were not detected in brine samples collected from any of the formations by agar plating methods whether or not the brine was heat-treated to stimulate germination of *Bacillus* spores. Aerobic and anaerobic most probable number analyses using Plate count broth (PCB) with or without 5% NaCl showed growth in low numbers (23 and 240 cells/ml) only in two of the formation fluids. Low numbers of heterotrophic microorganisms (ranging from 10 to 10^4 cells/ml) were detected with Peptone-Yeast Extract-Tryptone-Glucose (PYTG) and one-tenth-strength PYTG media with and without 10% NaCl, but no biosurfactant activity was detected. Biosurfactant activity was detected in enrichment cultures of all brines, but was much less than that observed when the enrichments were augmented with a known biosurfactant producer.
Molecular functional gene analysis showed the presence of surfactin or lichenysin (*srfA*/*licA*) and gyrase (*gyrA*) genes (indicating the presence of microorganisms related to *Bacillus subtilis* and/or *Bacillus licheniformis*) in only one enrichment culture. The *rhlR* gene (rhamnolipid gene regulator) was not detected in any of the enrichments. Nutrient amendment stimulated indigenous biosurfactant activity in all formation fluids. Maximum biosurfactant production was observed when brines were supplemented with nutrients and a known biosurfactant producer (in this case *B. licheniformis* strain RS-1). The nutrient combination that gave maximal biosurfactant production by *B. licheniformis* strain RS-1 was a mixture of trace metals, nitrate and either glucose or molasses. These results suggest that biosurfactant producers related to the *Bacillus subtilis-Bacillus licheniformis* are present but not numerous in the produced fluids from different oil reservoirs. Nevertheless, effective *in situ* biosurfactant production occurred when the brines were augmented with a known biosurfactant producer (*B. licheniformis* strain RS-1) and nutrients (glucose, nitrate, and metals).

Overview

Bacillus, *Clostridium* and *Pseudomonas* species have often been used in the MEOR process because of their ability to form spores (*Bacillus* and *Clostridium*), surfactants, gases, alcohols, solvents and sometimes polymers (22, 32, 41). Spores have the ability to withstand reservoir conditions and at the same time germinate and produce active microorganisms to produce the products that can be used for MEOR activities. The other products discussed earlier have significant use in the MEOR process. Kianipey et al. (69)

investigated the mechanisms of oil displacement by three different microorganisms (*Bacillus* sp., *Pseudomonas* sp. and *Clostridium* sp.) and found that *Bacillus licheniformis* strain JF-2, decreased surface tensions, increased the wettability and sweep efficiency of recovery fluids in unconsolidated sand-packed flow cells. The production of biosurfactants in addition to gas production, and the change in wettability by the strain JF-2 was believed to contribute to the reduction in the residual oil saturation. *Pseudomonas aeruginosa* was less effective for oil recovery because this bacterium had negligible gas production and no significant increase in the wettability of the sand matrix was observed. *Clostridium acetobutylicum* produced significant amounts of CO_2, which reacted with the brine forming carbonic acid, which could increase the wettability of the system. From these results, it is apparent *Bacillus* species have the most potential in reducing residual oil saturation.

Biosurfactant production by *Bacillus* species haven been used in innumerable cases for improving oil recovery due to its properties of reducing interfacial and surface tensions between oil and water phases. However other properties of the bacteria such as biomass formation have also been utilized in the MEOR process. Several researchers have used *Bacillus* species to improve sweep efficiency (2, 7). Zekri et al. (70) showed that microbial flooding with *Bacillus* species isolated from UAE hot water streams decreased the residual oil saturation to around 11% and also post microbial flooding the permeability dropped from 19 md to 2.4 md in six different limestone cores. Jenneman et al. (71) and Raiders et al. (72) showed that the in situ growth of *Bacillus* species markedly reduced permeability (almost by 100%) while *Pseudomonas* species caused change in the permeability of Berea sandstone cores.

Given the importance of biosurfactant production for oil recovery, it is important to know whether biosurfactant-producing bacteria are present in oil reservoirs. *Bacillus* species and *Pseudomonas* species have been isolated from a range of different environments. Bodour et al. (43), found that 11 out of 16 isolates from undisturbed and contaminated soils belonged to the *Bacillus* genus. These isolates also had surface tension activity of as low as 33.1mN/m. Four of the isolates were *Pseudomonas* species, two of which were positive for the presence of the one of the genes involved in rhamnolipid production (*rhlB*). *Bacillus licheniformis* strain BAS50 and *Bacillus* strain JF-2 were both isolated from oil reservoirs in Germany (7, 49) and Carter County, Oklahoma, (6, 50), respectively. Both produced biosurfactants that substantially lower the interfacial tension between water and crude oil under conditions that exist in the petroleum reservoir (6, 49, 50). Almeida et al. (13) have found strains of the genera *Bacillus*, *Pseudomonas* and *Micrococcus* species in an oil reservoir from the Recôncavo basin of Bahia, Brazil. Several *Bacillus* species and phylogenetically-related bacteria were found in production fluids from oil reservoirs in Russia (51, 52). While the above anecdotal observations suggest that *Bacillus* species potentially useful for oil recovery are present in oil reservoirs, clearly a more comprehensive study is needed that directly assesses the presence of biosurfactant producers in different petroleum reservoirs.
For *in situ* microbial processes such as biosurfactant production is to be successful, we must first determine whether stimulation of indigenous biosurfactant-producing microorganisms is possible or whether such microorganisms will have to be introduced.

Here, I use a cultivation-dependent approach to determine the presence of biosurfactant-producing *Bacillus* species in the oil reservoirs with varying salinity and lithologies. Biosurfactant-producing species were detected in only a few formations and only after enrichment indicating that their numbers are low when they are present. So, a nutrient mixture that would stimulate maximum biosurfactant production by a strain of *Bacillus licheniformis* that could serve as an inoculum was determined.

Materials and Methods

Sampling sites

Brine samples were collected from six different Oklahoma geological formations from the Pennsylvanian age. The Hunton and Wewoka formations are carbonate formations while the Earlsboro, Gilcrease, Skinner flood and Hart formations are sandstone formations (Table 3.1). However, intermingling of limestone with sandstone occurs in the Gilcrease formation.

Wells from these formations are considered marginal since the daily oil production was about 10 barrels or less per day. The Earlsboro and Skinner flood reservoirs were water flooded with Wilcox formation water and Layton sand formation water, respectively. The other formations were not water flooded. The injection wells for the Earlsboro formation were treated with biocides while the production wells were treated with anti-corrosion fluids (supplier's product number 1630). The injection wells are about 1 km away from the production wells so the biocide treatment was not expected to impact the microbiological analyses. The Gilcrease and Skinner flood wells were also treated with anti-corrosion fluids. All of the anti-corrosion fluids mentioned above are not listed as biocides and are not expected to impact the microbiological analyses.
Brine samples were collected from production wells that produced fluids from the specified and not from neighboring formations. None of the production wells were treated with biocides and each production well was running for 3-7 days prior to sampling. Ideally, two production wells from each formation were sampled for each formation. This was not possible for Wewoka, Gilcrease, and Hart formations because only one well produced enough brine for sampling or the other wells produced fluids from multiple formations.

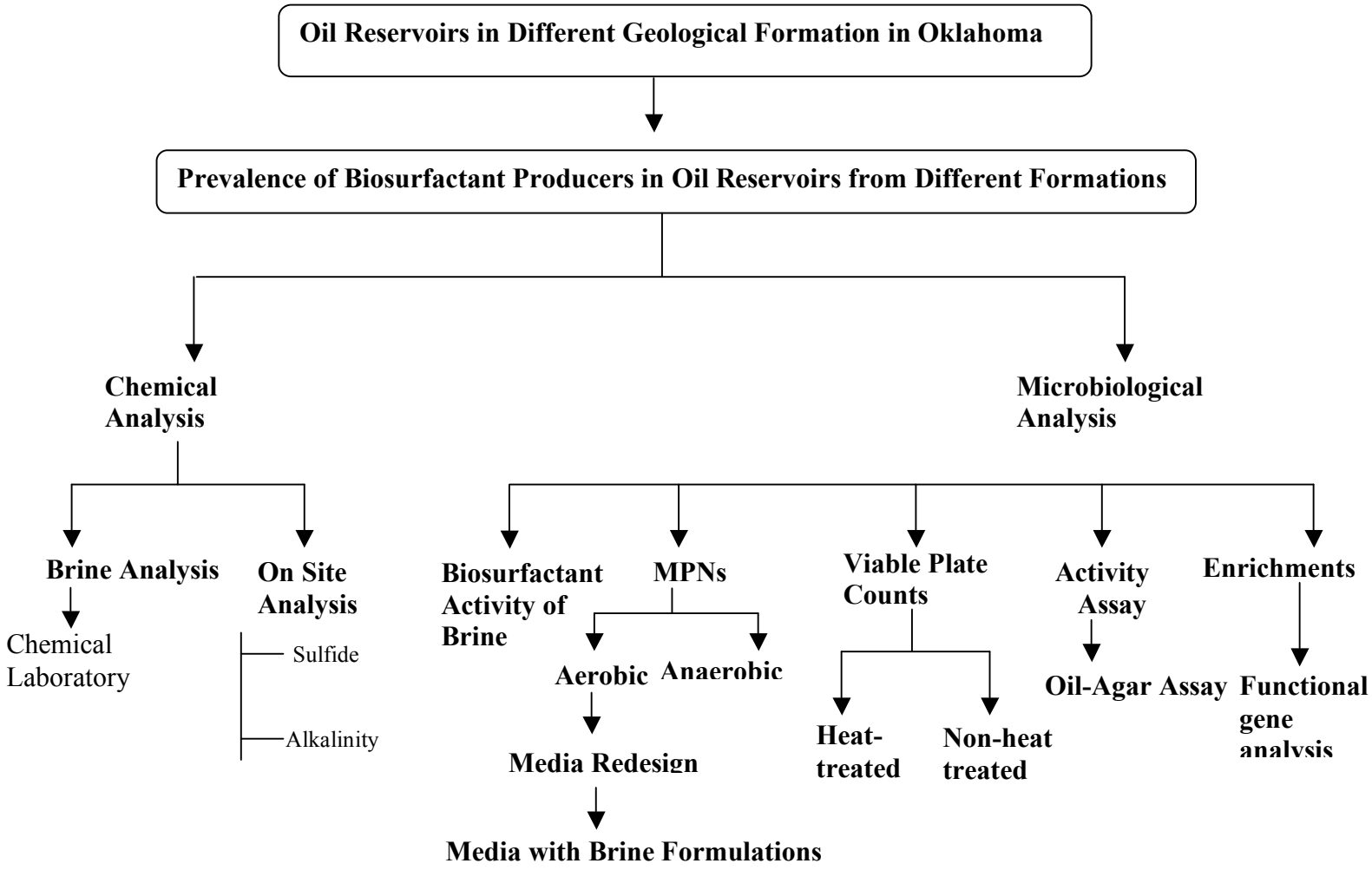
Sample Collection

The samples were collected in each of two sterile, 1.5-liter Biobags (Nalgene) and 2-liter separatory flasks for each well. The brine was allowed to overflow to flush out the entrapped air and to reduce the amount of crude oil in the container. The biobags were capped and the flasks were closed with rubber stoppers and immediately stored on ice during transit back to the laboratory. The samples were then stored at 4^{o}C until analyzed. The samples were processed for chemical, microbial and molecular analyses as illustrated in Figure 3.1.

Table 3.1. Characteristics of the oil reservoirs sampled.

Formation	County	Comment	Lithology	Well name	Legal Description	Total depth(m)	Avg. oil production (Barrels /day)
Hunton	Pontotoc County	Not water flooded	Carbonate	ROB11	SE.NE SW19-5N-5E	646.2-694.9	
				ROB2	S:19, T:5N, R:5E	728.2	10.5
Wewoka	Pontotoc County	Not water flooded	Carbonate	ROB14	S:19, T:5N, R:5E	818.4	10.5
Earlsboro	Seminole County	Naturally flooded with Wilcox formation water. Injection wells, but not production wells were treated with biocide. Anti-corrosion treated.	Sandstone	ERL1	S:13, T:9N, R:5E	n/a	1.2
				ERL5	S:18, T:9 N, R:6E	n/a	0.5
Gilcrease	Seminole County	Not water flooded. Anti-corrosion treated.	Sandstone Limestone	GIL1	S:6, T:11N, R:7E	n/a	n/a
Skinner flood	Logan County	Naturally flooded with Layton sand formation water. Production wells treated with anti-corrosion.	Sandstone	TUBER 1	SE:NW:SEC. 36-16N-3W	n/a	~5
				DAVIS1	NE:SE:SEC. 36-16N-3W	n/a	~5
Hart (Deese)	McClain County		Sandstone	HEWIIT 4-32	NW NE SEC. 32-5N-3W	n/a	

Figure 3.1. Flow chart illustrating the processing of samples from the different formations



Chemical Analysis:

Chemical analysis of the aqueous portion of the sample (brine) was done by the Environmental Resource Technologies, LLC, Ada, Oklahoma. The analyses of sulfide, CO_2 and dissolved oxygen measurements were carried out on site by using the Hach and Chemetrics methods (HACH Chemical Company, Loveland, Colorado). The rest of the tests and analyses were done off-site by using EPA certified methodologies.
To avoid any sulfide loss, 10µl and 100 µl of the brine samples were fixed with DMPD (*N,N- dimethyl- p- phenylenedi*amine sulfate) reagent (per liter, Zn $(CH_3COO)_2 \cdot 2H_2O$, 1g; DMPD•HCl, 1g; and concentrated H_2SO_4, 50 ml) on the site. Before reading the absorbance, 0.1 ml of a ferric chloride reagent (per liter, $FeCl_3 \cdot 6H_2O$, 250g) was added to each fixed brine sample to develop the color. The absorbance was measured at 660 nm after 10 min. The concentration of sulfide was calculated from a sulfide standard curve ranging from 0 to 50 mg/L of sulfide (53).

To remove particulates in brine, 5 ml of a brine sample was filtered by using a 0.45-µm filter. The alkalinity test was performed by the two-dye titration method by using the HACH kit (HACH Chemical Company. Loveland, Colorado). The filtrate was poured into the HACH titration bottle. The first indicator (phenolphthalein) did not change the color of the brine so the titration was done with concentrated H_2SO_4 after the addition of the second indicator, bromocresol green, until the sample turned pink. The end point was when the color did not change with further H_2SO_4 addition. The tube and the titration bottle were washed thoroughly with distilled water and the water was discarded.

Biosurfactant activity assay

Oil spreading assay as described by Youssef et al. (10) was used to determine the presence of surface-active compounds in each brine sample. In a large Petri dish (25cm in diameter), 50 ml of distilled water was added followed by the addition of 20 µl of crude oil. Next, triplicate 10 µl samples of un-filtered brine were carefully added onto the oil surface. The diameter of clear zone was measured in triplicate for each sample. An overnight culture of *Bacillus licheniformis* strain RS-1 was used as a positive control for biosurfactant activity. *B. licheniformis* strain RS1 produces a lipopeptide biosurfactant and was isolated from an oil field, (D. R. Simpson, unpublished data).

Enumeration Methods

The three-tube most probable number technique was used to enumerate heterotrophic organisms in the production fluids from the different formations. The procedure was modified to use 96-well plates where three wells filled with medium were used at each dilution, which ranged from 10^{-1} to 10^{-8}. Duplicate MPN series were done for each brine sample. The MPN numerations were performed with the following media: Plate Count Broth (PCB), (Difco, Inc.); PCB with 5% NaCl; Peptone-Yeast Extract-Glucose-Tryptone (PYTG) medium as described by Balkwill et al. (8); and one-tenth strength PYTG. To select for halotolerant, *Bacillus*-like biosurfactant producers, Medium E (a

medium with sucrose, yeast extract, and nitrate) (6) and Medium E without the addition of 5% NaCl were also used for the MPN enumerations. The analysis of the chemical composition of the brines from the Earlsboro and Gilcrease showed that $CaCO_3$ and NaCl were the major components of the brine (Table 4). For these two formations, MPN enumerations were also conducted with the one-tenth PYTG medium that was supplemented with $CaCO_3$ and NaCl that corresponded to the concentration present in each formation. These MPN series were serially ten-fold diluted to 10^{-6}. For the Skinner flood and Hart formations, the MPN analyses were also done with one-tenth strength PYTG medium that was supplemented with NaCl to match the chloride concentration of the brine.

The temperature of the brine samples ranged from 20^{o} C to 24^{o} C. Assuming that some heat was lost as the fluids were produced from the formation, it is likely that the reservoir temperatures were still within the mesophilic range for microbial growth. For this reason, each MPN series was incubated at 37^{0}C for 11 days. The highest dilutions that were positive for growth were tested for biosurfactant production by the oil-spreading assay as described earlier. *Bacillus licheniformis* strain RS-1 was used as a positive control for all of the above MPN enumerations. All of the above MPN enumerations were done aerobically. In addition, MPN enumerations with PCB and Medium E were also done anaerobically.

Anaerobic enumerations were achieved by using the modified 3 tube MPN method with 96 well plates. The medium used was PCB, PCB with 5% NaCl, Medium E, and Medium E with 5% NaCl. Each MPN series was done in duplicates. The medium was dispensed in the 96-well plate in an anaerobic chamber and the production fluids from each well were serially diluted from 10^{-1} to 10^{-8}. The plates were then placed in an airtight container, which was then removed from the anaerobic chamber and incubated at 37^{o} C for 5 days. Anaerobic overnight culture of *B. licheniformis* strain RS-1 culture grown in PCB was used as a positive control for all the anaerobic enumerations.
Enumerations were also done with the MOPS minimal medium described by Palmer et al. (57) to enumerate *Pseudomonas*-like biosurfactant producers in the production fluids from Skinner flood and Hart formations. *Pseudomonas aeruginosa* ATCC strain 10145 was used as the positive control for enumerations with the MOPS minimal medium. The 96-well plates were incubated aerobically at 37^{o} C for 11 days. The dilutions that were positive for growth were tested for biosurfactant production by the oil-spreading assay as described earlier.

Viable agar plate counts were done with Plate Count Agar (PCA) (Difco, Inc.) with and without 5% NaCl and with one-half strength PCA with and without 5% NaCl. An aliquot of each brine was serially, ten-fold diluted in phosphate buffer (per liter, K_2HPO_4, 13.9 g; KH_2PO_4 2.7 g; pH of 7.2) and 100 µl of each dilution from 10^{-1} to 10^{-4} was plated onto triplicate agar plates of the above media. *B. licheniformis* strain RS-1 was used a positive control and was streaked onto an agar plate of each medium. Agar plates that were streaked with sterile phosphate buffer served as the negative control.
To select for *Bacillus*-like, biosurfactant producers, one milliliter of each brine sample was incubated at 85^{0}C for 20 min to kill vegetative cells and to germinate any spores that

may be present in the brine. The heat-treated brine was then serially diluted and inoculated onto the agar medium described above. To screen for biosurfactant producers, an oil overlay medium was prepared as described by Morikawa et al. (54), except that Luria-Burtani (LB) agar was used. The plates were then inoculated with diluted brine or heat-treated brine as described above. After the agar solidified approximately 35 µl of sterile crude oil was spread over each LB agar plate.
All agar plates were incubated at 37^{o} C for at least 48 hr.

Enrichments

Since MPN analyses did not detect biosurfactant producers, enrichments were established by adding 10 ml of brine from to each of two bottles containing 20 ml of either full-strength Plate Count Broth (PCB), one-half strength PCB, or one-tenth strength PCB. A second series of enrichments with duplicate bottles for each of the above media was prepared and each bottle was inoculated with 500 µl of an overnight culture of *B. licheniformis* strain RS-1 to test for any inhibitory substances that may be present in the brine. Each enrichment was incubated aerobically at 37^{0} C for 11 days with shaking at 90 rpm. The oil-spreading assay as described by Youssef et al. (10) was used to determine biosurfactant activity. After 11 days of incubation, each enrichment was centrifuged (7000 rpm; 20 min; 4^{o}C) and the pellet was re-suspended in TE buffer and stored at -20^{o} C until used for DNA extraction and analysis.

Molecular approaches

DNA was extracted from the enrichment cultures by the conventional Phenol-Chloroform-Iso Amyl alcohol method (55).

Taq polymerase chain reaction (PCR) was done by using a thermocycler with the DNA extracted from the enrichment cultures. The universal eubacterial 16S ribosomal DNA gene primers, forward 27 F (5' AGA GTT TGA TCM TGG) and reverse 1492R (5' TAC CTT GTT ACG ACT T) were used to identify the presence of bacterial DNA. The *srfA/licA* primers, *sfrA/licA* F (5' CAA AAK CGC AKC ATA CCA CKT TGA G) and *srfA/licA* R (5' TCA TAR AGC GGC AYA TAT TGA TGC) were used to amplify the surfactin- and lichenysin-like genes that may be present. These primers were developed by Dr. D. Randall Simpson (personal communication). The *gyrA* primers, *gyrA* F (5' CAG TCA GGA AAT GCG TAC GTC CTT) and *gyrA* R (5' CAA GGT AAT GCT CCA GGC ATT GCT), designed by Roberts et al. (65) were used to amplify a portion of the gyrase gene to indicate the presence of *Bacillus subtilis* and related species. The *rhlR* primers, *rhlR* F (5' CTG CGC TCC WCG GAA ATG GTG) and *rhlR* R (5' TCT GGA TGW YCT TGW GGT GGA AGT TC), were used to detect the potential to make rhamnolipids found in *Pseudomonas* species. The *rhlR* primers were designed by D. R. Simpson (personal communication).

For the 16S rDNA PCR amplification, the reaction mixture consisted of 5µl of 10X buffer; 5µl of 5M Betaine; 4µl of 25mM $MgCl_2$; 1µl of 10mM dNTPs; 1.2 µl each of 5pmol/µl of 16S forward and reverse primer, 0.24 µl of Platinum *Taq* polymerase

(Invitrogen), 3 µl of 5ng/µl (15ng) of template DNA. The reaction was made up to 50µl reaction by the addition of 29.36µl of deionized water. The thermocycler was programmed for an initial denaturation for 5min at 94^{o}C, 30 cycles of 1min at 94^{o}C for denaturation, 2min at 42^{o}C for annealing, 2min at 72^{o}C for extension followed by a 6 min at 72^{o}C for a final extension. A 1465bp amplicon was expected at the end of the PCR reaction.
The reaction mixture for the *srfA/licA* PCR consisted of 5µl of 10X buffer, 2.5µl of 25mM $MgCl_2$, 1µl of 10mM dNTPs, 1µl each of 5pmol/µl of *srfA/licA* forward and reverse primers, 0.5µl of DMSO, 0.25 µl of Platinum *Taq* Polymerase (Invitrogen) and 3 µl of 5ng/µl (15ng) of template DNA. The reaction was made up to a 50µl reaction by the addition of 35.75µl of deionized water. The thermocycler was programmed for an initial denaturation for 5min at 95^{o}C, 10 cycles of 35seconds at 94^{o}C for denaturation, 35secondsmin at 55^{o}C to a touchdown to 50^{o}C for annealing, 45seconds at 72^{o}C for extension. An additional 23 cycles was programmed at 95^{o}C for denaturation, 35seconds at 50^{o}C for annealing, 45seconds at 72^{o}C for extension followed by 6 min at 72 ^{o}C for a final extension. A 273bp amplicon was expected at the end of the PCR reaction.
A reaction mixture that was used for the *srfA/licA* PCR was used for the *rhlR* PCR except for the different primers. The thermocycler was programmed for an initial denaturation for 5min at 95^{o}C, 10 cycles of 40seconds at 95^{o}C for denaturation, 40seconds at 57.3^{o}C to a one-degree touchdown to 52.3^{o}C each for 40seconds for annealing, 45seconds at 72^{o}C for extension. An additional 23 cycles was programmed at 95^{o}C for denaturation, 40seconds at 52.3^{o}C for annealing, 45seconds at 72^{o}C for extension followed by 7 min at 72^{o}C for a final extension. A 300bp amplicon was expected at the end of the PCR reaction.

The reaction mixture for the *gyrA* PCR consisted of 2.5µl of 10X buffer, 2µl of 25mM $MgCl_2$; 2µl of 10mM dNTPs; 0.5µl each of 5pmol/µl of *gyrA* forward and reverse primers, 0.125 µl of Platinum *Taq* polymerase (Invitrogen) and 2 µl of 5ng/µl (10ng) of template DNA. The reaction was made up to 25µl by the addition of 15.375µl of deionized water. The thermocycler was programmed for an initial denaturation for 4min at 94^{o}C, 35 cycles of 1 min at 94^{o}C for denaturation, 1min at 48^{o}C for annealing, 1min 30seconds at 72^{o}C for extension followed by a 10min cycle at 72^{o}C for a final extension. A 1024b amplicon was expected at the end of the PCR reaction.
To check for non-specific amplification, *B. licheniformis* strain RS-1 DNA was used as a positive control for all the PCR reactions except for *rhlR* PCR where *Pseudomonas aeruginosa* ATCC 10145 DNA was used. For all the PCR reactions deionized water was used as a negative control for all the reactions. Gel electrophoresis was performed on all the PCR end products to visualize the banding patterns. A 1% Agarose (Promega) gel was used for the electrophoresis process. A 1Kb Ladder was also used as a marker to identify the appropriate size of the band achieved.

Nutrient treatments

A series of nutrient components were tested to determine the optimal nutritional formulation needed to maximize the production of biosurfactant activity in the brine. The final concentration of each nutrient was: molasses (1%)(v/v); glucose (10g/L); proteose

peptone (10g/L); glycerol (10ml/L); NaCl (20g/L); $NaNO_3$ (1g/L); and K_2HPO_4 (1g/L). Combinations of each of the nutrients were also tested separately by deleting one or two components at a time. Single-component and two-component additions were also tested. The treatments where NaCl, $NaNO_3$ and K_2HPO_4 were the only nutrients present in the medium were not done, as there was no carbon source present. Each treatment was done in triplicate in 24-well plates in which brine from the formation was used to bring the final volume of each well to 2 ml. An identical set of nutrient treatments prepared in brine was inoculated with 200 µl of an overnight culture of *B. licheniformis* strain RS-1. As a positive control, each nutrient treatment was also prepared with sterile distilled water and inoculated with 200 µl of an overnight culture of *B. licheniformis* strain RS-1. For the Skinner flood formation, a reduced nutrient treatment regime was used where only molasses, glucose, and $NaNO_3$ were tested. Biosurfactant activity was measured by the oil-spreading assay after 3, 5, and 7 days of incubation.

Another series of nutrient treatments was conducted as described above except that oxygen was excluded. The final concentration of the nutrients tested was: molasses (1%) (v/v); glucose (10g/L); $NaNO_3$ (1g/L); and Wolins' metals solution (25ml/L) (56). Combinations of each of the nutrients were also tested separately by deleting one or two components at a time. Single-component and two-component additions were also tested. Each nutrient treatment was done in triplicate in Balch tubes and brine from the formation was used to bring the final volume to 5 ml. The headspace of the serum tubes was replaced with N_2 gas and was sealed with a rubber stopper after the addition of the nutrient components. An identical set of nutrient treatments was inoculated with 250 µl of an overnight culture of *B. licheniformis* strain RS-1 grown anaerobically in PCB. As a positive control, each nutrient treatment was also prepared with sterile deionized water and inoculated with 250 µl of an overnight culture of *B. licheniformis* strain RS-1 grown anaerobically in PCB. The uninoculated nutrients prepared in sterile deionized water served as the negative control. Anaerobic nutrient treatments were tested with the brines obtained from Hunton, Wewoka, and Earlsboro formations only. Biosurfactant activity was measured by the oil-spreading assay after 3, 5, 7 and 14 days of incubation.

Results

Chemical assays

In situ microbial processes require an understanding of the reservoir factors that control microbial growth (66). The composition and ionic strength of the aqueous phase controls both the physiological types of microorganisms present as well as their growth and activity. Table 3.3 summarizes the chemical composition of the produced brines from six Oklahoma formations. The dominant cations were sodium, calcium, potassium and magnesium with chloride being the dominant anion. The presence of these components along with magnesium and iron indicate that the brines contain the major inorganic nutrients required for microbial growth. In addition, the most commonly used microbial sulfur sources, sulfate and sulfide, were present in all of the brines. Many microorganisms require CO_2, bicarbonate, or carbonate for growth. The alkalinity values indicate that carbonate ions were present in all of the sampled brines. As expected, the

brines obtained from the carbonate formations (Wewoka and Hunton formations) had higher alkalinity values than those from sandstone formations, except the brine from the Hart formation also had high levels of alkalinity.

Assuming that chloride is the major ion that contributes to the salinity, the salinity of the sampled reservoir fluids ranged from 2.1% to 15.6%. Total dissolved solids of the brines ranged from about 39,700 to 259,500 mg/L. The wide range of salinity and total dissolved solids indicates that ionic strength will be an important factor that governs microbial growth and activity in Oklahoma reservoirs. It may be difficult to obtain a single biosurfactant producer that can function over this wide range of salinity so treatments may have to be tailored to match specific reservoir conditions.

Sulfate and sulfide were also analyzed to determine the potential for sulfate reduction, which often leads to souring and corrosion. Sulfate levels were low (less than 10 mg/L) in all but two of the brines. The brines from the Gilcrease and Skinner flood formations had sulfate concentrations ranging from 36.2 to about 180 mg/L, respectively. Sulfide concentrations were low (less that 4.0 mg/L) in all of the sampled brines, even in the brines that contained higher sulfate concentrations. These data indicate that the potential for sulfate reduction is low in all formations except the Skinner flood and Gilcrease formations where the high levels of sulfate may lead to an increase in sulfide production during nutrient treatments.

To determine if the sulfide concentration and alkalinity changed as a result of sample storage, analyses for these two brine components were also done on site immediately after sample collection. The on-site, sulfide concentrations (Table 3.4) agreed well with those obtained during laboratory analysis. Both protocols indicate low levels of sulfide were present in all of the brines that were analyzed. Alkalinity was measured on site with a HACH test kit (HACH chemical company), which is not as precise as the titration method used in the laboratory. However, there was good agreement between the on-site and in laboratory alkalinity data for four of the eight brine samples (Tables 3.3 and 3.4). For the other four brine samples, the laboratory analyses were lower by a factor of about 2 to 3 fold, indicating that the bicarbonate equilibrium had changed with sample storage.

Table 3.3. Temperatures and chemical composition of produced water samples.

Analysis	Formation and well designations								
	Hunton		Wewoka	Earlsboro		Gilcrease	Skinner flood		Hart
	ROB11	ROB2	ROB14	ERL1	ERL5	GIL1	TUBER1	DAV1	HEWITT 4-32
Temperature (^{o}C)	21.2	22.1	22.3	19.2	19.4	22.0	*N/D* [a]	*N/D*	*N/D*
pH	6.58	6.93	6.61	6.67	7.11	6.42	5.88	5.85	5.11
TDS (mg/L)	57674	62114	39662	179712	188462	218792	259460	257194	184600
CO_2 (mg/L)	40	35	75	33	21	23	N/D	N/D	N/D
Alkalinity (mg/L)	163	60	285	62.5	42.5	62	36.3	35	207.5
HCO_3 (mg/L)	163	60	285	62.5	42.5	62	36.3	35	207.5
SO_4 (mg/L)	1	1.4	0.5	6.6	0.5	36.2	181.8	178	7.4
Sulfide (mg/L)	3.5	1.4	4.0	0.3	0.1	0.2	*N/D*	*N/D*	*N/D*
Cl (mg/L)	32000	35000	21500	94500	102500	113500	159000	158000	130500
Na (mg/l)	14600	14200	10200	109000	92900	6190	72960	66710	50680
Ca (mg/L)	5740	3890	2600	13400	13400	7360	14050	13320	8544
K (mg/L)	2930	1480	1240	1270	352	673	697.2	800	470.1
Mg (mg/L)	863	850	395	2120	1130	0.506	2084	1990	1299
Mn (mg/L)	0.55	0.744	0.347	9.69	4	3.6	6.7	6	15.11
Fe (mg/L)	26.1	58.1	15.2	55.2	10.4	45.8	69.85	63.53	1009

[a] N/D Not done

Table 3.4. The concentrations of sulfide and alkalinity of reservoir fluids measured by on-site analyses.

Formation	Well Names	Sulfide Concentration (mg/L)	CO_3^{2-} (mg/L)
Hunton	ROB 11	2.5	180
	ROB 2	0.7	120
Wewoka	ROB 14	2.4	300
Earlsboro	ERL1	0	120
	ERL5	0	120
Gilcrease	GIL1	0	140
Skinner flood	TUBER1	0.1	60
	DAVIS1	0.1	40
Hart	HEWITT 4-32	*N/D* [a]	*N/D*

[a] N/D Not done

Presence of surface-active molecules in brine

To test for the presence of surfactant-like molecules, the oil-spreading assay described by Youssef et al. (10) was done with the production fluids from the different formations. Table 3.5 summarizes the results obtained from the oil-spreading assay. Earlsboro and Gilcrease formation fluids showed the presence of surface-active molecules due to the ability of the brine to clear the oil film on water. However, the brine from these formations had oil mixed with the brine and the hydrocarbon may have contributed to the oil spreading activity. Little or negligible oil-spreading activity was observed in brines from the other wells. As a comparison, a 24-hour culture fluid of a known biosurfactant producing bacterium, *B. licheniformis* strain RS-1 had an oil-spreading activity of 2.6 cm.

Table 3.5. Presence of surface-active materials in produced fluids from different formations.

Hart	HEWITT 4-32	0.3±0.14

Formation	Well Names	Diameter of clearing (cm)[a]
Hunton	ROB11	BDL[b]
	ROB2	BDL
Wewoka	ROB14	BDL
Earlsboro	ERL1	0.3±0.28
	ERL5	0.5 ±0
Gilcrease	GIL1	0.6 ±0.01
Skinner flood	TUBER1	BDL
	DAV1	BDL

[a] The positive control used was culture fluid of *Bacillus licheniformis* strain RS-1, which showed 2.6 cm of oil spreading.
[b] BDL Below detection limit. Oil spreading activity less than 0.2 cm was considered below detection limit.

Enumerations

Most probable number (MPN) enumerations were performed to estimate the numbers of heterotrophic organisms in the formation fluids. The Plate count broth (PCB) medium was used because other researchers were able to enumerate aerobic and anaerobic heterotrophic organisms from oil field brines with this medium (62). Medium E is known to support the growth of biosurfactant-producing bacteria in the genus *Bacillus* (56, 71). The results of the MPN are summarized in Table 3.6. Heterotrophic bacteria were not detected with either aerobic or anaerobic media in any of the brine samples except those obtained from the Skinner flood and Hart formations. Oil spreading assay done with distilled water gave a clearing of 0.1 to 0.2 cm. Clearing less than 0.2 cm was considered below detection limit.

No growth was observed at any dilution when Medium E with and without 5% NaCl was used. The highest cell number observed was about 10^2 cells/ml in brine from the DAVIS 1 well (Skinner flood formation) with PCB without salt under aerobic conditions. The brines from the TUBERVILLE 1 (Skinner flood formation) and HEWIIT 4-32 (Hart formation) wells had low levels of aerobic heterotrophic growth (23 cells/ml). No oil-spreading activity was observed in any of the medium wells that were positive for growth.

Balkwill et al. (8) and Musselwhite et al. (67) used one-tenth strength PYTG medium to enumerate microorganisms in aquifer sediments and found concentrations as high as 10^7 cells per gram. The MPN analysis of reservoir fluids was modified to use PYTG and one-tenth strength PYTG medium. With these media, heterotrophic bacteria were detected in all of the reservoir brine samples (Table 3.7). Microbial numbers were low, less than 100 cells/mL in most brine samples. The maximum number of cells detected was about 10^4 cells/ml in brine from the Robertson 11 well (Hunton formation). Of the five brine samples where the number of heterotrophs exceeded 100 cell/mL, four were from the low salinity reservoirs (<4% NaCl) (Hunton and Wewoka) and one was from a high salinity reservoir (>10% NaCl). It did not appear that the concentration of organic compounds (full strength versus one-tenth strength medium formulations) affected the enumeration microorganisms from the reservoir brines.

The one-tenth PYTG medium used for enumerations studies for the brines obtained from the Earlsboro and Gilcrease formations, was supplemented with 10% NaCl and the respective $CaCO_3$ concentration (1.3% for the Earlsboro formation and 0.5% for Gilcrease formation) to match the brine composition. Similar MPN values were obtained with supplemented or unsupplemented media (see Table 3.8). No oil-spreading activity was observed in the wells that were positive for growth.

Interestingly, when *B. licheniformis* strain RS-1 was used as the positive control, the cells counts were 2.4 x 10^5 and 2.4x10^4 with one-tenth strength PYTG supplemented with 10% NaCl and one-tenth strength PYTG supplemented with 10% NaCl and $CaCO_3$ concentration, respectively. These data indicate that *B. licheniformis* may be able to grow over a wide range of salinities.

Enumerations using one-tenth strength PYTG supplemented with NaCl to match the salinity of the brine were also done with samples from Skinner flood and Hart formations. Again, there was little difference in cell counts (23 to 43 cells/mL) regardless of whether the ionic strength of the medium reflected that of the brine (Table 3.9). Biosurfactant production measured by the oil-spreading assay in the wells that were positive for growth showed no detectable activity. *B. licheniformis* showed growth in medium with salt concentrations as high as 15%.

Standard plate counts using a variety of different media were also done to enumerate microorganisms in the brines. The brine samples were also heat-treated to germinate spores that may be present in the formation waters. The media with 5% NaCl was also used to select for halotolerant biosurfactant bacilli. However, none of these approaches was effective in enumerating microorganisms (Table 3.10). The oil agar (54) also did not detect the presence of any culturable microorganisms. This medium was used to help screen for the presence of biosurfactant producers, which would form a clearing in the oil layer around the colony.

MPN enumerations were also done with the MOPS minimal medium described by Palmer et al. (57) to detect the presence of *Pseudomonas*-like microorganisms in the produced fluids from Skinner flood and Hart formations. Low numbers of microorganisms were detected with this medium, 23 and 2.4 x 10^2 cells/ml for the Skinner flood and Hart formation brines, respectively (Table 3.11). Each dilution well that was positive for growth was checked for biosurfactant activity with the oil-spreading assay but no oil-spreading activity was detected.

Table 3.6. Most probable number of aerobic and anaerobic microorganisms in produced fluids from different formations with PCB and Medium E.

		Media							
		PCB[a]		**PCB+5%NaCl**		**Medium E**[b]		**Medium E+5%NaCl**	
Formation	Well Name	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
Hunton	ROB11	BDL[c]	BDL	BDL	BDL	BDL	BDL	BDL	BDL
	ROB2	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Wewoka	ROB14	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Earlsboro	ERL1	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
	ERL5	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Gilcrease	GIL1	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Skinner floods	TUBER1	23[e]	*N/D*[d]	*N/D*	*N/D*	BDL	*N/D*	NG	*N/D*
	DAV1	240[e]	*N/D*	*N/D*	*N/D*	BDL	*N/D*	NG	*N/D*
Hart	HEWITT 4-32	23[e]	*N/D*	*N/D*	*N/D*	BDL	*N/D*	NG	*N/D*

[a] PCB Plate count broth (Difco, Inc.) [b] Medium E is a minimal mineral medium with sucrose, nitrate and yeast extract
[c] BDL Below detection limit. Cell numbers less than 3 cells/ml were considered below detection limit.
[d] N/D Not Done. [e] Oil spreading assay showed no clearing in the tubes that were positive for growth

Table 3.7. Most probable number of aerobic microorganisms with PYTG and one-tenth-strength PYTG media as described by *Balkwill et al*. (8).

Formation	Well name	PYTG (cells/ml) [a]	Oil spreading (cm)	1/10 PYTG (cells/ml) [a]	Oil spreading (cm)
Hunton	ROB11	2.1×10^4	BDL[b]	3.6	BDL
	ROB2	11	BDL	1.1×10^2	BDL
Wewoka	ROB14	3.5×10^3	BDL	4.610^3	BDL
Earlsboro	ERL1	92	BDL	9	BDL
	ERL5	9	BDL	3.6	BDL
Gilcrease	GIL1	NG[c]	BDL	3.6	BDL
Skinner flood	TUBER 1	23	BDL	43	BDL
	DAVIS 1	2.9×10^2	BDL	23	BDL
Hart	HEWITT 4-32	23	BDL	23	BDL

[a] *B. licheniformis* was used a positive control showed cells numbers greater than 2.4×10^5 were obtained with PYTG and one tenth strength PYTG.
[b] BDL Below detection limit. Oil spreading activity less than 0.2 cm was considered below detection limit
[c] NG No growth

Table 3.8. Enumeration results by the MPN method with PYTG media supplemented with 10% NaCl and the respective brine concentration of $CaCO_3$.

Formation	Well names	1/10 PYTG +10% NaCl (cells/ml)	Oil spreading (cm)	1/10 PYTG + 10% NaCl +$CaCO_3$ (cells/ml)	Oil spreading (cm)
Earlsboro	ERL1	9.1	BDL[a]	3.6	BDL
	ERl5	28	BDL	2.1	BDL
Gilcrease	GIL1	15	BDL	NG[b]	BDL

[a] BDL Below detection limit. Oil spreading activity less than 0.2 cm was considered below detection limit
[b] NG No growth

Table 3.9. Enumeration results by the MPN method with one-tenth PYTG supplemented with 15% NaCl and 13% NaCl, respective to the brine concentration of Skinner flood and Hart formations.

Formation	Well names	1/10 PYTG + 15% NaCl[a] (cells/ml)	Oil spreading (cm)	1/10 PYTG + 13% NaCl (cells/ml)	Oil Spreading (cm)
Skinner	TUBER 1	23	BDL [c]	*N/D*	*N/D*
	DAVIS 1	23	BDL	*N/D*	*N/D*
Hart	HEWITT 4-32	*N/D*[b]	*N/D*	33	BDL

[a] Cell numbers of approximately 10^2 cells/ml were obtained with *B. licheniformis*, which was used a positive control.
[b] N/D Not done
[c] BDL Below detection limit. Oil spreading activity less than 0.2 cm was considered below detection limit

Table 3.10. Attempts to enumerate microorganisms by using viable plate count approaches. For each medium, the brine with and without heat treatment was used.

	Media							
	PCA[a]		PCA+5%NaCl		½PCA [b]		½PCA+5%NaCl	
Well Name	Heat-treated	Not-Heat treated	Heat-treated	Not-Heat treated	Heat-treated	Not-Heat treated	Heat-treated	Not-Heat treated
ROB11	BDL[c]	BDL	BDL	BDL	BDL	BDL	BDL	BDL
ROB2	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
ROB14	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
ERL1	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
ERL5	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
GIL1	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
TUBER1	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
DAV1	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
HEWITT 4-32	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL

[a] PCA Plate count agar (Difco Inc.)
[b] ½ PCA Half strength Plate count agar
[c] BDL Below detection limit. Plate counts less than 20 cells per plate were considered below detection limit

Table 3.11. MPN enumeration results with MOPS minimal medium described by *Palmer et al*. (57) to detect *Pseudomonas*-like microorganisms.

Formation	Well names	MOPS Minimal media [a] (cells/ml)	Oil Spreading (cm)
Skinner flood	TUBER 1	2.4×10^2	BDL [b]
	DAVIS 1	2.4×10^2	BDL
Hart	HEWITT 4-32	23	BDL

[a] *P. aeruginosa* ATCC strain 10145 was used a positive control and cells numbers greater than 2.4×10^5 cells/ml were obtained
[b] BDL Below detection limit. Oil spreading activity less than 0.2 cm was considered below detection limit

Enrichments

Enrichments to enhance aerobic biosurfactant activity were done using PCB (Plate count broth), one-half strength PCB and one-tenth strength PCB. Growth was observed in all of the above media, suggesting the presence of active microorganisms, but in low numbers. All of brines except the brine from the Skinner flood formation contained indigenous biosurfactant-producing microorganisms as indicated by the presence of oil-spreading activity in at least one of the media not inoculated with *B. licheniformis* (Table 3.12). Enrichments with the Earlsboro (ERL1) well brine with PCB medium had the highest indigenous oil spreading activity (1.1 cm). However low indigenous biosurfactant activity was stimulated in the enrichments from other formation fluids with the PCB, one half-strength or one tenth-strength PCB medium except in the Skinner flood formation fluids. The addition of a known lipopeptide biosurfactant producer *B. licheniformis* increased biosurfactant activity in brine enrichments from the Hunton and Gilcrease formations. *B. licheniformis* was apparently able to produce its biosurfactant in Skinner flood formation brine since indigenous oil-spreading activity was not detected (Table 3.12). These data show that many brines contain indigenous biosurfactant-producing microorganisms whose activity can be stimulated by the nutrients present in PCB (glucose, yeast extract and tryptone). Apparently, their numbers are too low to be detected by standard enumeration procedures. Bioaugmentation with a known biosurfactant producer (e.g., *Bacillus licheniformis*) was most effective in brines with low salinities (<5% NaCl).

Table 3.12. Enrichments to enhance biosurfactant activity in produced fluid samples from the different formations.

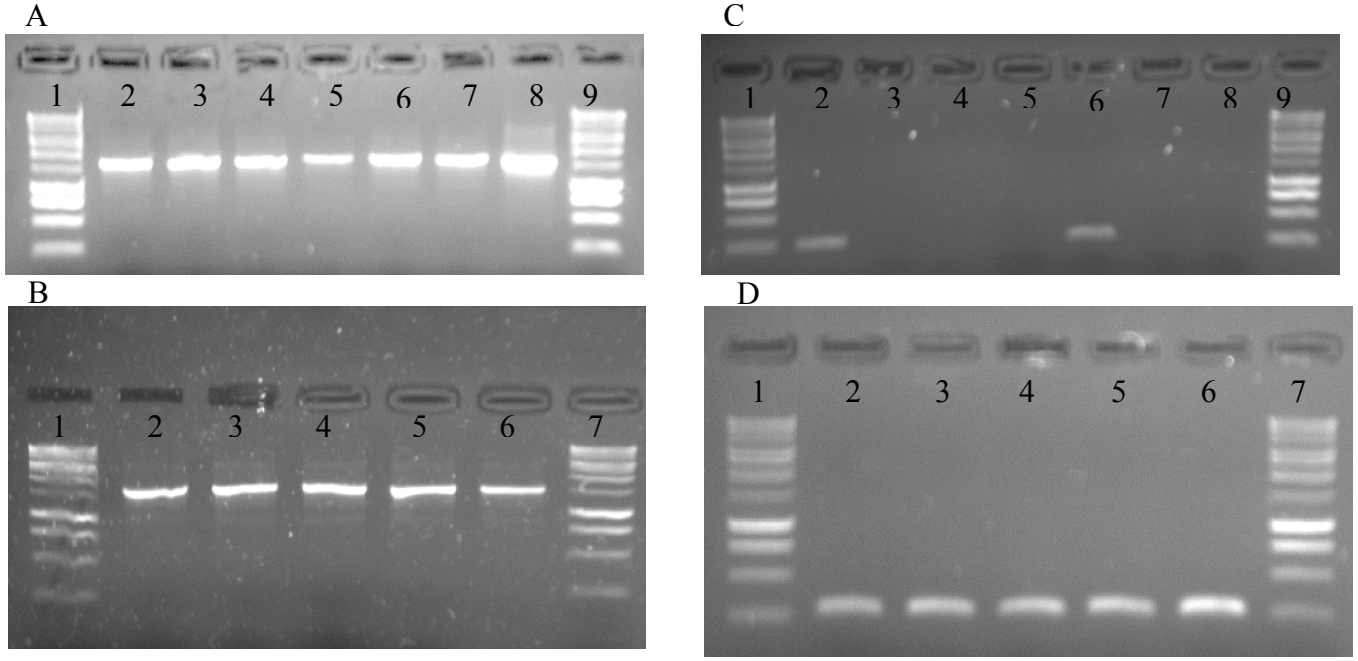
Oil Spreading (cm) of enrichment cultures of produced fluids							
Formation	**Well**	**PCB[a] + Brine**	**PCB + Brine+ Inoculum**	**½PCB [b] + Brine**	**½PCB + Brine+ Inoculum**	**1/10 PCB[c] + Brine**	**1/10 PCB + Brine+ Inoculum**
Hunton	ROB11	BDL [d]		BDL		BDL	
	ROB2	0.3 ±0.1	0.8±0.2[e]	BDL	1.0±0[e]	BDL	0.7 ±0[e]
Wewoka	ROB14	BDL		0.3 ±0.1		BDL	
Earlsboro	ERL1	1.1±0.2		0.4±0.1		BDL	
	ERL5	BDL	0.5±0.2[f]	0.3 ±0	0.3±0[f]	BDL	0.3 ±0[f]
Gilcrease	GIL1	0.4 ±0.0	0.6 ±0.1	0.5 ±0	0.9 ±0.17	BDL	BDL
Skinner flooc	TUBER1+DAV1	BDL	0.3±0.1	BDL	0.6±0.15	BDL	BDL
Hart	HEWITT 4-32	0.4±0.1	BDL	BDL	BDL	0.3±0.2	BDL

[a] PCB Plate Count Broth (Difco.); 10ml of brine was added to 20ml of PCB. [b] ½ PCB one-half strength PCB
[c] 1/10 one-tenth strength PCB
[d] BDL Below detection limit. Oil spreading activity less than 0.2 cm was considered below detection limit
[e] Brine from ROB11, ROB2 and ROB 14 were mixed when inoculum was added to the enrichment
[f] Brine from ERL1 and ERL5 were mixed when inoculum was added to the enrichment

Molecular Approaches

The enrichment cultures were further analyzed by culture-independent methods to detect the presence of genes for biosurfactant production such as those coding for the lipopetides surfactin and/or lichenysin made by microorganisms in the *Bacillus subtilis*-*Bacillus licheniformis* group (*srfA/licA* genes) and rhamnolipids made by *Pseudomonas* species (*rhlR* gene). DNA from all of the enrichments gave the expected PCR product with the universal eubacterial primers for the 16S ribosomal DNA gene (Figure 3.2A). When DNA from enrichments that were not inoculated with *B. licheniformis* (e.g., DNA from indigenous microorganisms) was screened, the *srfA/licA* gene was detected only in the Earlsboro 5 (ERL5) enrichment with PCB (Figure 3.2C). This enrichment also gave a PCR product when primers for the *gyrA* gene were used (Figure 3.3A). These molecular analyses indicate that presence of microorganisms related to the *B. subtilis*-*B. licheniformis* group. However, no oil-spreading activity was detected in this enrichment. Neither *srfA/licA* gene nor the *gyrA* gene was detected in any other enrichment that contained only indigenous microorganisms. However, these two genes were detected in all of the enrichments that were inoculated with *B. licheniformis* (Figures 3.2B, 3.2D and 3.3B). The *rhlR* gene (rhamnolipid gene regulator) was not detected in any of the enrichments.

Figure 3.2. PCR results of 16S rDNA and *srfA/licA* gene amplification for inoculated and uninoculated enrichment cultures.



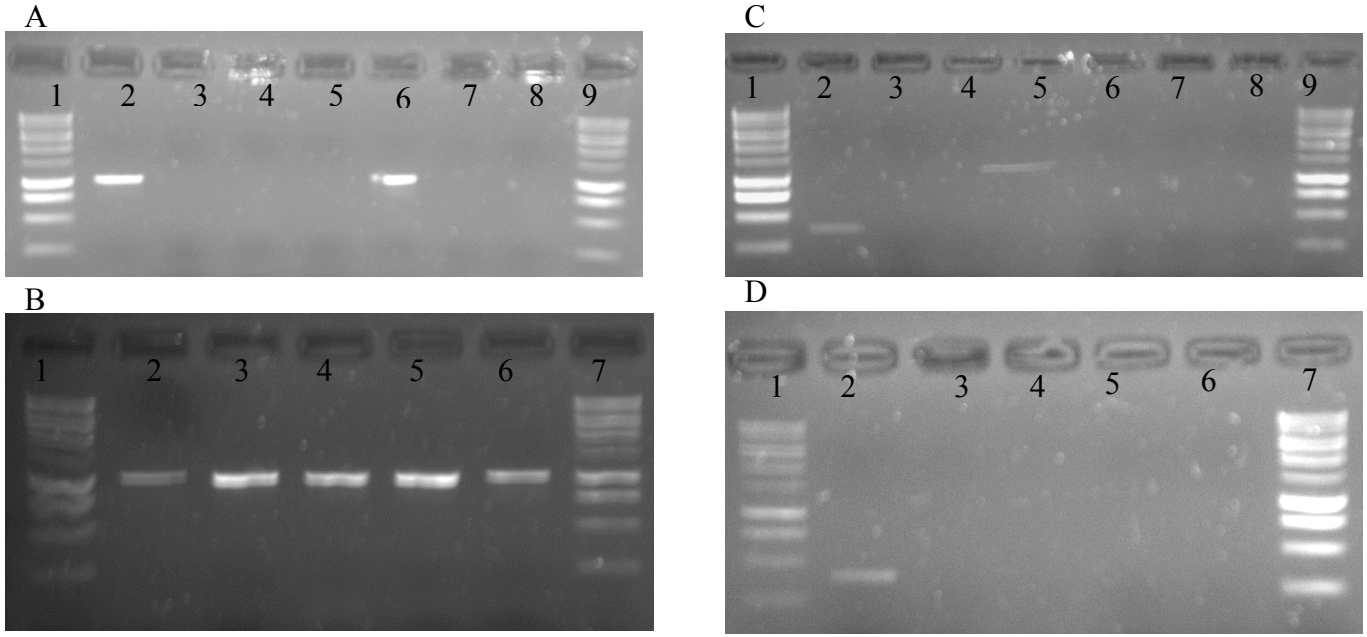
A. 16S rDNA PCR products with un-inoculated brine. Lane 1, 1Kb ladder; lane 2, *B. licheniformis* strain RS-1; lane 3, ROB11 (Hunton); lane 4, ROB 2 (Hunton); lane 5, ERL1 (Earlsboro); lane 6, ERL5 (Earlsboro); lane 7, GIL1 (Gilcrease); lane 8, TUBER1and DAV1 (Skinner flood); lane 9, 1Kb ladder.

B. 16S rDNA PCR products with brine inoculated with *B. licheniformis*. Lane 1, 1 Kb ladder; lane 2, *B. licheniformis* strain RS-1; lane 3, ROB11, ROB2, ROB14 and inoculum; lane 4, ERL1, ERL5 and inoculum; lane5, GIL1 and inoculum; lane 6, TUBER1, DAV1 and inoculum; lane 7, 1Kb ladder.

C. *srfA/licA* PCR products with un-inoculated brine. Lane 1, 1Kb ladder; lane 2, *B. licheniformis* strain RS-1; lane 3, ROB11 (Hunton); lane 4, ROB 2 (Hunton); lane 5, ERL1 (Earlsboro); lane 6, ERL5 (Earlsboro); lane 7, GIL1 (Gilcrease); lane 8, TUBER1 and DAV1 (Skinner flood); lane 9, 1Kb ladder.

D. *srfA/licA* PCR products with brine inoculated with *B. licheniformis* strain RS-1. Lane 1, 1 Kb ladder; lane 2, *B. licheniformis* strain RS-1; lane 3, ROB11, ROB2, ROB14 and inoculum; lane 4, ERL1, ERL5 and inoculum; lane5, GIL1 and inoculum; lane 6, TUBER1, DAV1 and inoculum; lane 7, 1Kb ladder

Figure 3.3. PCR results of *gyrA* and *rhlR* gene amplification for inoculated and un-inoculated enrichment cultures.



A. *gyrA* PCR products with un-inoculated brine. Lane 1, 1Kb ladder; lane 2, *B. licheniformis* strain RS-1; lane 3, ROB11 (Hunton); lane 4, ROB 2 (Hunton); lane 5, ERL1 (Earlsboro); lane 6, ERL5 (Earlsboro); lane 7, GIL1 (Gilcrease); lane 8, TUBER1and DAV1 (Skinner flood); lane 9, 1Kb ladder.

B. *gyrA* PCR products with brine inoculated with *B. licheniformis*. Lane 1, 1 Kb ladder; lane 2, *B. licheniformis* strain RS-1; lane 3, ROB11,ROB2, ROB14 and inoculum; lane 4, ERL1, ERL5 and inoculum; lane5, GIL1 and inoculum; lane 6, TUBER1, DAV1 and inoculum; lane 7, 1Kb ladder.

C. *rhlR* PCR products with un-inoculated brine. Lane 1, 1Kb ladder; lane 2, *P. aeruginosa* ATCC 10145; lane 3, ROB11 (Hunton); lane 4, ROB 2 (Hunton); lane 5, ERL1 (Earlsboro); lane 6, ERL5 (Earlsboro); lane 7, GIL1(Gilcrease); lane 8, TUBER1 and DAV1(Skinner flood); lane 9, 1Kb ladder.

D. *rhlR* PCR products with brine inoculated with *B. licheniformis* strain RS-1. Lane 1, 1 Kb ladder; lane 2, *P. aeruginosa* ATCC 10145; lane 3, ROB11, ROB2, ROB14 and inoculum; lane 4, ERL1, ERL5 and inoculum; lane5, GIL1 and inoculum; lane 6, TUBER1, DAV1 and inoculum; lane 7, 1Kb ladder.

Nutrient treatments

Since the enumeration and enrichment studies indicated that indigenous biosurfactant producers were detected only after enrichment and that the addition of *B. licheniformis* stimulated biosurfactant activity in some cases, a series of experiments was conducted to determine whether simple nutrient additions stimulate biosurfactant activity with or without an inoculum. Seven different nutrients were tested (glucose, molasses, glycerol, proteose peptone, $NaNO_3$, K_2HPO_4 and NaCl) in different combinations by single and double nutrient deletion or by single and multiple nutrient additions. Glucose and molasses are commonly used carbon and energy sources for MEOR (74). K_2HPO_4 serves as the phosphorous source for many microorganisms and is often absent in reservoir brines (71). Glycerol is commonly used in medium to select for *Pseudomonas*-like microorganisms (57). Also, Folmsbee et al. (56) found that Proteose peptone was needed for anaerobic growth of strains of *Bacillus subtilis* and *Bacillus mojavensis*.
The biosurfactant activity detected in brine samples from each formation with and without an inoculum of *B. licheniformis* for the single nutrient series is shown in Figure 3.4 to Figure 3.9. Data from all of the various treatments regimes are presented in Appendices I to V. Indigenous biosurfactant activity under aerobic conditions was detected only in Hunton formation production fluids. Maximum indigenous oil-spreading activity in Hunton formation production fluids was observed when phosphate but not the other six nutrients were deleted. The removal of nitrate and proteose peptone decreased the indigenous oil-spreading activity of brine indicating both of these nutrients were stimulatory for indigenous biosurfactant production. However, biosurfactant activity in Hunton formation production fluids was higher when the inoculum was present (Figure 3.4). In addition, the presence of an inoculum shortened the time needed before maximal oil-spreading activity was observed.

In brines from all of the other formations, maximum biosurfactant production was observed when the nutrient treatments were augmented with a known biosurfactant producer (in this case *B. licheniformis*). Generally, maximal biosurfactant activity occurred after five days of incubations. All the formations tested showed an oil-spreading activity of 1.10 cm to 3.17 cm when the brine contained molasses and $NaNO_3$ and *B. licheniformis* as the inoculum (Figures 3.4 to 3.7).

From the data, the nutrient combination that most often gave maximum biosurfactant production was glucose and/or molasses with $NaNO_3$. Proteose peptone was also highly effective for stimulating biosurfactant activity but only when used in combination with either glucose or molasses. The removal of proteose peptone from the treatment decreased oil-spreading activity, but when proteose peptone was the only organic carbon supplement, oil-spreading activity was low even if the other inorganic nutrients were present. These data suggest that proteose peptone is stimulatory when used in combination with another carbon source such as glucose or molasses. The addition of NaCl and K_2HPO_4 appeared to be inhibitory for the biosurfactant activity since the deletion of these nutrients often increased oil-spreading activity. Deleting glycerol from the mixture markedly decreased biosurfactant activity (Figure 3.4 to 3.7). However, little

or no oil spreading activity was observed when the brine was supplemented only with glycerol and $NaNO_3$. Thus, much like proteose peptone, glycerol is stimulatory for biosurfactant only when used in combination with another carbon source. In almost all cases, oil-spreading activity was high when the brines were supplemented with glucose and/or molasses and $NaNO_3$ (see Appendices) suggesting that additional carbon sources such as proteose peptone or glycerol may be beneficial but not required.

It was observed that *B. licheniformis* strain RS-1 required nutrient components like glucose/molasses, nitrate, proteose peptone and glycerol for maximum biosurfactant production in a medium prepared in sterile deionized water i.e. no brine added to the nutrient components. The deletion of each component decreased biosurfactant production remarkably suggesting that these components were stimulatory. As discussed earlier, proteose peptone and glycerol seemed to be stimulatory only in the presence of other carbon sources like glucose or molasses. These results again suggest that proteose peptone and glycerol serve as additional stimulatory components, but are not absolutely required.

The optimal nutrient formulation was also tested anaerobically for the Hunton, Wewoka and Earlsboro formations. Since Youssef et al. (unpublished data) found that in situ biosurfactant production occurred with the addition of an inoculum, glucose, nitrate and a mixture of metals, we tested whether this combination of nutrients would stimulate anaerobic biosurfactant activity in brines from other formations. Molasses was also tested since this is a cheap and readily available source of sugars.

The anaerobic biosurfactant activity in brine with or without inoculum is shown in Figures 3.8 and 3.9. The data from all the treatments are shown in the Appendix VI to Appendix VIII. Compared to aerobic treatments, none of the anaerobic treatments gave an oil-spreading activity greater than 1.0 cm, indicating that less active biosurfactants were made, the biosurfactant concentration was lower, or a combination of these two factors occurred under anaerobic conditions.

Anaerobic indigenous biosurfactant activity was observed in the treatments with the brine from the Hunton, Wewoka and Earlsboro formations (Figure 3.8, 3.9; Appendices VI to VIII). However, the indigenous biosurfactant activity was considerably less than that observed under aerobic conditions. The maximum indigenous biosurfactant production was observed when glucose, molasses, nitrate and metals were present in the medium. Deletion of one of the above components decreased the oil spreading activity, suggesting their importance for anaerobic biosurfactant production. The addition of inoculum increased the biosurfactant activity significantly (Figure 3.8, 3.9).

Maximum biosurfactant activity was observed when the medium contained glucose, molasses, nitrate and metals, as found with aerobic incubations. The Hunton and Wewoka formation fluids showed the highest oil spreading activity of 1.27 cm and 0.83 cm when molasses, nitrate and inoculum were present in the brine. For the Earlsboro formation fluids, the maximum oil spreading observed was only 0.50 cm and occurred when molasses, nitrate and an inoculum were present in the brine. Anaerobic

biosurfactant production as measured by oil spreading activity was maximal with molasses, nitrate and inoculum. The Earlsboro formation fluids showed the least oil-spreading activity (0.5cm or less; see Appendix VIII). The high salt concentration in the brine itself may have inhibited the biosurfactant production.

Figure 3.4. Aerobic nutrient treatment for the produced fluids from the Hunton formation.

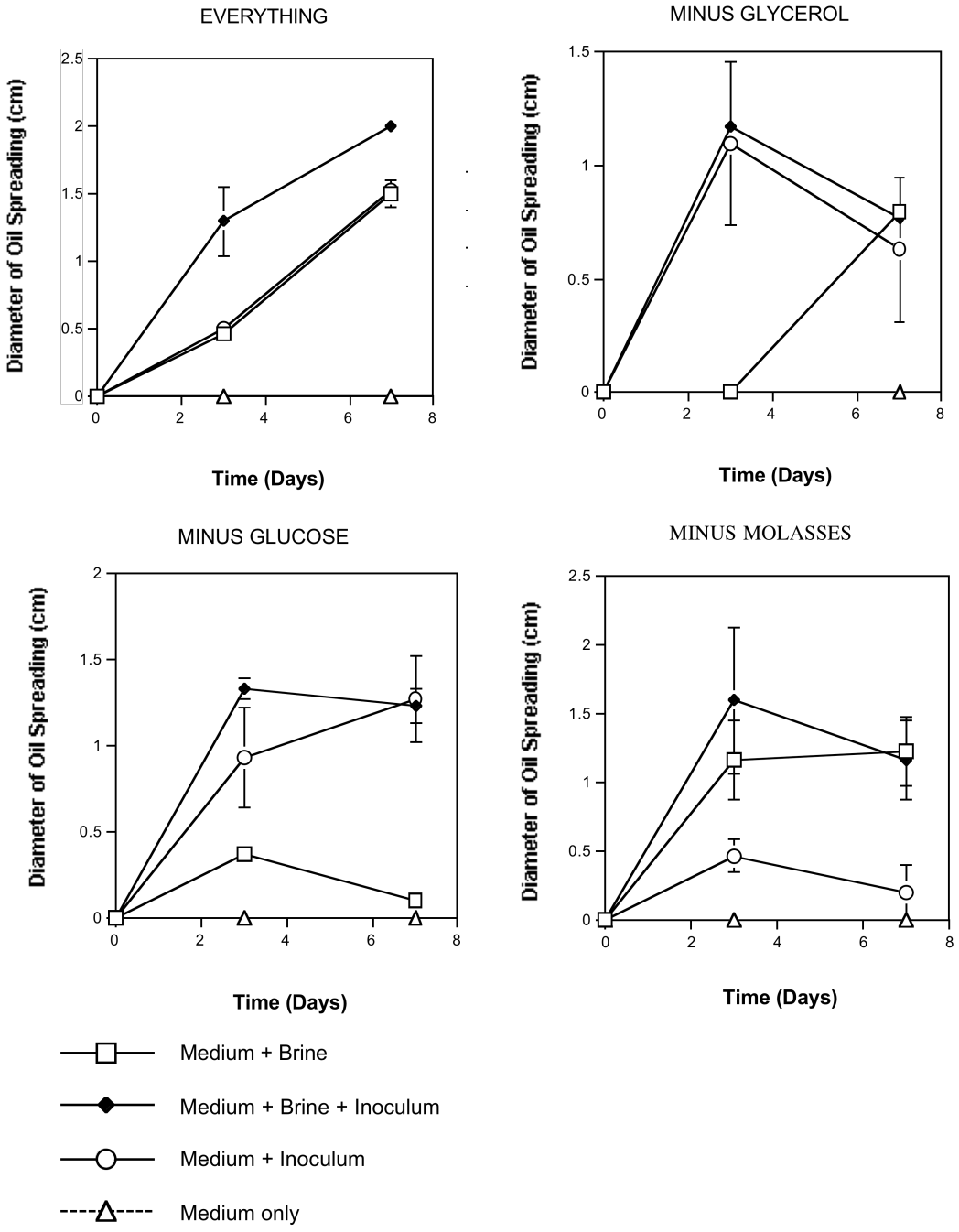
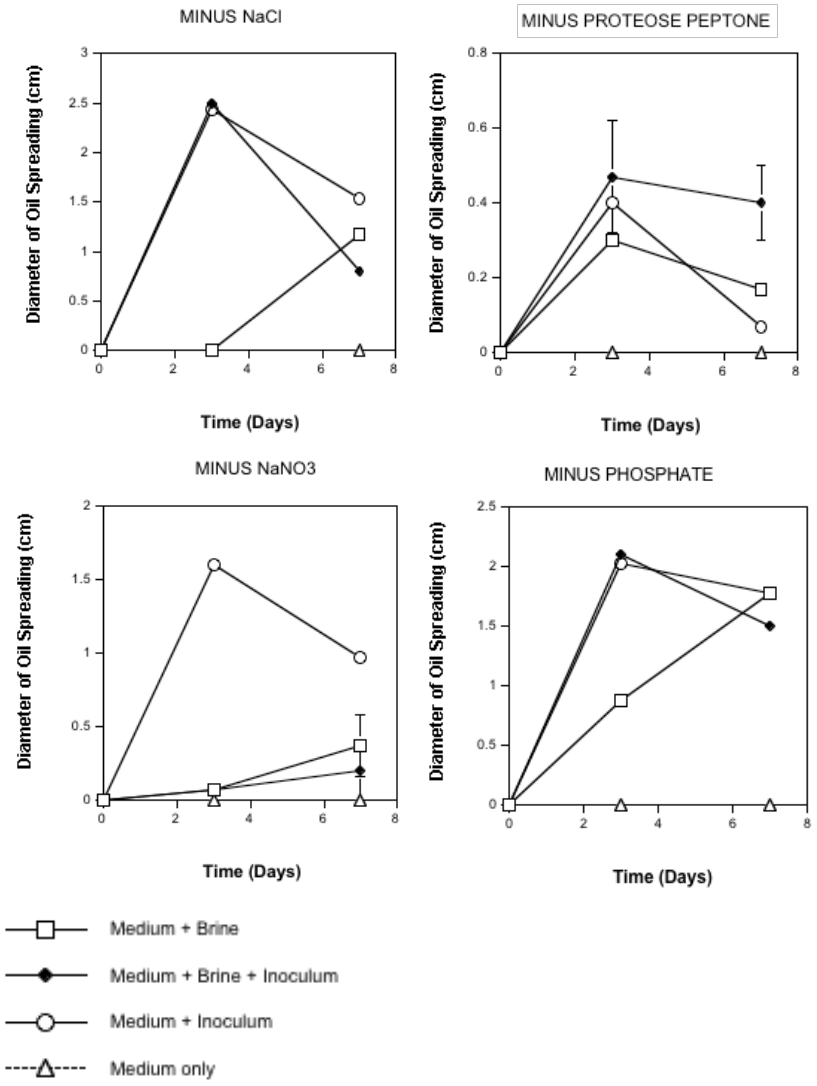


Figure 3.4 (contd). Aerobic nutrient treatment for the produced fluids from the Hunton formation.



Medium - includes the nutrient components in their respective concentration. The medium with single nutrient deletion consists of all other nutrient components except the nutrient that was eliminated.
Medium + Brine – The formation fluids tested for indigenous biosurfactant activity
Medium+Brine+Inoculum – The formation fluids tested for maximum biosurfactant production with bioaugmentation with *B. licheniformis* strain RS-1.
Medium+Inoculum - *B. licheniformis* strain RS-1 tested for biosurfactant production was used as a positive control
Medium only – Nutrient components in deionized water was used as a negative control

Figure 3.5. Aerobic nutrient treatment for the produced fluids from the Wewoka formation.

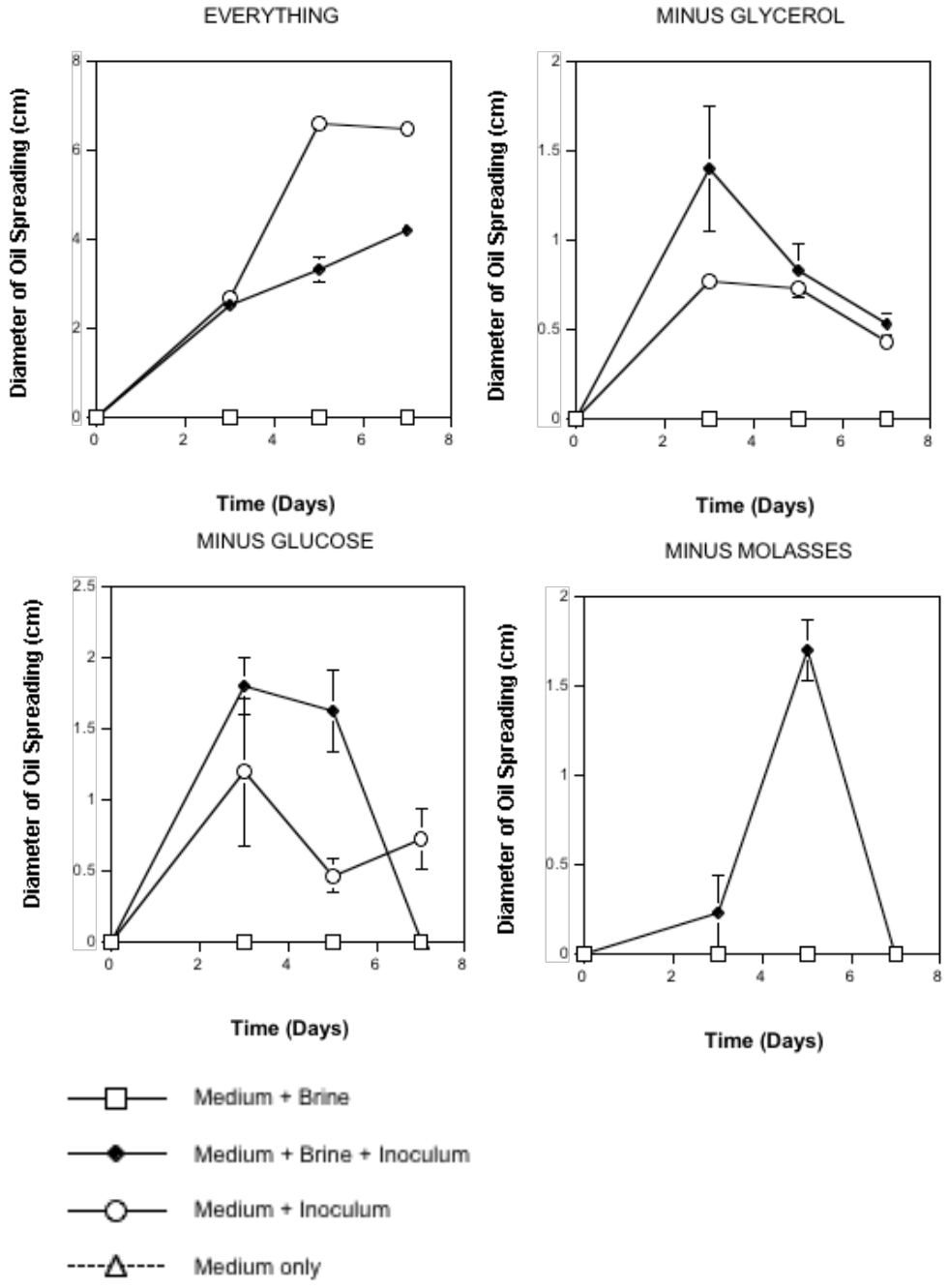
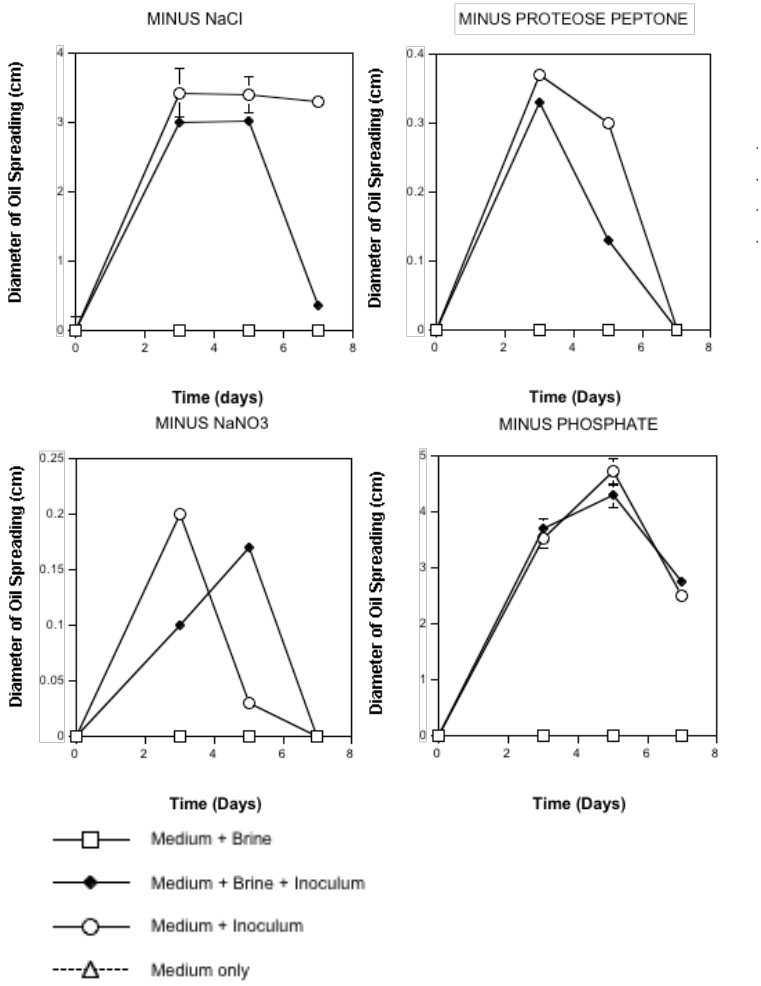


Figure 3.5 (Contd.). Aerobic nutrient treatment for the produced fluids from the Wewoka formation



Medium - includes the nutrient components in their respective concentration. The medium with single nutrient deletion consists of all other nutrient components except the nutrient that was eliminated.
Medium + Brine – The formation fluids tested for indigenous biosurfactant activity
Medium+Brine+Inoculum – The formation fluids tested for maximum biosurfactant production with bioaugmentation with *B. licheniformis* strain RS-1.
Medium+Inoculum - *B. licheniformis* strain RS-1 tested for biosurfactant production in deionized water (used as a positive control)
Medium only – Nutrient components in deionized water (used as a negative control)

Figure 3.6. Aerobic nutrient treatment for the produced fluids from the Earlsboro formation

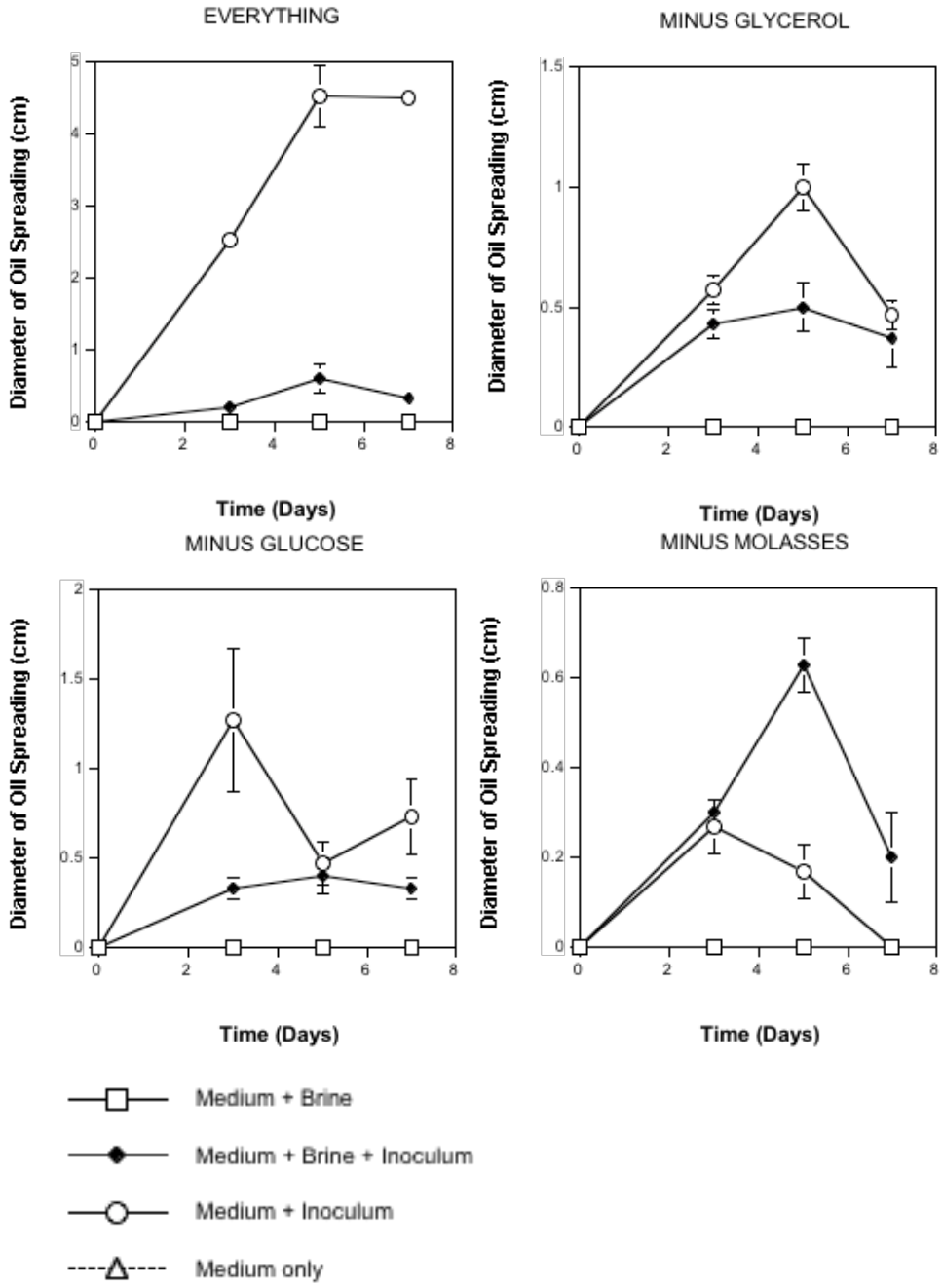
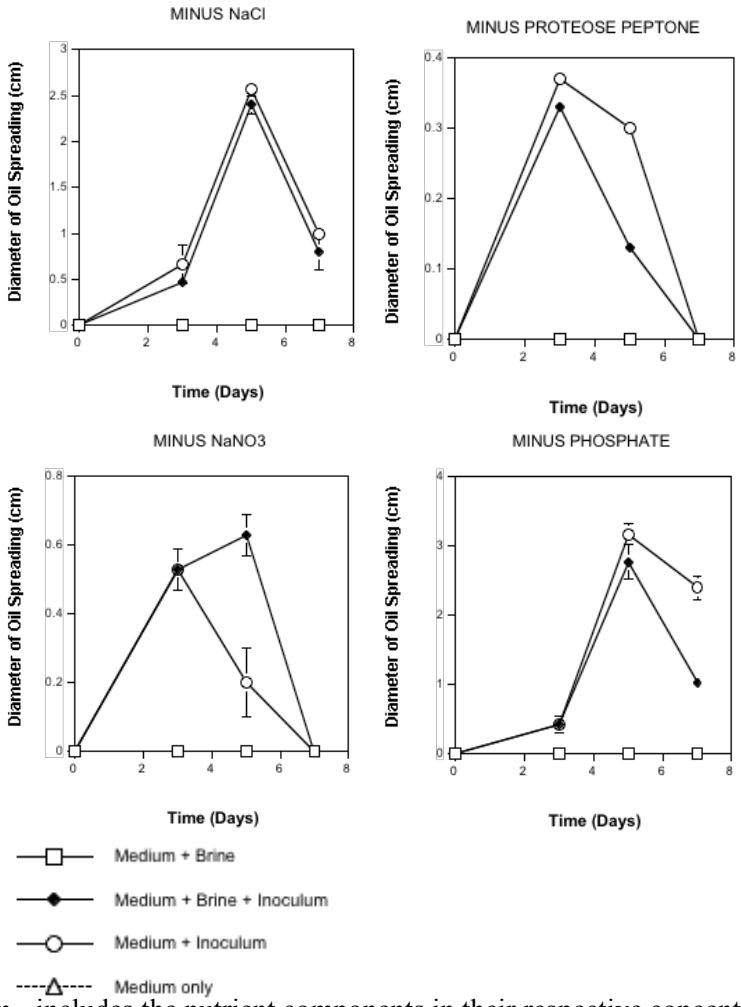


Figure 3.6 (Contd.). Aerobic nutrient treatment for the produced fluids from the Earlsboro formation.



Medium - includes the nutrient components in their respective concentration. The medium with single nutrient deletion consists of all other nutrient components except the nutrient that was eliminated.

Medium + Brine – The formation fluids tested for indigenous biosurfactant activity

Medium+Brine+Inoculum – The formation fluids tested for maximum biosurfactant production with bioaugmentation with *B. licheniformis* strain RS-1.

Medium+Inoculum - *B. licheniformis* strain RS-1 tested for biosurfactant production was used as a positive control

Medium only – Nutrient components in deionized water was used as a negative control

Figure 3.7. Aerobic nutrient treatment for the produced fluids from the Gilcrease formation.

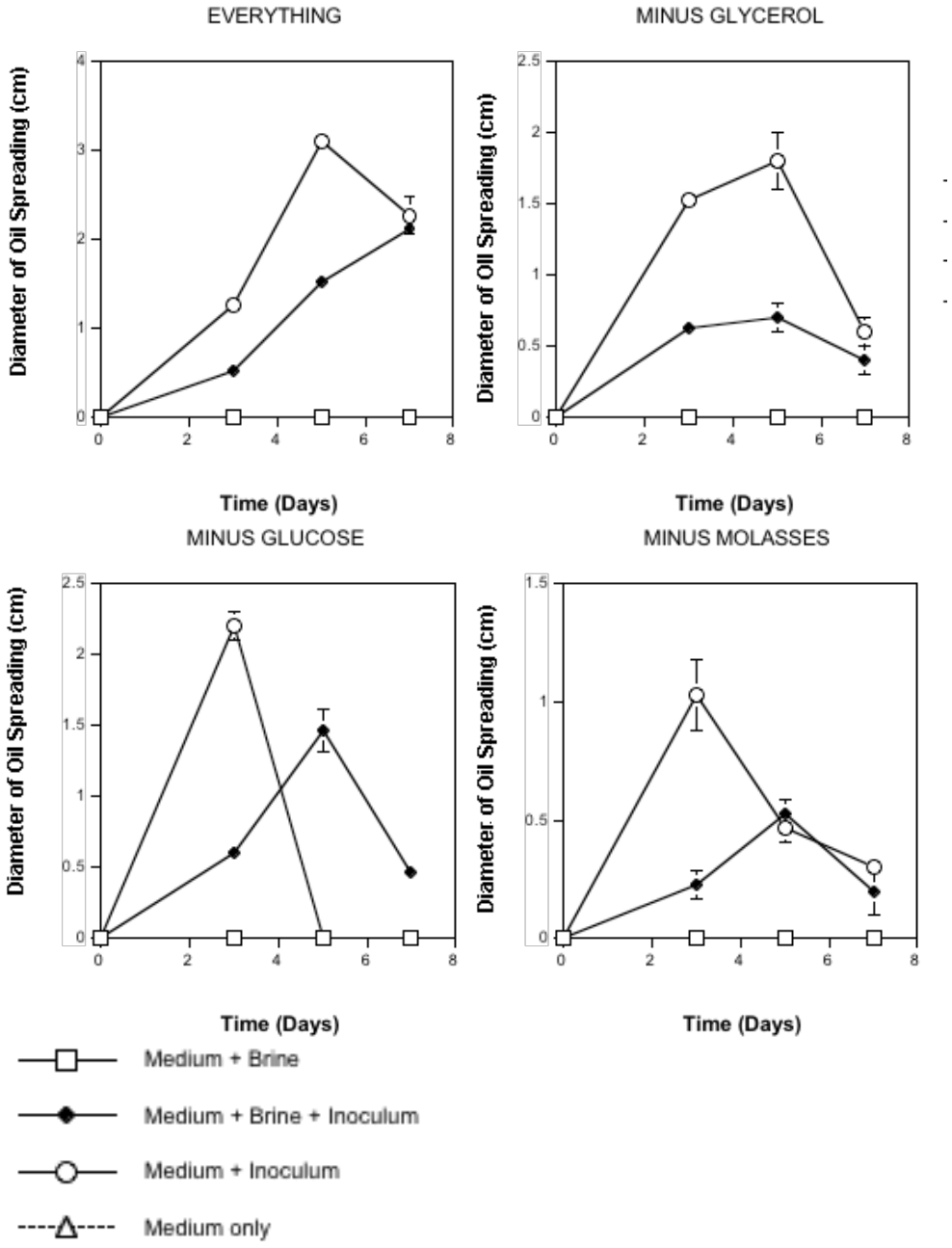
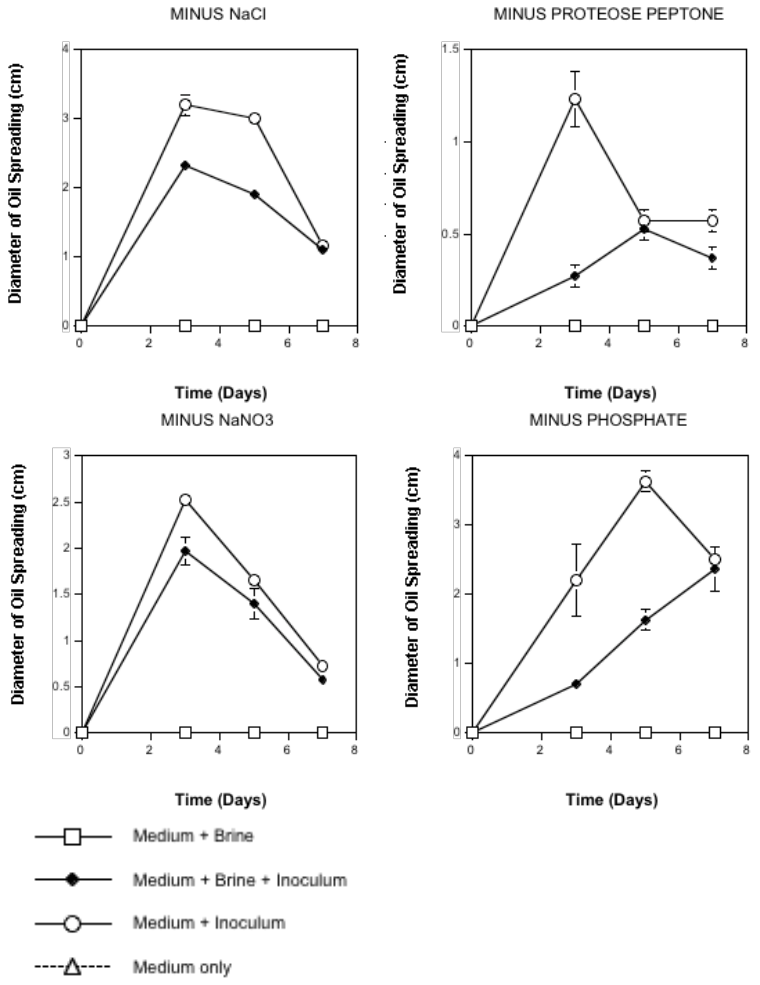
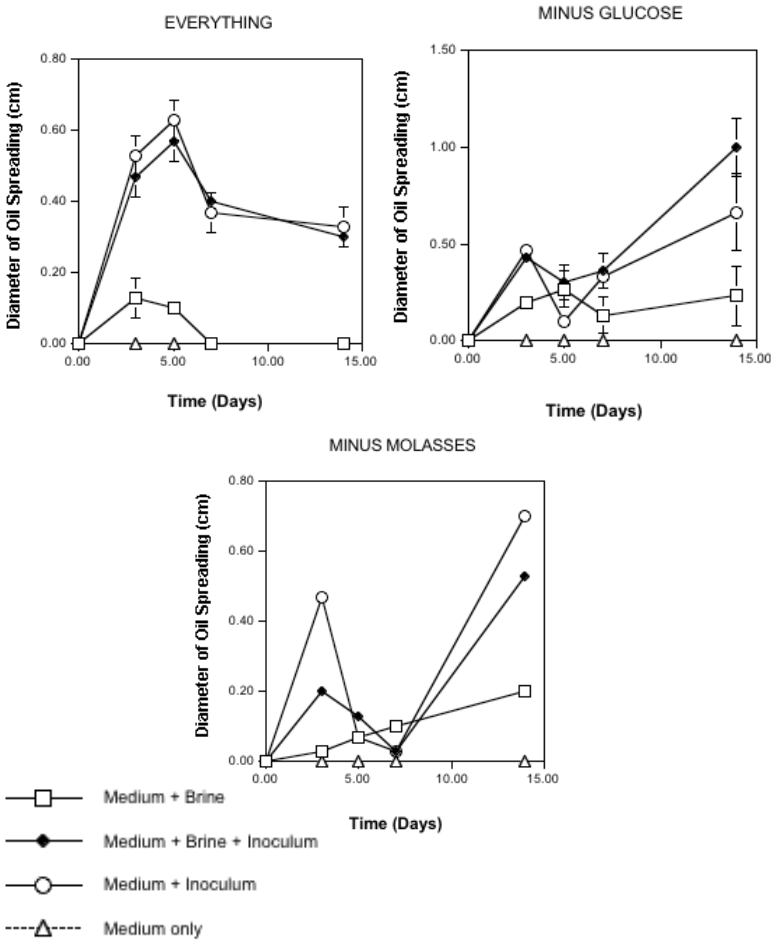


Figure 3.7 (Contd.). Aerobic nutrient treatment for the produced fluids from the Gilcrease formation



Medium - includes the nutrient components in their respective concentration. The medium with single nutrient deletion consists of all other nutrient components except the nutrient that was eliminated.
Medium + Brine – The formation fluids tested for indigenous biosurfactant activity
Medium+Brine+Inoculum – The formation fluids tested for maximum biosurfactant production with bioaugmentation with *B. licheniformis* strain RS-1.
Medium+Inoculum - *B. licheniformis* strain RS-1 tested for biosurfactant production was used as a positive control
Medium only – Nutrient components in deionized water was used as a negative control.

Figure 3.8. Anaerobic nutrient treatment for the produced fluids from the Hunton formation.



Medium - includes the nutrient components in their respective concentration. The medium with single nutrient deletion consists of all other nutrient components except the nutrient that was eliminated.
Medium + Brine – The formation fluids tested for indigenous biosurfactant activity
Medium+Brine+Inoculum – The formation fluids tested for maximum biosurfactant production with bioaugmentation with *B. licheniformis* strain RS-1.
Medium+Inoculum - *B. licheniformis* strain RS-1 tested for biosurfactant production was used as a positive control
Medium only – Nutrient components in deionized water was used as a negative control

Figure 3. 8 (Contd.). Anaerobic nutrient treatment for the produced fluids from the Hunton formation.

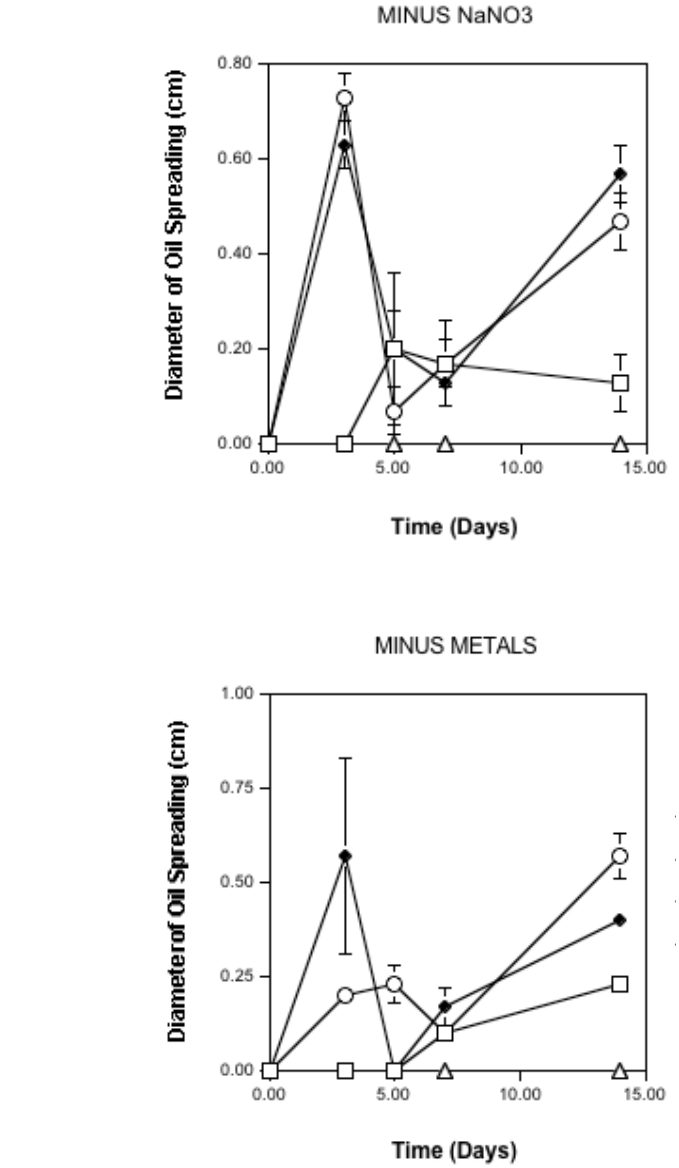
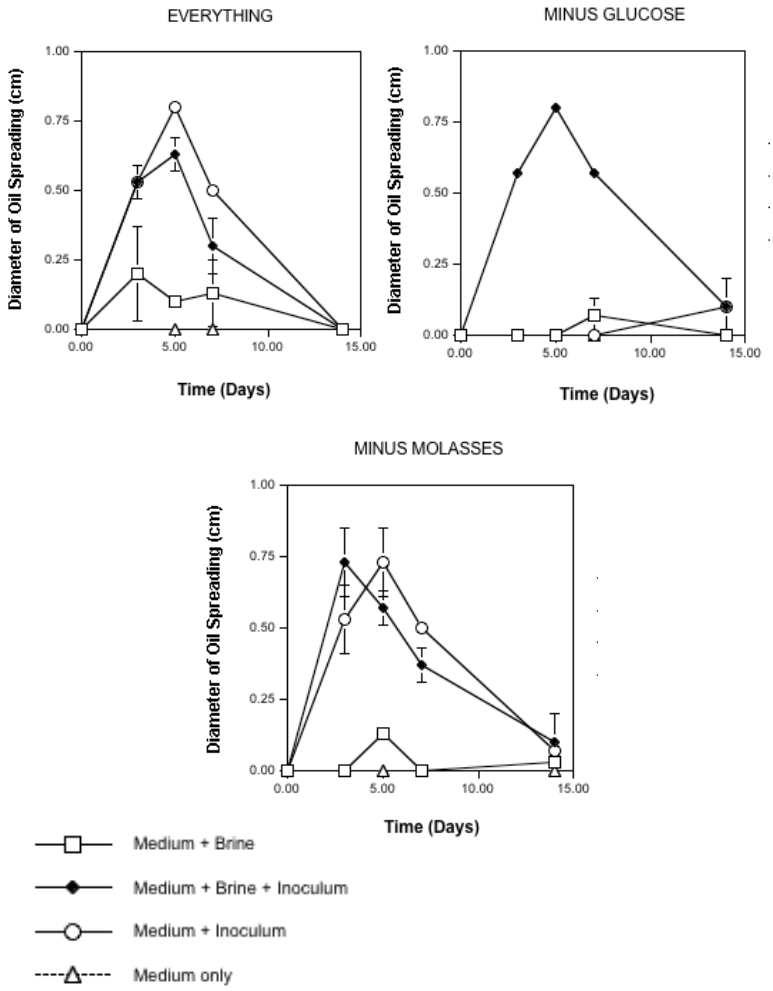
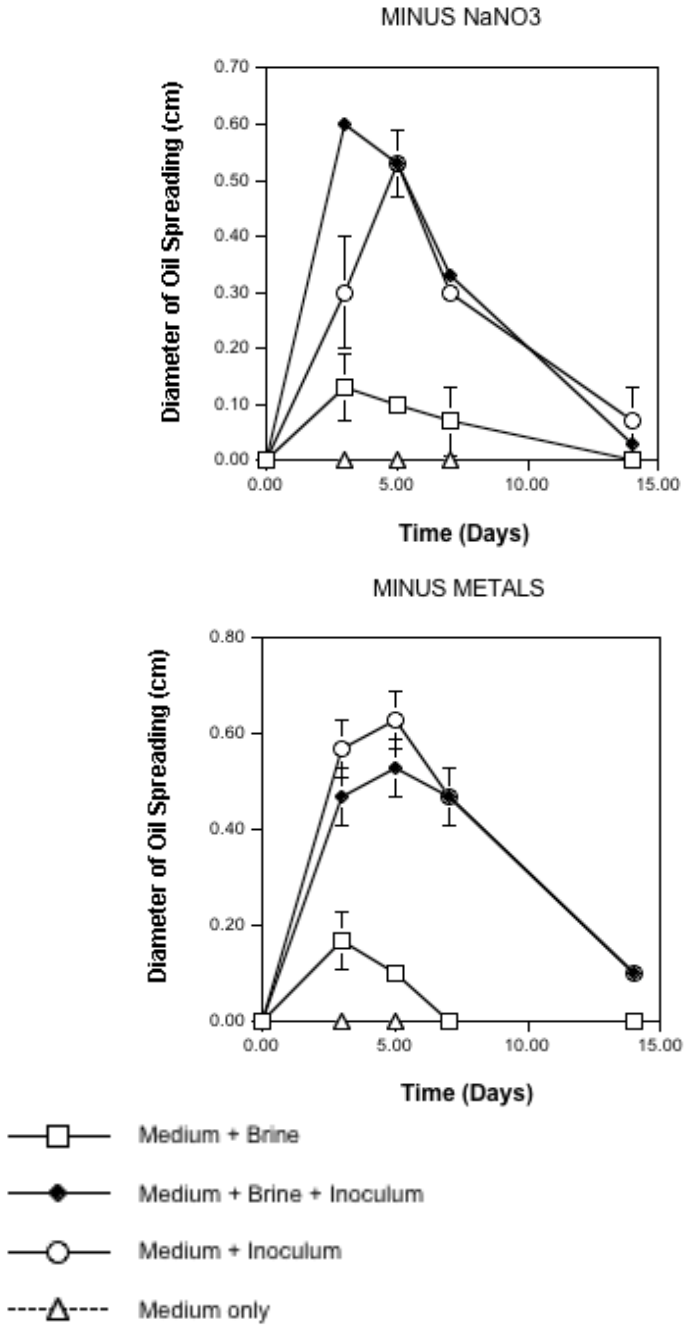


Figure 3.9. Anaerobic nutrient treatment for the produced fluids from the Wewoka formation.



Medium - includes the nutrient components in their respective concentration. The medium with single nutrient deletion consists of all other nutrient components except the nutrient that was eliminated.
Medium + Brine – The formation fluids tested for indigenous biosurfactant activity
Medium+Brine+Inoculum – The formation fluids tested for maximum biosurfactant production with bioaugmentation with *B. licheniformis* strain RS-1.
Medium+Inoculum - *B. licheniformis* strain RS-1 tested for biosurfactant production was used as a positive control
Medium only – Nutrient components in deionized water was used as a negative control

Figure 3.9 (Contd.). Anaerobic nutrient treatment for the produced fluids from the Wewoka formation.



Discussion and Conclusions

This study focused on answering two important questions needed to implement in situ biosurfactant-mediated oil recovery. The first question is whether the reservoir contains biosurfactant-producing microorganisms? If not, then an appropriate inoculum must be supplied. Secondly, what nutrients are needed to stimulate biosurfactant production either by indigenous or introduction biosurfactant producers? Culture-dependent approaches were used to answer these questions, which complement the on going culture-independent approaches used in Chapter 3. The media used for enumeration and enrichment studies were known to support the growth of the most commonly studied biosurfactant producers, lipopeptide producers of the *Bacillus subtilis-Bacillus licheniformis* group and rhamnolipid producers in the genus *Pseudomonas* (1). Both lipopeptide and rhamnolipid biosurfactants are known to generate the ultra-low interfacial tensions against crude oil and benchmark hydrocarbons required for significant recovery of entrapped oil (3, 4, 5). Oil reservoirs are known to contain diverse and active microbial communities (61, 62, 63, 64) and *Bacillus* species have been isolated from the oil field brines (13, 43, 49). However, a systematic study of their presence in produced fluids obtained from different geological formations that have different salinities has not been conducted. Previous surveys have shown that many Oklahoma oil field have brines with high salinities (>10% NaCl) and the median salinity of Oklahoma oil field brines is about 5% NaCl (Jenneman et al. 1981). Our analyses included brines from both sandstone and carbonate formations with salinities ranging from 2% to 15% (Table 3.3). Thus, we were able to sample brines that captured the range of lithologies and salinities found in Oklahoma oil reservoirs.

MPN and viable plate count approaches using PCB and medium E, which are known to support the growth on heterotrophic biosurfactant producers, did not detect any microorganisms except in brines from the Skinner Flood and Hart formations (Table 3.6 and 3.10). The enrichments with PCB, one-half strength PCB and one-tenth strength PCB showed growth with all the formation fluids, implying that heterotrophic microorganisms capable of growing in these media were probably in low numbers that were not detectable by the enumeration methods. However, MPN enumerations with PYTG medium did detect low numbers of heterotrophic bacteria in all of the brines regardless of whether full-strength or dilute media were used (Table 3.7). Low numbers of heterotrophic organisms capable of growing at high salt concentrations (10 and 15%) were detected in brines when enumerated using the PYTG medium. Adjusting the medium to match the salinity and alkalinity of the brine did not increase the cell counts, suggesting that if halophilic indigenous microorganisms were present, they were not cultured with the PYTG medium. Bhupathiraju et al. (62) and Adkins et al. (34) also detected low numbers of heterotrophic organisms (ranging in the order of 10 to 10^2 cells/ml) when using PCB medium. However, Bhupathiraju et al. (62) found increased cell counts when the salt concentration increased.

While biosurfactant production by the indigenous heterotrophic microorganisms was not detected by the oil-spreading assay with any of the MPN or viable plating methods, other

lines of evidence suggest that indigenous biosurfactant producers are present in produced fluids from Oklahoma oil formations. Oil-spreading activity was detected in brines collected from the Earlsboro and Gilcrease formations. The presence of hydrocarbons in the brines may have contributed to the oil-spreading activity. However, enrichments of Earlsboro brine with PCB had very high levels of oil-spreading activity (Table 3.12), suggesting that the oil-spreading activity detected in the brine itself may have been the result of microbial activity. Indigenous biosurfactant production was detected in enrichment cultures with PCB and dilute PCB media (Table 3.12) and when specific nutrients were added directly to brine (Figures 3.4 to 3.9; see Appendices). Interestingly, specific nutrient additions only stimulated indigenous aerobic oil-spreading activity in Hunton formation brine (Figure 3.4) but low levels of indigenous oil-spreading activity were detected in enrichments with either PCB or dilute PCB with brines from all formations except from the Skinner flood (Table 3.12). Low levels of anaerobic indigenous oil-spreading activity was detected in brines from the Hunton, Wewoka, and Earlsboro formations with the addition of glucose and/or molasses with nitrate and trace metals (Figure 3.8, 3.9; see appendix VIII). The varied response to nutrient treatment may indicate the presence of different kinds of biosurfactant producers whose metabolic activity is triggered with different nutrient additions. Functional gene analysis supports this view. The *srfA/licA* and *gyrA* genes were detected in the Earlsboro 5 (ERL5) brine enrichment with PCB (Figure 3.2), indicating the presence of a member of the *B. subtilis-B. licheniformis* group. However, oil-spreading activity was not detected in this enrichment but was detected in the Earlsboro 1 (ERL1) brine enrichment. Neither the *srfA/licA* gene nor the *gyrA* gene was detected in Earlsboro 1 (ERL1) brine enrichment. Thus, a microorganism different from the *B. subtilis-B. licheniformis* group must have be responsible for oil-spreading activity in the Earlsboro 1 (ERL1) brine enrichment. No evidence for the presence of rhamnolipid-producing, *Pseudomonas* species was obtained.

The difficulty in detecting culturable indigenous biosurfactant-producing bacteria and the low indigenous oil-spreading activity often observed argues for bioaugmentation. A fortuitous discovery of this work was the finding that *B. licheniformis* strain RS-1 was able to grow and produce its biosurfactant in brines with salinities ranging from 1.3 to 15%. These data show that strain RS-1 is robust enough to serve as an inoculum in diverse oil reservoirs. The addition of *B. licheniformis* RS-1 enhanced oil-spreading activity in low salinity brine enrichments. In most cases, greater oil-spreading activity was observed when *B. licheniformis* RS-1 was added to nutrient-supplemented brines compared to nutrient-supplemented brines without *B. licheniformis* RS-1 addition. These date suggest that the bioaugmentation with the known biosurfactant producers may be the appropriate MEOR strategy and that *B. licheniformis* RS-1 is a likely candidate for use as an inoculum.

Glucose and/or molasses, proteose peptone, and nitrate were the critical nutrient components needed for aerobic biosurfactant production. Phosphate and salt were the inhibitory to biosurfactant production. This probably explains the presence of very low biosurfactant production in the Skinner floods (15% salinity observed in the Skinner flood [see Table 3.3]). The high salt concentration present naturally in this brine probably inhibited the production of biosurfactant, although growth was observed when *B.*

licheniformis was added as an inoculum. Anaerobic conditions are probably more reflective of the actual environmental conditions that exist in the reservoir. The information obtained with the aerobic nutrient screening allowed me to narrow the number of nutrients to be tested to glucose, molasses, nitrate and a mixture of trace metals. The highest oil-spreading activities were observed when glucose and/or molasses were present and that nitrate and the trace metals mixture stimulated oil-spreading activity. Youssef et al. (unpublished data) found that the addition of glucose, nitrate and trace metals stimulated *in situ* biosurfactant production by *B. licheniformis* RS-1 and *Bacillus subtilis* subsp. *spizizenii* strain NRRLB-23049 in a carbonate formation. Our work shows that this simple nutrient formulation is effective in stimulating anaerobic biosurfactant formation by *B. licheniformis* RS-1 in several brines that vary in salinity. The maximum anaerobic oil-spreading activity even in the presence of *B. licheniformis* RS-1 never exceeded 1.0 cm. Indigenous anaerobic oil-spreading activity was much lower than that observed under aerobic conditions. These data effectively argue that effective *in situ* biosurfactant production requires bioaugmentation.

A major concern with a bioaugmentation approach is the multiple selective pressures that act against the establishment of the introduced strains. These factors include competition with indigenous microorganisms for nutrients or other resources and possibly predation or susceptibility to viral attack. The work by Youssef et al. (unpublished data) showed that injected biosurfactant-producing strains were metabolically active. Here, I have shown that *B. licheniformis* RS-1 is metabolically active in the presence of indigenous microbial oil field communities from a number of brines. Apparently, the nutrient supplementation regime creates a niche that allows *B. licheniformis* RS-1 to establish and become metabolically active.

Our work shows that biosurfactant producers related to the *Bacillus subtilis-Bacillus licheniformis* are not very prevalent in the produced fluids from a number of different oil reservoirs. Only in one case was strong evidence obtained for the presence of these organisms (Earlsboro 5 brine enrichment; Figure 3.3) and this was only after enrichment by growth in PCB. However, several lines of evidence suggest that these brines do contain indigenous biosurfactant producers since oil-spreading activity was detected after various nutrient amendments. However, since most reservoirs are anaerobic, it is clear that effective in situ biosurfactant production will require bioaugmentation with a known biosurfactant producer such as *Bacillus licheniformis* strain RS-1 along with the addition of the appropriate nutrients (glucose, nitrate, and metals).

4. Molecular methodologies to detect and characterize biosurfactant producers and their use to determine the prevalence of biosurfactant producers in oil reservoirs

Abstract

In-situ MEOR treatment strategy relies on a determination of whether biosurfactant producing microorganisms are indigenous to a specific oil reservoir or if bioaugmentation with such bacteria is required. We developed a culture-independent approach to detect the presence of genes involved in lipopeptide biosurfactant biosynthesis (*srfA* and *licA* genes) (surfactin and lichenysin, respectively), and the *rhlR* gene expressing a rhamnolipid biosurfactant transcriptional regulator in *Pseudomonas aeruginosa*. The PCR protocols had a high degree of accuracy in detecting biosurfactant-producing known strains. In addition, these approaches successfully detected the presence of two biosurfactant-producing *Bacillus* strains in produced fluids from an oil reservoir inoculated with these two strains. The PCR approaches were used to survey seven different oil formations that had widely different salinities. Genes for lipopeptide biosynthesis were detected in six of the seven formations. This finding was corroborated by the concomitant detection of members of the *Bacillus subtilis-Bacillus licheniformis* group, a group known to contain many biosurfactant producers, by *gyrA* gene amplification and sequencing. 16S rRNA gene sequence analysis also detected members of this group in formations that had chloride concentrations greater than 10%. 16S rRNA gene sequences related to heterotrophic, lactic acid-producing bacteria were commonly detected in clone libraries obtained from samples of reservoirs that were treated with a mixture of glucose and nitrate or in fluids collected from highly saline reservoirs. Culture-independent approaches support the conclusions of culture-dependent approaches that biosurfactant production is prevalent in many oil formations, even over a wide range of salinity.

Introduction

Biosurfactant production is has been used to enhance hydrocarbon mobilization in subsurface environments by increasing the apparent aqueous solubility of hydrocarbons [100-105]. There are several biosurfactants that generate the low interfacial tensions between the hydrocarbon and the aqueous phases required to mobilize entrapped hydrocarbon [106, 107]. The lipopeptide biosurfactant produced by *Bacillus* species [108-110] and the rhamnolipid produce by various *Pseudomonas* species [102] reduce the interfacial tension between the hydrocarbon and aqueous phase to very low levels (<0.01 mN/m) [106, 111]. The generation of ultra-low interfacial tension results in the enhanced mobilization and recovery of oil. In addition, the critical micelle concentrations for rhamnolipids and lipopeptides are low (20 to 50 mg/L), indicating that these biosurfactants are effective at low concentrations [106]. Several U.S. Department of Energy sponsored field trials have shown the potential for biosurfactant-based microbial flooding processes [112-115].

An important question that must be answered if biosurfactant-mediated oil recovery is to be widely accepted is whether oil formations contain indigenous biosurfactant producers or whether biosurfactant producers will have to be introduced into the formation. To

answer this question, one must be able to detect and track biosurfactant-producing microorganisms. Since it is often difficult to culture microbes from extreme environments such as oil reservoirs, it is necessary to develop cultivation-independent approaches. Such tools provide the opportunity not only to detect and identify biosurfactant-producing microorganisms but also to monitor the growth of biosurfactant-producing strains such as *Bacillus* strain and competing bacteria *in-situ* during an ongoing MEOR treatment. Molecular tools will also assist in the characterization and classification of biosurfactant-producing isolates from oil formations. Commonly expressed in *Bacillus* are the *srfA* gene and the *licA* genes for the biosynthesis of the lipopeptide biosurfactants, surfactin and lichenysin, respectively. Conversely, *P. aeruginosa* species often express the *rhlR* transcriptional regulator for the *rhlAB* gene system responsible for biosynthesis of a rhamnolipid biosurfactant. Identification of either of these biosurfactant functional genes would establish whether indigenous species were present. Phylogenetic information from genes such as the 16S rRNA gene and *gyrA* especially if specifically designed for biosurfactant-producing genera would also enhance monitoring of strains as well as assist in documenting any competing microbial community changes resulting from bioamendment and/or bioaugmentation. The ability to distinguish between similar species of biosurfactant-producing isolates indigenous to oil formations using phylogenetic typing and REP-PCR fingerprinting could help in identifying a group of species with characteristics vital to the success of MEOR, especially if this group has activity over the broad range of salt concentration, temperature and pH that exist in oil formations. Ultimately, we must prove the effectiveness of these molecular tools in monitoring biostimulated and/or bioaugmented wells during the time course of an actual field test, as well as utilize them to successfully identify isolates that can proliferate and produce biosurfactant *in-situ* of an oil formation.

Here, we report the development and use of functional genes to determine the prevalence of biosurfactant producers in oil formations with a wide range of salinity. These approaches successfully detected the presence of two *Bacillus* biosurfactant-producing strains used as an inoculum in produced fluids from the reservoir and delineated the changes in the microbial community after the addition of the glucose/nitrate-based nutrient mixture. Finally, we utilize culture-independent clone sequences from these formations along with phylogenetic typing of a putative *B. licheniformis* RS-1 strain and Sonoran Desert isolates to identify a group of related strains that have characteristics useful MEOR because they have a broad range of salt tolerance and ability to produce biosurfactants.

Materials and Methods

Development and testing of srfA3R2 *functional gene primer.*

As a result of the near 100% identity at the 3' end particularly for the *srfA3/licA3*R described in the 2005 Annual Report [115], a new primer *srfA3*R2 was developed (Table 4.1) based on the same *srfA3* and *licA3* gene sequences available in the NCBI GenBank. In am attempt to enhance the specificity of the primer set to amplify only *srfA3* and *licA3* homologues, the primer *srfA3*R2 was moved 5 bp toward the 3'-end in relation to *srfA3*R

resulting in an expected product size of 268 bp. This relocation left the first 5 bp of the 3'-end of *srfA3*R2 with no sequence identity to the putative esterase gene amplified by the original *srfA3/licA3*R primer set.

Table 4.1. PCR primer sequences utilized for detection and identification of microorganisms.

Primer	Sequence	Reference
27F	5'-AGAGTTTGATCMTGG-3'	[1]
1492R	5'-TACCTTGTTACGACTT-3'	
GM5F	5'GCCCGCCGCGCCCGCGCCCGTCCCGCCGCCCCC GCCCGCCTACGGGAGGCAGCAG-3'	[4, 5]
907R	5'-CCGTCAATTCCTTTRAGTTT-3'	
*srfA3/licA3*F	5'-CAAAAKCGCAKCATACCAAKTTGAG-3'	[6, 7]
*srfA3/licA3*R	5'-TCATARAGCGGCAYATATTGATGC-3'	
*srfA3/licA3*R2	5'-AGCGGCAYATATTGATGCGGYTC-3'	
gyrA F	5'-CAGTCAGGAAATGCGTACGTCCTT-3'	[2]
gyrA R	5'-CAAGGTAATGCTCCAGGCATTGCT-3'	
rhlR F	5'-CTGCGCTCCWCGGAAATGGTG-3'	[8-11]
rhlR R	5'-TCTGGATGWYCTTGWGGTGGAAGTTC-3'	
BOXA1R	5'-CTACGGCAAGGCGACGCTGACG-3'	[3]

Identical reaction conditions and mixtures were used for the original *srfA3/licA3*F and *srfA3/licA3*R primers. The reaction mix consisted of 5 μL of 10X PCR buffer, 5 μL of 5 M betaine, 4 μL of 50 mM magnesium chloride, 1 μL of 10 mM dNTP mix, 1 μL of 5 pmol/μL of both *srfA3/licA3* F and R2 primers, 4 μL of cell template, 0.25 μL of Platinum *Taq* polymerase (Invitrogen) and 28.75 μL of sterile deionized water forming a 50 μL reaction volume. The thermal cycler was again programmed for 5 min at 94^{o}C for initial denaturation, 30 cycles of 35 s at 94^{o}C for denaturation, 35 s at 50^{o}C for annealing, 45 s at 72^{o}C for extension subsequently followed by a final extension at 72^{o}C for 6 min and a 4^{o}C quench.

A subset of type strains including the two field strains (*B. subtilis* subsp. *spizizenii* NRRL B-23049 and *B. licheniformis* strain RS-1) and three Gram-negative strains (*Escherichia coli* OU8739, *Klebsiella pneumoniae* OU10149 and *Salmonella typhi* OU10209) were then PCR amplified to test the *srfA3/licA3*F and *srfA3/licA3*R2 primers. In addition, amplification of several other species of the *B. licheniformis/ subtilis* clade were attempted to determine the specificity range of the *srfA3/licA3* F and R2 primer set. Four strains were isolates from the Sonoran Desert including T89-11, TG2-32, TE-48 and RL-1 [116, 117]. Other strains included *B. licheniformis* ATCC 14580, *B. sonorensis* NRRL

B-23154, *B. mojavensis* ROB2, *B. mojavensis* NRRL B-1469, *B. mojavensis* JF2, *B. subtilis* subsp. *subtilis* strain 168, *B. subtilis* OKB105, *B. vallismortis* NRRL B-4890, *B. pumilis* ATCC 7061, *B. atrophaeus* NRRL NRS B213, *B. cereus* ATCC 14579, *B. magaterium* ATCC 14581, and *B. amyloliquefaciens* CBD566. *B. fusiformis* strain RS-2 isolated from Robertson/Parrish Tank Battery oil field brine near Oil Center, OK was tested against the primers. Only *srfA3/licA3* sequences for *B. licheniformis* ATCC 14580 and *B. subtilis* subsp. *subtilis* strain 168 had previously been identified in the NCBI GenBank.

Purification of the PCR product and cloning into the pGEM vector was performed on each of the *srfA3/licA3* F and R2 positive strains. A total of 3 clones were sequenced using both the T7 and SP6 vector primers for each amplicon by the Oklahoma Medical Research Facility (OMRF) Sequencing Laboratory (Oklahoma City, OK). The OMRF provided all DNA sequencing services described in this report. A consensus sequence was determined after sequence assembly and multiple sequence alignment by DNAMAN (Lynnon Biosoft) and visual inspection. Each of these consensus sequences will be deposited into the NCBI GenBank.

The oil drop assay based on an existing static biofilm formation assay as described in the 2005 Annual Report (28) was again utilized to correlate *srfA3/licA3* presence with qualitative measurement of biosurfactant production in a strain. A modified Bacillus Biofilm Growth Medium (BBGM) consisting of Luria Bertani broth (LB), 0.1% dextrose, 150mM ammonium sulfate, 34mM sodium citrate, 100mM potassium phosphate buffer and 1 g/L sodium nitrate was the medium used in a 96-well plate format [118]. After static growth, strains were scored positive for biosurfactant production if there was dissipation or condensation of the oil drop. Surface activity of each strain was also determined by hemolytic reaction on tryptic soy agar plates with 5% sheep blood [119].

Development and testing of rhlR *functional gene primer.*

A degenerate primer set was also developed and tested against type strains for *rhlR* which expresses a rhamnolipid biosurfactant regulator in *Pseudomonas aeruginosa* [8-11]. Cell or DNA template from strains of several *Pseudomonas* species as well as other Gram-negative strains from other genera and the Gram-positive *B. subtilis* subsp. *subtilis* strain 168 were subjected to PCR amplification with the *rhlR* and *rhlF* primer set. The reaction mix consisted of 5 μL of 10X PCR buffer, 0.5 μL DMSO, 2.5 μL of 50 mM magnesium chloride, 1 μL of 10 mM dNTP mix, 1 μL of 5 pmol/μL of both *rhlR*F and *rhlR*R primers, 4 μL of cell template, 0.25 μL of Platinum *Taq* polymerase (Invitrogen) and 34.75 μL of sterile deionized water forming a 50 μL reaction volume. The thermal cycler was programmed for 5 min at 95^{o}C for initial denaturation, 10 touchdown cycles of 40 s at 95^{o}C for denaturation, 40 s at 57.3^{o}C minus 0.5^{o}C per cycle to 52.3^{o}C for annealing, 45 s at 72^{o}C for extension followed by 23 cycles of 40 s at 95^{o}C for denaturation, 40 s at 52.3^{o}C for annealing, 45 s at 72^{o}C for extension and finally a final extension at 72^{o}C for 7 minutes and a 4^{o}C quench.

Each of the strains available in culture was also tested for rhamnolipid activity using the oil spread assay using a *Pseudomonas* Biofilm Medium (PBM) [120] which consisted of 50mM MOPS, 2.5 g/L sodium chloride, 5.0 g/L ammonium chloride, 0.3 g/L dibasic potassium phosphate, 0.01% yeast extract, 0.5 % glucose and 0.5% casamino acids.

Detection of injected strains in produced fluids.

DNA templates extracted from time course brine for ROB 15 and ROB 13 at 14:00MT as described in the 2005 Annual Report (28) were then re-amplified with the new *srfFA3/licA3* F and R2 primers. PCR mix and reaction conditions were the same except that 5 μL of extracted DNA template and 27.75 μL of sterile deionized water were utilized in the 50 μL reaction. The products were excised and extracted (Qiagen) from a 1.5% agarose gel, cloned into pGEM (Promega) then plasmid amplified and purified (Qiagen). Clones were sequenced in both directions by using the T7 and SP6 primers and assembled using DNAMAN.

In addition, the same DNA extracted from time course brine for ROB 15 and ROB 13 at 14:00MT were then amplified with the *gyrA*F and R primers. Except for the inclusion of only the *gyrA* primer set, the PCR mix was identical in formulation to that used above for *srfA3/licA3* amplification. However, the thermocycler was programmed for 4 min at 94^{o}C for initial denaturation, 35 cycles of 1 min at 94^{o}C for denaturation, 1 min at 48^{o}C for annealing, 1.5 min at 72^{o}C for extension subsequently followed by a final extension at 72^{o}C for 8 min. and a 4^{o}C quench.

The products were extracted (Qiagen) from a 1.0% agarose gel, cloned into pGEM (Promega) then plasmid amplified and purified (Qiagen) and sequenced in both directions by using the T7 and SP6 primers. DNAMAN was used to assemble the sequences.

Effect of treatments on the oil reservoir microbial community.

In addition to the pGEM clones excised from the DGGE gel discussed in the 2005 Annual Report [115], 27F and 1492R primers for PCR amplification 16S rRNA gene in eubacteria were utilized with the DNA extracted from the time course brine samples before and after treatment. The reaction mix consisted of 5 μL of 10X PCR buffer, 5 μL of 5 M betaine, 4 μL of 50 mM magnesium chloride, 1 μL of 10 mM dNTP mix, 1 μL of 5 pmol/μL of both 27F and 1492R primers, 6 μL of DNA template, 0.25 μL of Platinum *Taq* polymerase (Invitrogen) and 26.75 μL of sterile deionized water forming a 50 μL reaction volume. The thermal cycler was programmed for 5 min at 95^{o}C for initial denaturation, 30 cycles of 1 min at 94^{o}C for denaturation, 2 min at 42^{o}C for annealing, 2 min at 72^{o}C for extension subsequently followed by a final extension at 72^{o}C for 8 minutes and a 4^{o}C quench. The amplicons were gel extracted and cloned into pGEM for sequencing. Approximately 8 clones were sequenced for each of the 5 wells before and after treatment (14:00MT). Sequences were assembled using DNAMAN and compared to GenBank submissions with BlastN results. The most prevalent clones from this very limited 16S survey were compared in a multiple sequence alignment using DNAMAN

which was then used to assemble a phylogenetic tree using the observed divergence method with 1000 bootstrap trials.

srfA3/licA3 and *gyrA* bands from culture-dependent enrichment DNA were also PCR amplified, cloned and sequenced.

Prevalence of biosurfactant producers in various oil formations.

A 2L sample of produced brine was collected from 11 wells from 7 formation types with a range of [Cl^-] concentrations (Table 4.2). For the culture-independent approach, DNA was extracted in accordance with the protocol used for the field test time course analysis described on page 19 of the 2005 Annual Report [115]. Several extractions did require the addition of a concentration step from 100 μL to 30 μL final volume by rotary vacuum concentrator. DNA formation samples were PCR amplified with the *srfA3/licA3* E and R2 degenerate primers. The amplicons were excised from 1.5% agarose gels, gel extracted (QIAquick Gel Extraction Kit) and pGEM (Promega) cloned. Three individual clones were subsequently amplified for each formation band by growth in LB broth containing 100 μg/l of ampicillin. After plasmid purification (QIAprep Spin Mini Prep Kit) and A_{260}/A_{280} quantification, sequencing was performed by OMRF. The formation clones (shown in red) were then aligned together with the type consensus sequences (shown in blue) in a multiple sequence alignment for phylogenetic tree construction using the Observed Divergency Distance Method and 1000 bootstrap trials.

Table 4.2 Formation types sampled and their salinity.

Formation Type	Well Number	[Cl-] (mg/L)
Viola	Robertson 15	3775
	Robertson 13	3900
Wewoka Sand	Roberston 14	21,500
Hunton	Robertson 11	32,000
	Roberston 2	35,000
Earlsboro	Tract 8 Well 1	94,500
	Tract 5 Well W1-3	102,500
Gilcrease	Freeze 1	113,500
Hart	Hewitt 1	130,000
Skinner Flood	Davis 1	158,000
	Turbeville 1	159,000

In a similar fashion, the *gyrA* and 16S rRNA gene amplicons were obtained in a similar fashion from the culture-independent DNA templates then cloned, sequenced and compiled into a phylogenetic tree. The PCR reaction mix for *srfA3/licA3*, *gyrA* and 16S rRNA gene included 5 μL of DNA template per 25 μL reaction in order to obtain an amplicon from most formation extractions. The reaction mix and thermocycler conditions were otherwise as previously described.

Results

Development and testing of srfA3R2 *functional gene primer on type strains.*

Sequencing of pGEM clones derived from gel extractions of DNA samples from ROB 15 and ROB 13 produced fluids (28) did not result in the expected *srfA3* and *licA3* fragment sequences of 274 bp for the field strains *B. subtilis* subsp. *spizizenii* NRRL B-23049 and *B. licheniformis* strain RS-1 respectively [115]. Instead a 280 bp fragment from a putative esterase with 85% identity to an *Escherichia coli* W3110 gene (AP009048) was obtained for the sequence of each clone. For the 25 bp *srfA3/licA3*F primer, 8 of 8 bp on the 3'-end matched the sequence of the putative esterase gene. In addition, for the 24 bp *srfA3/licA3*R primer, 13 of 14 bp on the 3'-end matched this gene sequence. This result could be explained by the fact that lipopeptide synthetases such as those expressed by *srfA* and *licA* have a thioesterase activity to move the peptide chain from domain to domain. The 3'-end of a primer is responsible for anchoring to the DNA template during

PCR amplification. As a result of the near 100% identity at the 3' end for each primer particularly for *srfA3/licA3*R, a new primer *srfA3*R2 was developed based on the same *srfA3* and *licA3* gene sequences available in the NCBI GenBank. In an attempt to enhance the specificity of the primer set to only amplify *srfA3* and *licA3* homologues, the primer *srfA3*R2 was moved 5 bp toward 3'-end in relation to *srfA3*R resulting in an expected product size of 268 bp. This relocation left the first 5 bp of the 3'-end of *srfA3*R2 with no sequence identity to the putative esterase gene. The primer set yielded an approximately 269 bp product for the two field strains and no product for the three Gram-negative strains (Figure 4.1).

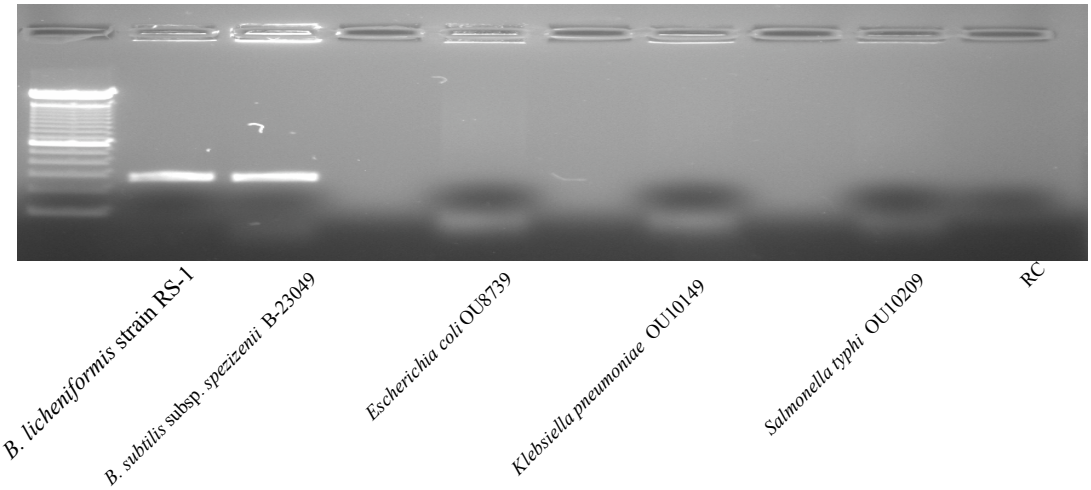


Figure 4.1 *srfA3/licA3* F and R2 specificity for field strains versus Gram-negative strains

Table 4.3 Summary of *srfA3/licA3* F and R2 Amplicons, β-hemolysis and Oil Spread

Bacterium	*srfA3/licA3 F and R2* PCR Product (268 bp)	Blood Agar -Hemolysis	Oil Spreading Assay (BBGM)
B. licheniformis ATCC 14580	+	+	+
B. licheniformis strain RS-1	+	+	+
TG2-32	+	+	+
T89-11	+	+	+
TE-48	+	+	+
RL-1	+	+	+
B. sonorensis NRRL B-23154	+	+	+
B. mojavensis ROB2	+	+	+
B. mojavensis NRRL B-14698	+	+	+
B. mojavensis JF2 (volcanic)	+	+	+
B. vallismortis NRRL B-14890	+	+	+
B. amyloliquefaciens CBD 566	+	+	+
B. pumilus ATCC 7061 (CBD 400)	-	-	-
B. atrophaeus NRRL NRS B-213	-	+	+
B. cereus ATCC 14579 (CBD 55)	-	+	-
B. megaterium ATCC 14581	-	+	+
B. subtilis subsp. *subtilis* 168	+	-	-
B. subtilis subs. *subtilis* OKB105	+	+	+
B. subtilis subsp. *spezizenii* NRRL B-23049 (MT-1)	+	+	+
B. fusiformis strain RS-2	-	+	+
Escherichia coli OU8739	-	-	-
Klebsiella pneumoniae OU10149	-	-	-
Salmonella typhi OU10209	-	-	-

+ strong reaction, + weak reaction, - no reaction

Similar amplicons were visible for each *Bacillus* strain except for *B. vallismortis* NRRL B-4890, *B. pumilis* ATCC 7061, *B. atrophaeus* NRRL NRS B213, *B. cereus* ATCC 14579, *B. magaterium* ATCC 14581 and *B. fusiformis* strain RS-2. In addition, oil spreading and β-hemolysis was indicated for all *srfA3/licA3* F and R2 positive strains except *B. subtilis* subsp. *subtilis* strain 168 which has an inactive *sfp* gene required for post translational modification of SrfA/LicA to an active form (Table 4.3). In addition, *srfA3/licA3* F and R2 negative *B. atrophaeus* NRRL NRS B213, *B. megaterium* ATCC 14581, and *B. fusiformis* strain RS-1 resulted in both oil spreading and β-hemolysis indicating the biosynthesis of a surface active compound of some type for these *srfA3/licA3* F and R2 negative strains. This result could indicate that either the *srfA3/licA3* F and R2 primers do not complement the srfAlic3 homologous genes for these strains or they produce a different surface-active compound.

Development and testing of rhlR *degenerate primer on type strains.*

The *rhlR* primer set resulted in strong bands of approximately 399 bp for all *P. aeruginosa* strains tested and faint bands for *P. pseudoalcaligenes* ATCC 17440 and *K. pneumoniae* OU10149 (Figure 4.2). Oil spreading indicating biosurfactant activity corresponded identically with the strong *rhlR* product obtained from each of the *P. aeruginosa* strains. Three clones were sequenced in both directions for each amplicon using the T7 and SP6 primers. A consensus sequence was determined by sequence assembly, multiple sequence alignment by DNAMAN (Lynnon Biosoft), and visual inspection. *P. aeruginosa* PAO1 and *P. aerugionsa* PA14 consensus sequences matched with 100% identity to their corresponding NCBI GenBank submissions. Each of the three remaining consensus sequences had a 99% nucleotide identity with *P. aeruginosa*

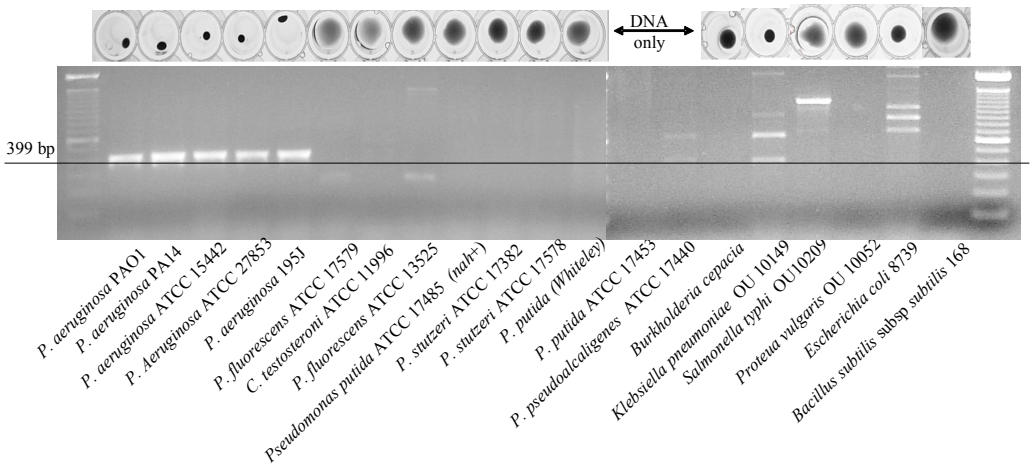


Figure 4.2 Specificity of *rhlR* Degenerate Primers Compared to Microtiter Plate Oil Spread Assay

PAO1. The new consensus sequences will be deposited into the NCBI GenBank except for the already existing sequences present in that database. The *K. pneumoniae* OU10149 product had an 86% identity to a *Yersinia pestis* putative membrane protein (AL590842).

Verification of the presence of the field strains in time course field test brine.

To verify the efficacy of the *srfA3/licA3* primers, these primers were tested with DNA extracted from produced fluids collected from ROB 15 well, which had been inoculated with two biosurfactant-producing *Bacillus* strains. Six of the seven clones submitted for sequencing matched the sequence of *B. licheniformis* strain RS-1, and one was *B. subtilis* subsp. *spizizenii* NRRL B-23049 (99% identity from NCBI BlastN analysis; Table 4.3). Furthermore, amplicons were obtained after PCR amplification of DNA obtained from the produced fluids from the other inoculated well, ROB 13. Again, 6 of the 7 clones submitted for sequencing were *B. licheniformis* strain RS-1 with 99% identity, and one was an unidentifiable product based on GenBank analysis (Table 4.3).

Table 4.4 BlastN Summary of ROB 15 at 14:00MT Clones from *srfA3/licA3* amplification.

Clone	Gene (Blastn)	Species	% Identity	Species
A	surfactin synthetase C	*Bacillus subtilis* subsp.*spizizenii* NRRL B-23049	100%	*Bacillus subtilis* subsp. *subtilis* str. 168
B	lichenysin synthetase C	*Bacillus licheniformis* RS1	100%	*Bacillus licheniformis* ATCC 14580
C	lichenysin synthetase C	*Bacillus licheniformis* RS1	100%	*Bacillus licheniformis* ATCC 14580
D	lichenysin synthetase C	*Bacillus licheniformis* RS1	100%	*Bacillus licheniformis* ATCC 14580
E	lichenysin synthetase C	*Bacillus licheniformis* RS1	100%	*Bacillus licheniformis* ATCC 14580
F	lichenysin synthetase C	*Bacillus licheniformis* RS1	100%	*Bacillus licheniformis* ATCC 14580

Table 4.5 BlastN Summary of ROB 13 at 14:00MT Clones from *srfA3/licA3* amplification.

Clone	Gene (Blastn)	Species	% Identity	Species	Product Size
A	lichenysin synthetase C	*Bacillus licheniformis* RS1	99%	*Bacillus licheniformis* ATCC 14580	269bp
B	lichenysin synthetase C	*Bacillus licheniformis* RS1	100%	*Bacillus licheniformis* ATCC 14580	269bp
C	Na+ driven multidrug efflux pump			*Clostridium tetani* E88	284bp
D	lichenysin synthetase C	*Bacillus licheniformis* RS1	100%	*Bacillus licheniformis* ATCC 14580	269bp
E	lichenysin synthetase C	*Bacillus licheniformis* RS1	100%	*Bacillus licheniformis* ATCC 14580	269bp
F	lichenysin synthetase C	*Bacillus licheniformis* RS1	100%	*Bacillus licheniformis* ATCC 14580	269bp

To confirm that the produced fluids contained microorganisms in the same taxonomic group as the injected strains, PCR analysis of DNA obtained from the produced fluids after inoculation was conducted by using primers to detect the *gyrA* gene. The gyrA gene is used to distinguish closely related *Bacillus* species. For both ROB 15 and ROB 13 samples collected 14 hours after the pumps were turned on (14:00MT), three of the four clones submitted for sequencing were *B. licheniformis* strain RS-1, and one was *B. subtilis* subsp. *spizizenii* NRRL B-23049 with 99% identity from NCBI BlastN analysis (Table 4.6 and 4.7).

Table 4.6 BlastN Summary of ROB 15 at 14:00MT Clones from *gyrA* amplification.

Clone	Gene (Blastn)	Species	% Identity
A	DNA gyrase subunit A	*Bacillus licheniformis* RS1	99%
B	DNA gyrase subunit A	*Bacillus licheniformis* RS1	99%
C	DNA gyrase subunit A	*Bacillus subtilis* susp. *spizizenii* NRRL B-23049	99%
D	DNA gyrase subunit A	*Bacillus licheniformis* RS1	99%

Table 4.7 BlastN Summary of ROB 13 at 14:00MT Clones from *gyrA* amplification.

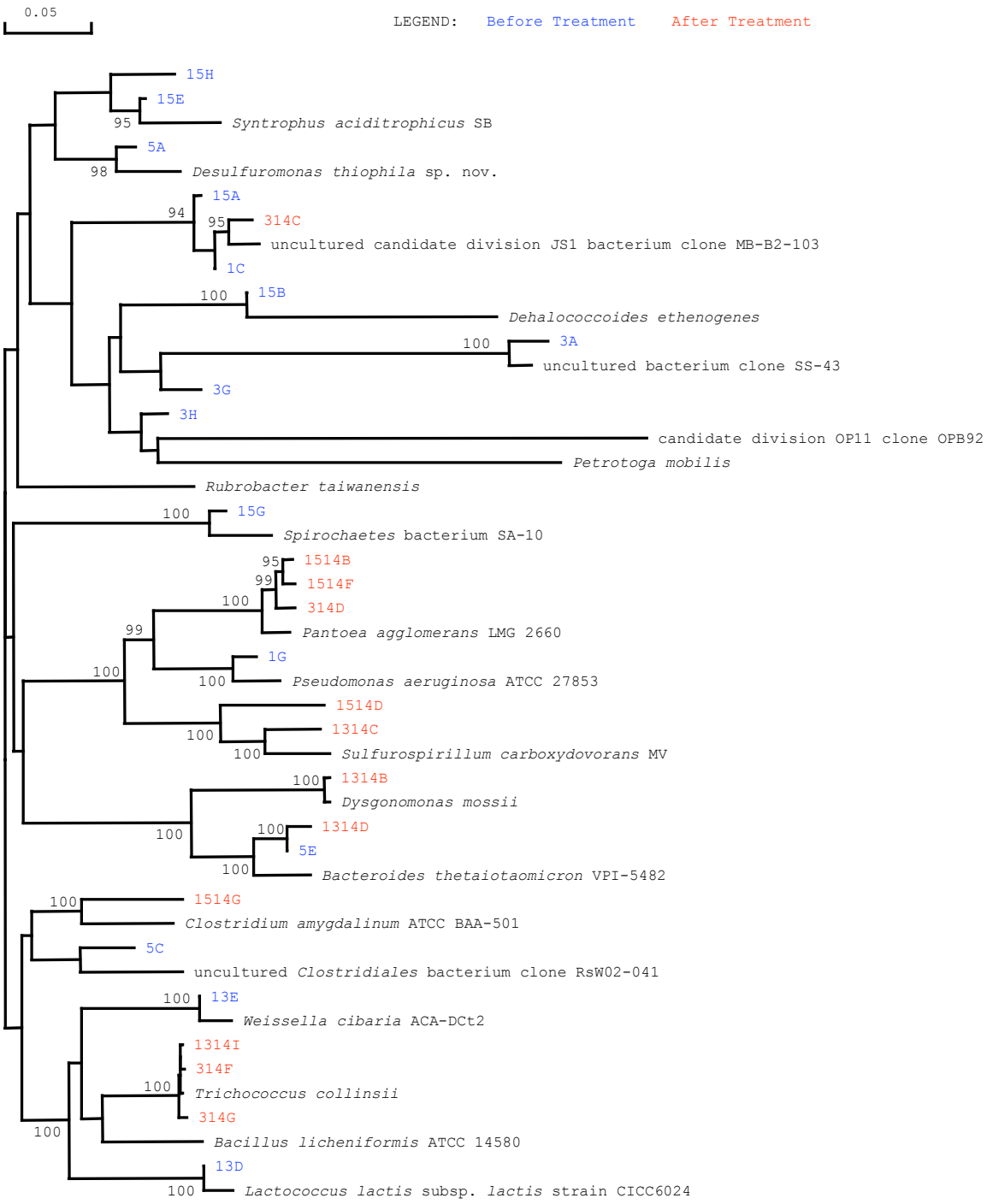
Clone	Gene (Blastn)	Species	% Identity
A	DNA gyrase subunit A	*Bacillus licheniformis* RS1	99%
B	DNA gyrase subunit A	*Bacillus subtilis* susp. *spizizenii* NRRL B-23049	100%
C	DNA gyrase subunit A	*Bacillus licheniformis* RS1	99%
D	DNA gyrase subunit A	*Bacillus licheniformis* RS1	99%

Thus, we were able to verify the post-MEOR treatment persistence of our two field strains by culture-independent molecular methods even through the biosurfactant producers only accounted for about 0.01% of the cultivable microbial population. However when the DNA extracted from time-course brine samples for ROB 15 and ROB 13 at 14:00MT were amplified with the *rhlR*F and *rhlR*R primer set, no PCR products were observed on a 1.5% agarose gel. Since ROB 15 and ROB 13 were not bioaugmented with any *Pseudomonas aeruginosa* strains, this result could indicate that *Pseudomonas aeruginosa* strains were not indigenous to the reservoir formations and were not enriched by the MEOR nutrient amendment in significant numbers.

Effect of treatments on the oil reservoir community.

Culture-independent approaches were used to determine how the injection of an inoculum and nutrients affected the indigenous microbial populations. DNA extracted from the produced fluids before and after treatment was PCR amplified by using primers for the 16S rRNA gene and the amplicons were cloned and sequenced. Before and after treatment clones are identified in blue and red, respectively, to indicate changes in the microbial community resulting from the bioaugmentation and nutritional amendment (Figure 4.3).

Although a much larger number of clones would be required to ascertain statistically relevant conclusions, several observations can be made from the very limited sequence analysis. The most prevalent clones before treatment appear to be similar to 15A, 15B and 15H identified samples from ROB 15 well, which are each related to uncultured microorganisms. Clone 15A has a 97% nucleotide identity with an uncultured bacterium clone Amsterdam-2B-48 (NCBI Accession AY592406) identified from a deep sea mud volcano perhaps related to candidate division JS1. Clone 15B has a 90% nucleotide identity with an uncultured bacterium clone MB-C2-127 identified from methane hydrate-bearing, deep marine sediments (NCBI Accession AY093480). Clone 15H has a 97% nucleotide identity with an uncultured bacterium clone ODP1230B18.24 from a sub-sea floor community with methane hydrate. After treatment, the clones in highest numbers could be similar to 1314I, 314G, 1314D, 1314C and 314F found in Figure 4.3 16S rRNA gene phylogenetic tree comparing clones obtained before and after treatment.


0.05
LEGEND: Before Treatment After Treatment
15H
15E
95
Syntrophus aciditrophicus SB
5A
98
Desulfuromonas thiophila sp. nov.
15A
94
95
314C
uncultured candidate division JS1 bacterium clone MB-B2-103
1C
100
15B
Dehalococcoides ethenogenes
100
3A
uncultured bacterium clone SS-43
3G
3H
candidate division OP11 clone OPB92
Petrotoga mobilis
Rubrobacter taiwanensis
100
15G
Spirochaetes bacterium SA-10
95
1514B
99
1514F
100
314D
Pantoea agglomerans LMG 2660
99
1G
100
100
Pseudomonas aeruginosa ATCC 27853
1514D
100
1314C
100
Sulfurospirillum carboxydovorans MV
100
1314B
Dysgonomonas mossii
100
100
1314D
5E
100
Bacteroides thetaiotaomicron VPI-5482
100
1514G
Clostridium amygdalinum ATCC BAA-501
5C
uncultured Clostridiales bacterium clone RsW02-041
100
13E
Weissella cibaria ACA-DCt2
1314I
314F
100
Trichococcus collinsii
314G
100
Bacillus licheniformis ATCC 14580
13D
100
Lactococcus lactis subsp. lactis strain CICC6024

Legend

15: Viola/ Robertson 15 before treatment
13: Viola/ Robertson 13 before treatment
3: Viola/ Robertson 3 before treatment
1: Viola/ Parrish before treatment
5: Viola/ Robertson 5 before treatment
1514: Viola/ Robertson 15 after treatment with cells and nutrients (14:00MT)
1314: Viola/ Robertson 13 after treatment with cells and nutrients (14:00MT)
314: Viola/ Robertson 3 after treatment with nutrients only (14:00MT)
114: Viola/ Parrish 1 after treatment with nutrients only (14:00MT)
514: Viola/ Robertson 5 after treatment with brine only (14:00MT)

Blue : formation clone before treatment

Red : formation clone after treatment

ROB13 and ROB3 with high homology to uncultured clones from a low temperature, biodegraded oil reservoir, close in identity to the genus *Trichococcus*. This genus consists of lactic acid-producing fermentative bacteria. Interestingly, these clones were founds both in samples from wells that received nutrients and inoculum and from wells that received nutrients only. Clones 1314D and 1314C were detected after treatment and showed near homology to *Bacteroides* and *Sulfospirillum*, respectively [121, 122]. The low number of *Bacillus* present in the inoculum may have precluded their detection by this approach. Analysis of additional clones will be needed to determine statistically the effect of treatment on the microbial community.

Prevalence of biosurfactant producers in oil formations with varying salinity.

We surveyed the produced fluids collected from oil formations that differed in lithology and geochemistry to determine if they contained indigenous biosurfactant producers (i.e. *Bacillus* species). Such information is needed to determine whether bioaugmentation is required for MEOR treatment and to determine whether any bioaugmentation and/or nutritional amendment would select for biosurfactant-producing microbes such as *Bacillus* species. Mid-continent reservoirs especially in central Oklahoma have wide ranges of salinity with some oil reservoirs having very high salinities. Thus, salinity is an important factor that would control microbial growth and activity. For this reason, we sampled produced fluids from reservoirs with a wide range of salt concentrations [Cl^-] (Table 4.2).

First we tested whether we could detect the functional genes for lipopeptide biosurfactant production in the DNA extracted from these produced fluids. The presence of these genes would indicate the potential to make lipopeptide biosurfactants in situ. We used the *srfA3/licA3* primers to detect lipopeptide biosynthetic genes. We found *srfA3/licA3* amplicons from the DNA extracted from nine of the eleven wells (six of seven

formations). The *gyrA* amplicons were detected in samples from seven of the eleven wells (six of seven formations). Furthermore, 16S rRNA gene amplicons were obtained by PCR using the 27F and 1492R primers in all of the samples (11 wells; 7 formations) (Table 4.6). The latter result shows that we successfully extracted DNA from each formation and that the sample did not contain inhibitory substances that interfered with the PCR reactions. The presence of the *srfA3/licA3* genes indicates the genetic potential to make lipopeptide biosurfactants is present in six of the seven formations. The detection of the *gyrA* gene indicates that *Bacillus* species phylogenetically related to the *Bacillus subtilis-Bacillus licheniformis* group, a group known to contain many biosurfactant producing strains, is present in six of the seven formations and corroborates the findings of the functional gene analysis with the *srfA3/licA3* primers.

Table 4.6 Genetic potential to make lipopeptide biosurfactants in oil formations with wide range of salinity.

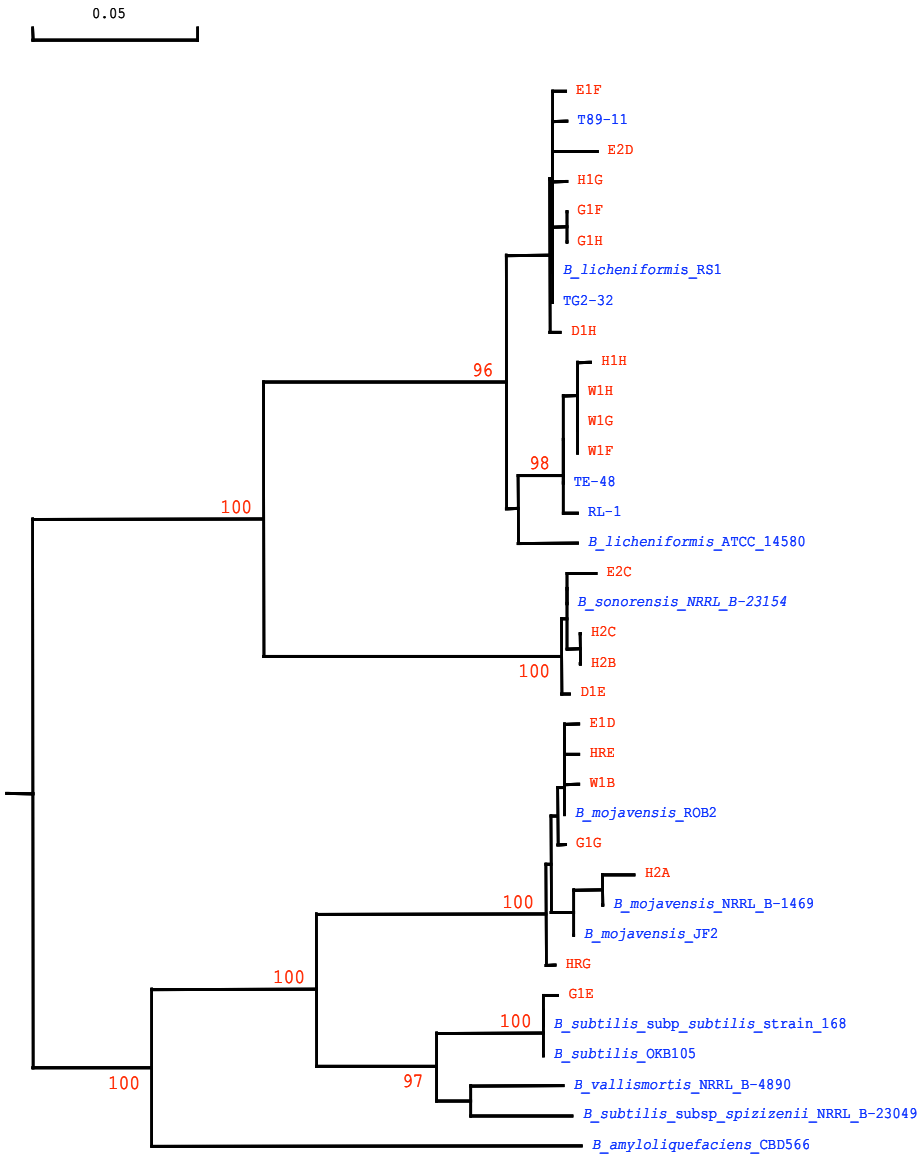
Formation Type	Well Number	*srfA3/licA3* amplicon	*gyrA* amplicon	16S rRNA gene amplicon
Viola	Robertson 15	-	-	+
	Robertson 13	-	-	+
Wewoka Sand	Roberston 14	+	+	+
Hunton	Robertson 11	+		+
	Roberston 2	+	+	+
Earlsboro	Tract 8 Well 1	+	+	+
	Tract 5 Well W1-3	+	+	+
Gilcrease	Freeze 1	+	+	+
Hart	Hewitt 1	+	-	+
Skinner Flood	Davis 1	+	+	+
	Turbeville 1	+	+	+

The sequences of the amplicons were determined to confirm that the correct gene product had been obtained. Twenty-one clones obtained from sequences amplified by the *srfA3/licA3* primers had high sequence similarity to *Bacillus* species that commonly contain biosurfactant-producing strains (Figure 4.4). Only the two Viola formations (V1 and V2) did not produce PCR products with the *srfA3/licA3* primers. As a result, none of our trageted *Bacillus* species could be identified from these two formations. Negative results were also obtained with the Viola samples when tested with the *gyrA* primers, which also detected the same *Bacillus* species. These results suggest that these *Bacillus* species may not be significant members of the microbial community in this reservoir. Interestingly, the Viola formations had the lowest chloride concentration, at approximately 0.4%.

A variety of *B. licheniformis*, *B. sonorensis*, *B. mojavensis* and a single *B. subtilis* strain were revealed from the cloning and sequencing analyses (Figure 4.4). The most commonly detected sequence (n=10) was similar to members of *B. licheniformis*. Many

of these showed significant homology to strains previously isolated from the Sonoran desert (TG2-32, T89-11, TE48 and RL-1). These strains are known to be halotolerant and grow in NaCl concentration up to 10 to 15% [116]. The formations in which *B. licheniformis*-related sequences were detected had chloride concentrations from 2.2% to 16%. Six sequences were related to members of *B. mojavensis* and were detected in four different formations that ranged from approximately 2.2% to 13% chloride concentration. One sequence was related to *B. subtilis* subsp. *subtilis*, which was obtained from the Gilcrease formation that had approximately 11.4% [Cl-]. Some sequences (100% nucleotide identity) were detected multiple times. G1F and G1H were identical to each other as were W1F, W1G, and W1H and H2B and H2C.

Figure 4.4 Phylogenetic tree of *srfA3/licA3* clones from different oil formation brines.



Legend for Figure 4.4, 4.5 and 4.6. Phylogenetic trees of genes of interest (*srfA3/licA3*, *gyrA,* 16S rRNA)

V1: Viola/ Robertson 15
V2: Viola/ Robertson 13
H1: Hunton/ Robertson 11
H2: Hunton/ Robertson 2
W1: Wewoka Sand/ Robertson 14
E1: Earlsboro 1
E2: Earlsboro 2 (Tract 5)
G1: Gilcrease/ Freeze 1
T1: Skinner Flood/ Turbeville 1
D1: Skinner Flood/ Davis 1
HR: Hart/ Hewitt 1

Blue: type strain consensus sequence
Red: formation clone sequence

Figure 4.5 Phylogenetic tree of *gyrA* clones recovered from different oil formation brines.

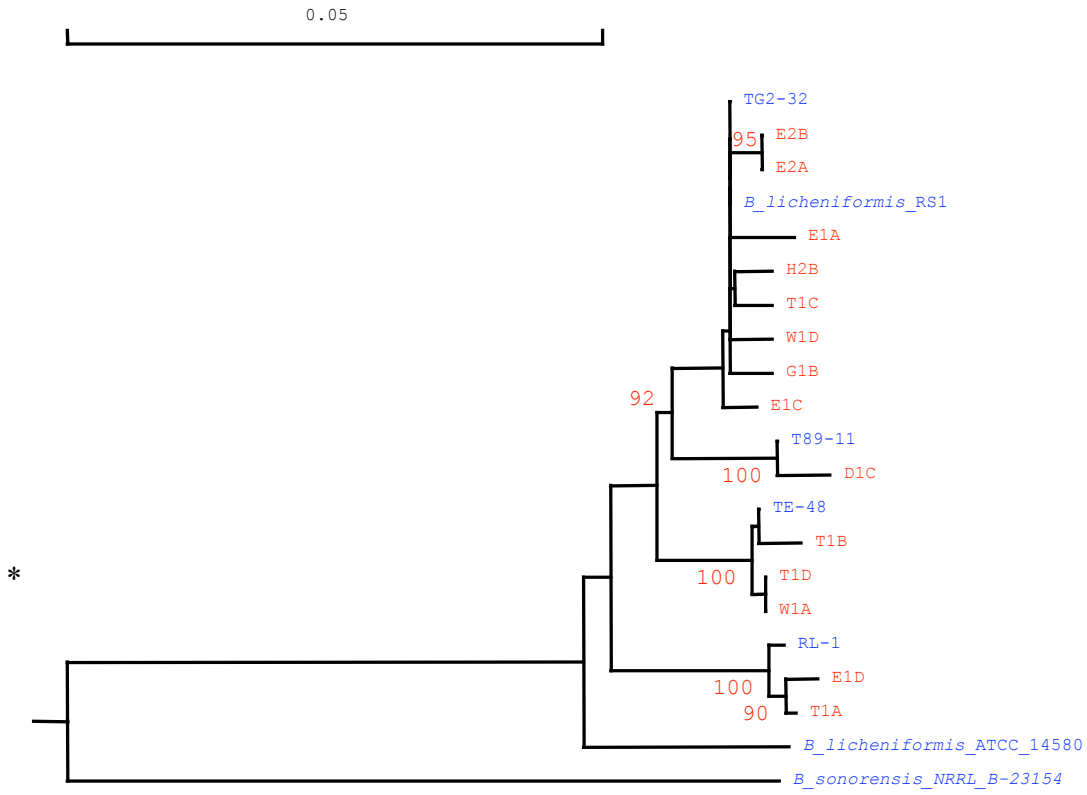
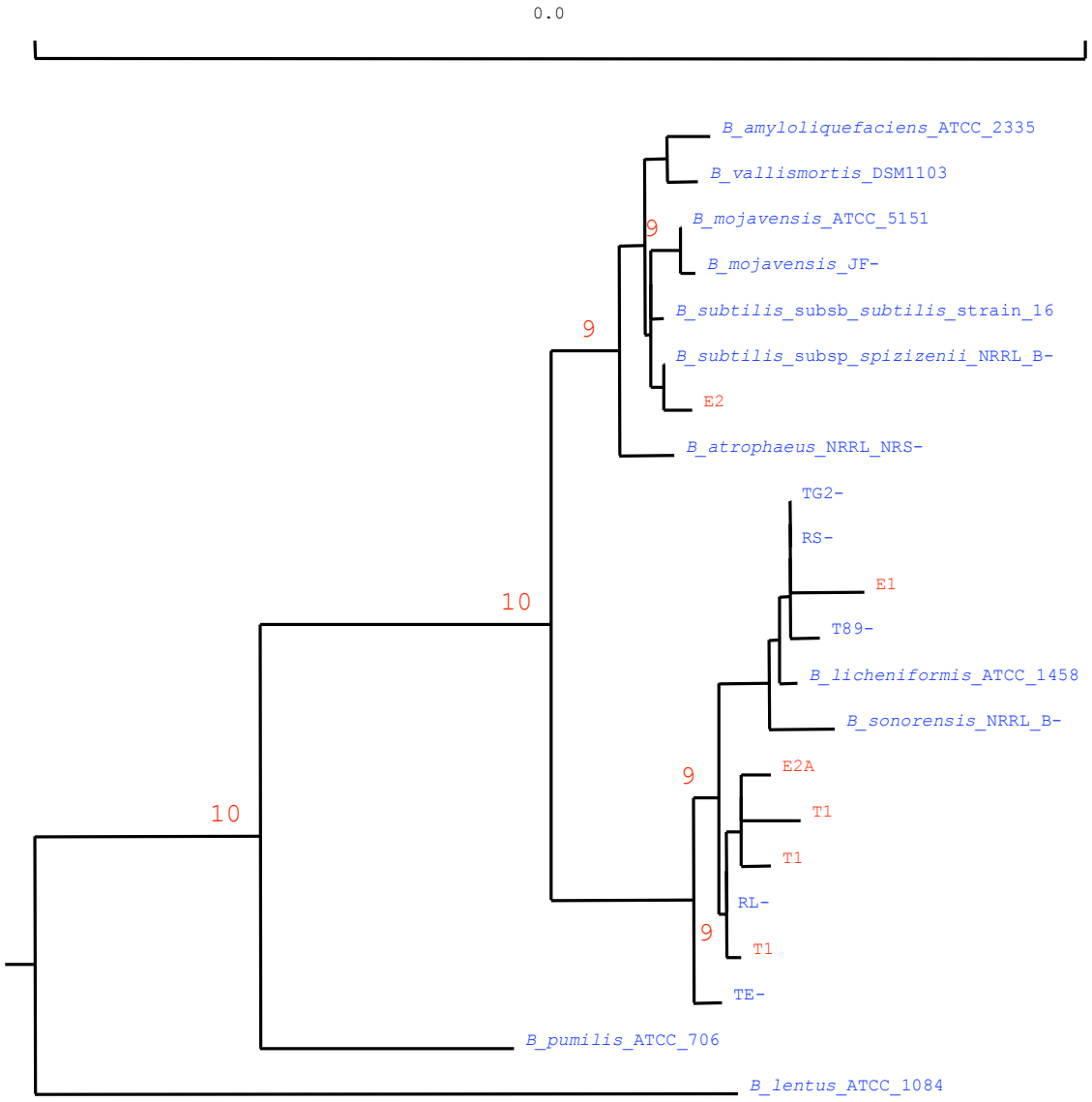


Figure 4.6 Phylogenetic tree of 16S rRNA gene clones related to the genus *Bacillus* from different oil formation brines.



A total of 14 clones with similarity to *Bacillus* species that commonly contain biosurfactant producers were identified by culture-independent methods using the *gyrA* primer set (Figure 4.5). Again, only the two Viola formations samples (V1 and V2), which had low salinity, 0.4% [Cl-], did not result in a PCR product. This is consistent with the finding with the *srfA3/licA3* PCR results and suggests that these *Bacillus* species are not indigenous to this formation. The distribution of *gyrA* clones was much narrower than for *srfA3/licA3* in that only sequences with similarity to *B. licheniformis* species were detected. Many of these sequences showed significant homology to strains isolated from the Sonoran Desert. Formations in which *B. licheniformis*-related sequences were obtained ranged from 2.2% to 16% [Cl^-]. Nine sequences grouped with strains TG2-32, T89-11 and RS-1. The E2A and E2B sequences were found in the same formation and had 100% nucleotide identity. Conversely, T1D and W1A had identical sequences but were found in different formations. Thus, species of the *B. licheniformis –B. subtilis* clade, which is known to contain biosurfactant producers, can be identified in a variety of formations so long as the salinity is above 0.4% in [Cl^-].

Six clones with similarity to *Bacillus* species were detected in clone libraries obtained after amplification with the eubaterial 27F and 1492R primer set to detect the 16S rRNA gene (Figure 4.6). Five clones had sequences that grouped them with *B. licheniformis*, and the remaining clone had a sequence that was similar to *B. subtilis* subsp. *spizizenii*. The sequences of four clones were similar to the Sonoran Desert *B. licheniformis* strains RL-1 and TE-48, and one clone grouped with strainsTG2-32, T89-11 and RS-1. Interestingly, 16S rRNA gene sequences similar to *Bacillus* species were obtained only from Earlsboro and Turbeville brine samples. These two formations had salinities of 9.4% to 16% [Cl^-]. However, 16S rRNA gene sequences were obtained from all eleven formations, showing again that the PCR protocols were effective. The gene sequences of these clones will be discussed below.

Other sequences obtained from 16S rRNA gene amplification from selected formations are tabled below (Tables 4.7, 8, 9, 10, 11, 12). Since only 6 to 10 clones were obtained from each of the seven formations, statistical analysis techniques are limited. However some putative trends may be ascertained form the data. None of the clones in the Viola formation, which has approximately 0.4% [Cl-], are homologues to NCBI GenBank sequences of know halotolerant microbes or sequences obtained from high salt environments (Table 4.7). Seven sequences were most similar to those of uncultured microbes, many of which were obtained from methanogenic and dechlorinating environments. Conversely, most of the sequences from the Hunton (Robertson 11 well) formation (3.2% Cl^-) have high identity with uncultured clones from brackish or hyperaline environments associated with sulfate-reducing conditions (Table 4.8). Clones C and F had sequences with 99% nucleotide homology to a cultured strain related to the sulfate reducer *Desulfotignum balticum* DSM7044, which was isolated from an oil reservoir model column [123].

As previously described in the 16S rRNA gene phylogenetic tree (Figure 4.6), Earlsboro 1 and Earlsboro 2 samples contained sequences related to the *Bacillus licheniformis-B. subtilis* clade. Interestingly, a 16S rRNA gene sequence (clone B) obtained from the

Earlsboro 1 sample had 99% identity to the sequence from *B. licheniformis* strain M1-1 (Table 4.9) [124]. The Earlsboro 1 (clone B) sequence differs by 5 bp from that of strain RS-1. The Earlsboro 16S rRNA gene sequence (clone B) could correspond to the *srfA3/licA3* gene sequence detected in this sample (Figure 4.4). Earlsboro 2 samples contained a 16S rRNA gene sequence with 99% nucleotide identity to that of *B. licheniformis* strain ACO1 (a halo-thermotolerant strain from a Persian petroleum reservoir) and to *B. subtilis* strain MO2 (a halotolerant, aerobe from the Great Salt Plains of Oklahoma) (Table 4.10) [125, 126]. Furthermore, multiple 16S rRNA gene sequences with 98% or higher identity to those of lactic acid-producing fermenters such as *Lactococcus lactis* and *Weissella confusa* were found. In addition, Earlsboro 1 sample contained a 16S rRNA gene sequence similar to that of *Ralstonia mannitolilytica* strain AU428, which forms biofilms (Table 4.9) [127]. The Gilcrease 1 samples had five 16S rRNA gene sequences similar to that of *Ralstonia* sp. (Table 4.11). In samples from the Skinner Flood/Turbeville 1 formation, 3 of the 8 clones had 16S rRNA gene sequences with 99% nucleotide identity to the same *B. licheniformis* strain ACO1 isolated from a halo-thermotolerant isolate from Persian petroleum reservoir (Table 4.12) [125]. Four clones had 16S rRNA gene sequences that were similar to that of lactic acid-producing fermenters in the genus *Lactobacillus.* Earlsboro 1, Gilcrease 1/Freeze 1 and Skinner Flood/Turbeville 1 samples yielded clones of the lactic acid producing fermenting *Leuconostoc* genus. These reservoirs had [Cl^-] from 9.4% to 16%.

Table 4.7 16S rRNA gene clone summary for the Viola (Robertson 15 well) formation (0.4% m/V of [Cl-]).

Clone	16S rDNA (blastn)	Accession	% Identity
A	Uncultured bacterium clone SHD-231 (1,2-dichloropropane dechlorinator)	AJ306798	96%
B	Uncultured bacterium clone PHB07 (propionate-degrading methanogenic consortium)	AB232821	97%
C	*Olavius algarvensis* sulfate-reducing endosymbiont (Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm)	AF328857	91%
D	Uncultured bacterium clone: ODP1230B18.24 (methane hydrate-bearing deep marine sediments)	AB177162	96%
E	Uncultured bacterium clone PHB07 (propionate-degrading methanogenic consortium)	AB232821	97%
F	Uncultured bacterium clone TANB44 (dechlorinating community resulting from in situ biostimulation in a trichloroethene-contaminated deep, fractured basalt aquifer)	AY667258_	89%
G	Uncultured anaerobic bacterium clone A-2A (Anaerobic Swine Lagoons)	AY953190	88%
H	Uncultured bacterium clone PHB07 (propionate-degrading methanogenic consortium)	AB232821	97%

Table 4.8 16S rRNA gene clone summary for the Hunton (Robertson 11 well) formation (3.2% m/V of [Cl-]).

Clone	16S rDNA (blastn)	Accession	% Identity
A	Uncultured Clostridia (German Wadden Sea)	AY370633	93%
B	Uncultured delta proteobacterium clone LA30-B27 (Hypersaline Lake Laysan and a brackish pond on Pearl and Hermes Atoll)	AF513951	94%
C	Delta proteobacterium S2651 (sulfate-reducing bacterium isolated from an oil reservoir model column)(also homologus to *Desulfotignum balticum* DSM 7044)	AF177429	99%
D	Uncultured bacterium clone ODP1230B23.08 (methane hydrate-bearing deep marine sediments on the Pacific Ocean Margin)	AB177179	96%
E	Uncultured Clostridiales bacterium clone D1Dbac (halophilic sulfate-reducing prokaryotes from an extreme hypersaline sediment in Great Salt Lake)	DQ386212	98%
F	Delta proteobacterium S2651 (sulfate-reducing bacterium isolated from an oil reservoir model column)(also homologus to *Desulfotignum balticum* DSM 7044)	AF177429	99%

Table 4.9 16S rRNA clone summary for the Earlsboro 1 formation (9.5% m/V of [Cl-]).

Clone	16S rDNA (blastn)	Accession	% Identity
A	*Leuconostoc citreum* IH22 (fermented cabbage product)	AF111949	99%
B	*Bacillus licheniformis* strain M1-1 (cellulose degrading mixed culture)	AB039328_	99%
C	*Ralstonia mannitolilytica* strain AU428 (secretions of cystic fibrosis patients)	AY043378_	99%
D	*Tepidimonas arfidensis* (leukemia bone marrow)	AY594193	99%
E	Uncultured bacterium clone EV818BHEB5102702SAS62 (subsurface water of Khalahari Shield)	DQ256349	99%
F	Uncultured *Anaerococcus* sp. clone ML2-55 (bacterial biota of normal human forearm skin)	DQ847450	97%
G	Uncultured gamma proteobacterium clone C-CL42 (surrogate minerals incubated in an acidic uranium-contaminated aquifer)	AY622230	95%
H	Uncultured gamma proteobacterium clone C-CL42 (surrogate minerals incubated in an acidic uranium-contaminated aquifer)	AY622230	95%

Table 4.10 16S rRNA gene clone summary for the Earlsboro 2 formation (10.3% m/V of [Cl-]).

Clone	16S rDNA (blastn)	Accession	% Identity
AA	*Bacillus licheniformis* strain ACO1 (halothermotolerant isolate from Persian petroleum reservoir)	DQ228696	99%
A	*Bacillus subtilis* strain MO2 (Halotolerant Aerobic Heterotrophic Bacteria from the Great Salt Plains of Oklahoma)	AY553095	99%
B	*Lactococcus lactis* subsp. *lactis* IL1403	AE006456	99%
D	*Acinetobacter calcoaceticus*	AJ888984_	99%
E	*Lactococcus lactis* clone 8C3 (breast milk of healthy women)	AM157424_	99%
F	*Lactococcus lactis* clone 8C3 (breast milk of healthy women)	AM157424_	99%
G	*Halanaerobiaceae* bacterium Benz1 (halophilic sulfate-reducing prokaryotes from an extreme hypersaline sediment in Great Salt Lake)	DQ386220	97%
H	*Weissella confusa* strain Inje LM S-338 (lactic acid bacterium)	DQ321751	98%

Table 4.11 16S rRNA gene clone summary for the Gilcrease/Freeze 1 formation (11.4% m/V of [Cl-]).

Clone	16S rDNA (blastn)	Accession	% Identity
AA	*Leuconostoc citreum* IH22 (fermented cabbage product)	AF111949	99%
BB	*Ralstonia* sp. AU378 (Respiratory Secretions of Cystic Fibrosis Patients)	AY043380	99%
A	*Ralstonia* sp. AU378 (Respiratory Secretions of Cystic Fibrosis Patients)	AY043380	99%
B	*Ralstonia* sp. AU378 (Respiratory Secretions of Cystic Fibrosis Patients)	AY043380	99%
C	*Ralstonia* sp. AU378 (Respiratory Secretions of Cystic Fibrosis Patients)	AY043380	99%
D	Uncultured bacterium clone EV818BHEB5102702SAS62 (subsurface water of Khalahari Shield)	DQ256349	99%
E	*Propionibacterium acnes* isolate WD1	AY642054_	99%
F	*Ralstonia* sp. AU378 (Respiratory Secretions of Cystic Fibrosis Patients)	AY043380	99%
G	*Propionibacterium acnes* isolate WD1	AY642054_	99%
H	Uncultured *Desulfohalobiaceae* bacterium clone J2Dbac (halophilic sulfate-reducers from an extreme hypersaline sediment in Great Salt Lake)	DQ386183	97%

Table 4.12 16S rRNA clone summary for the Skinner Flood/Turbeville 1 formation (15.9% Cl-).

Clone	16S rDNA (blastn)	Accession	% Identity
A	*Bacillus licheniformis* strain ACO1 (halothermotolerant isolate from Persian petroleum reservoir)	DQ228696	99%
B	*Lactococcus lactis* clone 8C3 (breast milk of healthy women)	AM157424_	99%
C	*Lactococcus lactis* clone 8C3 (breast milk of healthy women)	AM157424_	99%
D	*Bacillus licheniformis* strain ACO1 (halothermotolerant isolate from Persian petroleum reservoir)	DQ228696	99%
E	*Bacillus licheniformis* strain ACO1 (halothermotolerant isolate from Persian petroleum reservoir)	DQ228696	99%
F	*Leuconostoc mesenteroides* strain LM2 (fermented Korean traditional foods)	AY675249	99%
G	*Lactobacillus fermentum* strain SFCB2-6c (Lactic Acid Bacteria Community SFC-2 and Effects on Straw Fermentation)	DQ486144	99%
H	*Lactococcus lactis* clone 8C3 (breast milk of healthy women)	AM157424_	99%

Discussion

We found the genetic potential to make lipopeptide biosurfactants in six of the seven formations tested. In three formations, it was possible to sample multiple wells and in each case where multiple samples were obtained from the same formation, the results between the samples were reproducible. As discussed in Section 3 of this report, cultivation-dependent approaches showed that biosurfactant producers were not numerous in these formations. However, if the appropriate nutrients were used, biosurfactant activity could be stimulated. The detection of the lipopeptide genes was corroborated with the concomitant detection of the *gyrA* gene specific for the *Bacillus subtilis-Bacillus licheniformis* group. We did not obtain evidence for the presence of rhamnolipid-producing microorganisms either by culture-dependent or by culture-independent methods. The detection of the lipopeptide gene and evidence of the presence of members of the *Bacillus subtilis-Bacillus licheniformis* group by *gyrA* gene analysis in all but one of the formations sampled suggests that biosurfactant producers related to the *Bacillus subtilis-Bacillus licheniformis* group are prevalent in oil reservoirs with a wide range of salinity. Thus, it may not be necessary to inoculate such reservoirs. However, the addition of an inoculum could reduce shut-in times and result in more reproducible performance.

As a result, development of PCR primers for biosurfactant functional genes for specific *Bacillus* and *Pseudomonas* species typically capable of biosurfactant production was utilized to probe a variety of petroleum formations in central Oklahoma. The goal was to determine distinguish which petroleum formations would require bioaugmentation for successful MEOR treatment from those that may not. Although no indigenous *Pseudomonas* species clones were identified using *rhlR* or 16S rRNA gene primers for any formation type, all formations except the Viola provided clones indicative of *B. licheniformis/ subtilis* clade species. Thus, Viola formation wells may indeed require bioaugmentation with biosurfactant producers for any MEOR process to succeed. Conversely, oil wells in the Earlsboro or Turbeville formation types yielded *B. licheniformis/ subtilis* clade clones homologous to oil field isolates even with the non-specific, 16S rRNA primers and most likely would not require an inoculum. In addition, all formation production waters were above 2% [Cl^-] except for the Viola formation, which had approximately 0.4% [Cl^-]. Earlsboro and Turbeville production waters were approximately 10% and 16%, respectively with Turbeville being the formation with the highest [Cl^-] sampled. Since many *Bacillus* species are at least moderately halophilic, our findings indicate that formations with at least 2% [Cl^-] may have indigenous, biosurfactant producing *Bacillus* sp. present.

Since biosurfactant activity does not appear to be indigenous to the Viola formation wells utilized in our field test, bioaugmentation and nutritional amendment were required for MEOR treatment. Even though the environmental conditions were altered by the glucose-based nutritional amendment in attempt to select for our bioaugmented field strains, *B. licheniformis* strain RS-1 and *B. subtilis* subsp. *spizizenii* NRRl B-23049, the normal homeostatic mechanism within the reservoir microbial community did not prevent their establishment and persistence. Both the *srfA3/licA3* and *gyrA* primer sets successfully identified our bioaugmented strains after treatment.

Given that oil reservoirs have diverse and metabolically active microbial communities, care must be taken to ensure that the nutrient regime does not stimulate detrimental activities such as souring and corrosion or competition by non-biosurfactant producing heterotrophic fermenters that contribute negatively to the MEOR effects [114]. Minimally, one will need to know what organisms are present and the factors that influence their growth and activity to exploit their activities in the reservoir. Many fermentative anaerobes produce large amounts of acids such as acetic, lactic, and butyric acids; solvents such as ethanol, acetone, butanol and 2, 3-butanediol; and gases such as CO_2 and H_2 from readily fermentable carbohydrate feedstocks such as molasses (30). Organic acid production can lead to the dissolution of carbonates in source rocks, enhancing permeability and porosity [128, 129]. Solvents can alter the wettability of the oil-rock interface, releasing oil from the porous matrix. Due to its preferential solubility in oil, CO_2 production may swell the oil and reduce its viscosity, which would make the oil more mobile. *Leuconostoc* species produce dextran, an a-1, 4-D-glucan [130] that effectively reduces the permeability of fused-glass columns [131, 132]. In addition, a variety of *Bacillus* species that grow anaerobically and produce extracellular polysaccharides at temperatures up to 50^oC and salinities up to 10% NaCl have been

isolated [133]. These types of bacteria are found in a variety of environments including oil reservoirs, which suggests that the injection of these bacteria into oil reservoirs may not be needed. All that would be required is the injection of nutrients to stimulate their in situ growth and metabolism.

Fermentative heterotrophic bacteria appeared to a predominant group of bacteria based on the analysis of clones obtained after nutrient treatment. They were also commonly detected in clone libraries of untreated formations with chloride concentrations above 10%. *Trichococcus* species that have been identified previously in high numbers from gas condensate contaminated aquifers [134] and are known lactic-acid producing, sugar fermenters [121]. Other sequences detected after nutrient treatment include those from heterotrophic fermentative microorganisms similar to *Spirochaeta*, *Lactococcus*, *Clostridium and Weisella*. Both *Sulfospirillum* and *Bacteroides* have previously been identified in oil reservoirs [122]. Recently, a culture-dependent and culture-dependent investigation of the microbial diversity of production waters in low temperature and low salinity petroleum reservoir that had not been subjected to water injection identified *Spirochaeta, Sulfospirillum and Clostridium* as several of the dominant genera of the cultivable population [122]. Furthermore, *Lactobacillus*, *Lactococcus* and *Leuconostoc* were prevalent in our survey in petroleum formations with chloride concentrations above 10%. *Acinetobacter* sp. clone was identified in production water from the Earlsboro 2 formation. Thus heterotrophic fermenters that can positively affect MEOR by means other than biosurfactant production were found to be indigenous to many oil formations in this study.

Whether heterotrophic fermentative microorganisms are truly indigenous in origin or are introduced into the subsurface by drilling or production operations (i.e. water-flooding) is debatable [135, 136]. However, lactic acid producing fermenters appear to be present especially in formations with >10% [Cl^-] and could compete for the glucose-based nutritional amendment in the field test production wells. As a result, the establishment of the biosurfactant-producing *Bacillus* species used as an inoculum needs to be optimized and the overall effect of competing heterotrophic fermenters needs to be considered. However as [Cl^-] increases, indigenous *Bacillus* species typical of petroleum environments were commonly detected in clone libraries perhaps lessening the requirement for bioaugmentation. Heterotrophic fermenters could also be present and compete for the glucose-based nutritional amendment. As a result, an inoculum with biosurfactant producing *Bacillus* species might still be warranted to ensure adequate numbers and ultimately MEOR success. Since no *Bacillus* species were detected by culture-dependent or culture-independent in the Viola formation, bioaugmentation in addition to nutritional amendment could be considered the MEOR treatment of choice. The impact of bioaugmentation and biostimulation on the structure of microbial communities has been demonstrated in several studies [115, 137, 138]. We show here the first successful utilization of molecular tools such as degenerate PCR primers, DGGE and 16S rRNA gene clone libraries to verify bioaugmented strain persistence and microbial community responses in a MEOR treatment.

5. Characterization of *Bacillus licheniformis* strain RS-1 and related strains for suitability in MEOR bioaugmentation in diverse oil formations

Abstract

An isolate, strain RS-1, grew and produced a biosurfactant anaerobically. RS-1 was able to grow aerobically in medium with 15% NaCl added, and biosurfactant production occurred in medium with up to 10% NaCl added. Strain RS-1 also grew over a wide range of pH values from 2 to 10. Genetically, strain RS-1 was related to several *Bacillus licheniformis* strains isolated from the Sonoran Desert. This group of bacteria shared characteristics important for microbial oil recovery including growth at high salinity and the ability to produce a biosurfactant anaerobically. Furthermore, we determined that *B. licheniformis* strain RS-1 is a Group 1 *B. licheniformis* species from 16S rRNA gene sequencing. The genetic fingerprinting technique called REP-PCR was utilized to confirm the phylogenetic placement of RS-1 at the subspecies level in Group 1.

Introduction

A microbial candidate for MEOR must be capable of growth and biosurfactant production over a wide range of physical and geochemical conditions such as salt (NaCl) concentration, pH, temperature and redox potential [139]. Various strains of *Bacillus*, and others, have been studied as possible candidates [107, 110, 139]. A putative *B. licheniformis* strain RS-1 isolated from a limestone oil reservoir in central Oklahoma was tested for its ability to grow under the environmental conditions found in many oil reservoirs. Several isolates from the Sonoran Desert have also been shown to be biosurfactant producers and have a broad tolerance to salt, pH and temperature under aerobic conditions [116]. As a result of their phenotypic similarities, we wanted to determine if these strains were closely related genetically. Such a finding would indicate that a phylogenically coherent group of strains exists with the characteristics useful for MEOR.

Materials and Methods

Aerobic growth.

Starter cultures of *Bacillus licheniformis* strain RS-1, *Bacillus subtilis* subsp. *subtilis* strain 168 and *Bacillus subtilis* subsp. *spizizenii* ATTC B23459 were grown in 5 mL of sterile Luria Bertani Broth (LB) in a 20mL test tube. The tubes were inoculated with an isolated colony from Plate Count Agar and incubated overnight at 37^{o}C with shaking (200rpm). A sterile 500 mL baffled flask with HEPA filter and 50 mL of LB was subsequently inoculated with starter culture to give an initial A_{600} of 0.02 (determined spectrophotometrically). The A_{600} was measured every hour or ½ hour (in log phase). Once the cultures reached stationary phase, A_{600} was read at 24 and 48 hours. The growth curve was then plotted as A_{600} versus time. The growth rate constant and doubling time were calculated during log phase growth with the following formulae:

Instantaneous growth rate constant: $\mu = \ln(X_t) - \ln(X_o) / (t - t_o)$ and average growth rate constant: $k = \mu/\ln 2$.

Phenotypic tests.

B. licheniformis strain RS-1, *Bacillus licheniformis* ATCC 14580, *Bacillus sonorensis* NRRL B23154, *Bacillus mojavensis* NRRL B 14698, *B. subtilis* subsp. *subtilis* strain 168, and *B. subtilis* subsp. *spizizenii* B23049, were tested for protease activity by streaking on skim milk plates (1% skim milk, 0.1% yeast extract, and 1.5% agar) [140]. The plates were incubated for 48 hours at 37ºC then measured for clearing diameter.

The above strains were also tested for nitrate reduction by inoculating 4 mL of nitrate broth in duplicate, and incubating for 24 hours at 37ºC. The cultures were treated with NO_3 Reagent A (0.6 g N, N-Dimethyl-α-naphthylamine in 100mL of 5N Acetic Acid) and NO_3 Reagent B (0.8 g Sulfanilic acid in 100 mL 5N Acetic Acid).

Aerobic NaCl tolerance and the effects on biosurfactant production.

The strains tested for their aerobic NaCl tolerance were: *B. licheniformis* strain RS-1, *B. licheniformis* ATCC 14580, *B. sonorensis* NRRL B23154, *B. mojavensis* NRRL B 14698, *B. subtilis* subsp. *subtilis* strain 168, and *B. subtilis* subsp. *spizizenii* B23049. LB was again utilized as the base medium and NaCl concentration was varied. The positive and negative culture controls were LB with no NaCl at pH 7. For this experiment, 20 mL test tubes containing 4 mL of LB covering the NaCl range of 0%, 1%, 2%, 5%, 8%, 10%, and 15% were inoculated with a loopful of overnight culture in LB without NaCl addition. The cultures were incubated at 37ºC with shaking (200 rpm). The growth was qualified for five days by visual inspection using by a combination of turbidity and pellicle size. The surfactant production was checked every day with the oil spread assay according to Youssef *et al.* [139].

Anaerobic NaCl tolerance and the effects on biosurfactant production.

The strains tested for anaerobic NaCl tolerance were: *B. licheniformis* strain RS-1, TG2-32, T89-11, RL-1 and TE-48. Medium E was utilized as the base medium and the NaCl concentration was varied. The positive and negative culture controls were Medium E with no NaCl addition at pH 7. Balch tubes containing 10 mL of anaerobically prepared medium E covering the NaCl range of 0%, 1%, 2%, 5%, 8%, 10%, 15% and 18% were inoculated with an overnight Medium E culture to an A_{600} of 0.05. The cultures were incubated at 37ºC and static conditions. The growth was qualified for five days by a spectrophotometer at A_{600}. The surfactant production was checked each day with the oil spread assay according to Youssef *et al.* [139].

Aerobic pH Tolerance of B. licheniformis-B. subtilis *clade strains.*

The strains tested for pH tolerance were: *B. licheniformis* strain RS-1, *B. licheniformis* ATCC 14580, *B. sonorensis* NRRL B23154, *B. mojavensis* NRRL B 14698, *B. subtilis* subsp. *subtilis* strain 168, and *B. subtilis* subsp. *spizizenii* NRRL B23049. The pH of

nutrient Broth (NB) was adjusted with HCl or NaOH to give a pH range of 2, 4, 6, 8, and 10. In duplicate, 4 mL of the pH media was inoculated with a loopful of overnight culture of each strain grown in NB (pH 7). The 20 mL tubes were allowed to incubate aerobically for 72 hours at 37ºC under static conditions. Growth was determined qualitatively by relative estimation of turbidity and pellicle formation.

Genotypic and genetic fingerprinting analysis of B. licheniformis *strain RS-1 and Sonoran Desert isolates.*

Phenol/chloroform extraction from a 50 mL overnight cell cultures of *B. licheniformis* strain RS-1, TG2-32, T89-11, RL-1, TE-48 grown in LB as previously described at 37^{o}C/200 rpm were used to obtain DNA template. PCR amplification of each strain was performed by using *srfA3/licA3*, *gyrA* and 16S rRNA gene primer sets (Table 5.1). PCR mix and reaction conditions were the same as previously described (Section 4) with approximately 500ng of DNA used as template in each reaction. The products were excised and extracted (Qiagen) from a 1.0 to 1.5% agarose gel, pGEM cloned (Promega) then plasmid amplified and purified (Qiagen). Clones were sequenced in both the T7 and SP6 directions by OMRF and assembled as well as compared in a multiple sequence alignment using DNAMAN to obtain a 3 clone consensus sequence for each gene per strain. Finally, all strains and genes were compared in phylogenetic trees constructed using DNAMAN and the Observed Divergency Distance Method with 1000 bootstrap trials.

Table 5.1 PCR primer sequences utilized for detection and identification of microorganisms.

Primer	Sequence	Reference
27F	5'-AGAGTTTGATCMTGG-3'	[1]
1492R	5'-TACCTTGTTACGACTT-3'	
GM5F	5'GCCCGCCGCGCCCGCGCCCGTCCCGCCGCCCCC GCCCGCCTACGGGAGGCAGCAG-3'	[4, 5]
907R	5'-CCGTCAATTCCTTTRAGTTT-3'	
*srfA3/licA3*F	5'-CAAAAKCGCAKCATACCAAKTTGAG-3'	[6, 7]
*srfA3/licA3*R	5'-TCATARAGCGGCAYATATTGATGC-3'	
*srfA3/licA3*R2	5'-AGCGGCAYATATTGATGCGGYTC-3'	
gyrA F	5'-CAGTCAGGAAATGCGTACGTCCTT-3'	[2]
gyrA R	5'-CAAGGTAATGCTCCAGGCATTGCT-3'	
rhlR F	5'-CTGCGCTCCWCGGAAATGGTG-3'	[8-11]
rhlR R	5'-TCTGGATGWYCTTGWGGTGGAAGTTC-3'	
BOXA1R	5'-CTACGGCAAGGCGACGCTGACG-3'	[3]

DNA template from each of the 5 strains as well as from type strains *B. licheniformis* ATCC 14580 and *B. sonorensis* NRRL B-23154 were also amplified in a REP-PCR reaction to obtain a genetic fingerprint comparison of the species. The reaction mix consisted of 5 μL of 10X PCR buffer, 5 μL of 5 mM betaine, 4 μL of 50 mM magnesium chloride, 1 μL of 10 mM dNTP mix, 4 μL of 50 pmol/μL of BOXA1R primer, 3 μL or approximately 500 ng of cell template, 0.25 μL of Platinum *Taq* polymerase (Invitrogen) and 29.75 μL of sterile deionized water forming a 50 μL reaction volume. The thermal cycler was programmed for 5 min at 95^{o}C for initial denaturation, 30 cycles of 1 min at 94^{o}C for denaturation, 1 min at 50^{o}C for annealing, 2 min at 72^{o}C for extension subsequently followed by a final extension at 72^{o}C for 8 min and a 4^{o}C quench.

Results

Aerobic growth curve of B. licheniformis *RS-1 in Luria Bertani Broth.*

From log phase growth*, B. licheniformis* strain RS-1 resulted in an aerobic, instantaneous growth rate constant (μ) of 0.551 hr^{-1} in LB (Figure 5-1). This was comparable to *B. subtilis* subsp. *subtilis* strain 168 at μ = 0.525 hr^{-1} but less than *B. subtilis* subsp. *spizizenii* ATCC B23459 with μ = 0.196 hr^{-1}. However we can conclude

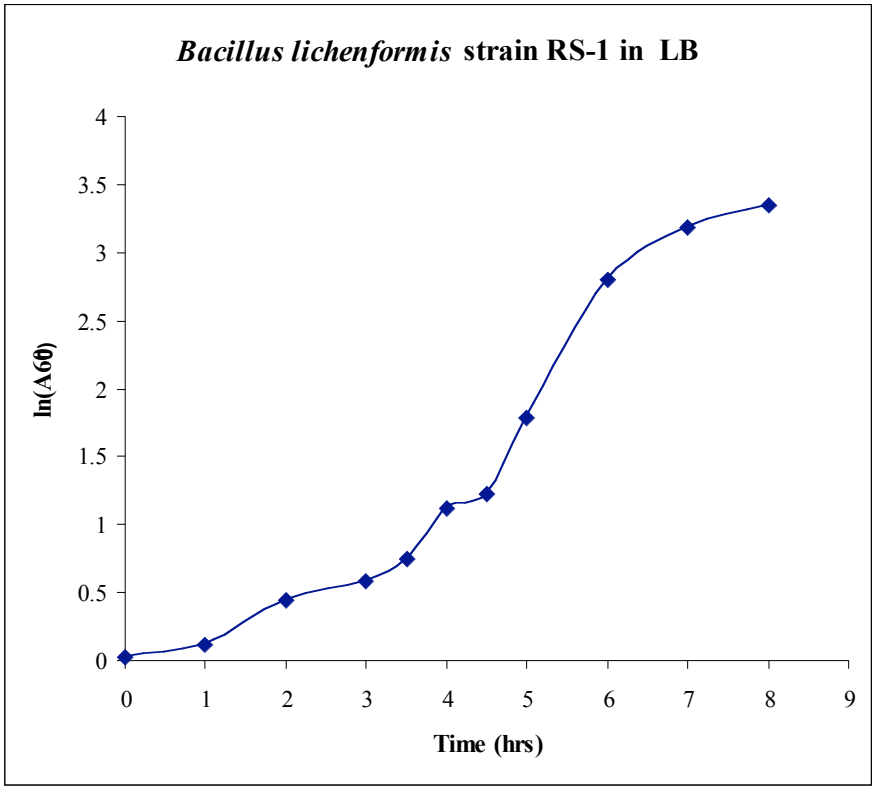


Figure 5-1 Aerobic growth curve for *B. licheniformis* strain RS-1 in LB

that *B. licheniformis* RS-1 is a relatively fast growing aerobic bacterium when a rich medium is used.

Phenotypic tests.

All the strains showed protease activity (Table 5-2). *B. sonorensis* (NRRL B23154) had very little activity, 0.2 cm clearing on the skim milk plates, while, *B. mojavensis* (NRRL B 14698), *B. subtilis* (168), and *B. subtilis* subsp. *spizizenii* (B23049) had the most protease activity (1.9 cm, 1.7 cm, and 1.8 cm clearings, respectively). Thus based on this protease activity assay, *B. licheniformis* RS-1 is closer to the phenotype of *B. licheniformis/ sonorensis* group as opposed to the phenotype of the *B. subtilis* group. All of the strains were capable of nitrate reduction, which proved to be a non-distinguishing phenotype.

Table 5-2 Comparison of protease activity on skim milk plates.

Strain	Clearing
***B. licheniformis* RS-1**	0.45 cm
***B. licheniformis* ATCC 14580**	0.4 cm
***B. sonorensis* NRRL B23154**	0.2 cm
***B. mojavensis* NRRL B 14698**	1.9 cm
***B. subtilis* 168**	1.7 cm
***B. subtilis spizizenii* B 23049**	1.8 cm

Aerobic NaCl tolerance and the effects on biosurfactant production.

Analysis of growth after five day of incubation showed that strain *B. licheniformis* (RS-1) had the remarkable ability to grow in medium with 0 to 15% NaCl concentration. This salt tolerance was also observed for the type strain of *B. licheniformis* (ATCC 14580). *B. subtilis* subsp. *spizizenii* (B23049) also had growth from 0 – 15% NaCl (Table 5-3). The other strains were limited to growth from 0 – 10% NaCl. The optimal growth varied with each strain. *B. licheniformis* (RS-1) showed optimal growth from 0 – 5%. The type strain for *B. licheniformis* (ATCC 14580) had the best growth from 0 – 8% NaCl. *B. sonorensis* (NRRL B23154) had optimal growth from 0 to 5%. *B. mojavensis* (NRRL B 14698) and *B. subtilis* subsp. *spizizenii* (B23049) showed the best growth at 2 and 5% NaCl. *B. subtilis* subsp. *subtilis* (168) had optimal growth at 0%. Most of the strains developed pellicles, interpreted as biofilms. The pellicles formed at the surface of the media. The thickness of the pellicles ranged from 0.1 cm to 0.4 cm. *B. subtilis* subsp. *subtilis* strain 168 did not show significant pellicle formation.

The surfactant production during the NaCl tolerance ranges from 0 to 0.9 cm (Table 5-4). The pinnacle of surfactant production was after 72 hours of incubation. The best production was found at the lower salt concentrations (≤ 2% NaCl). *B. mojavensis* NRRL B 14698 had the most surfactant production (0.9 cm) on day 3. *B. licheniformis* strain RS-1 had its best surfactant production (0.85 cm) on day three, as well. The rest of the strains had less production, with clearings ≤ 6 cm. *B. subtilis* subsp. *subtilis* strain 168 showed no surfactant production, which was expected since it has an inactive *sfp* gene for the post translational modification of surfactin required for activity.

Table 5-3. NaCl tolerance of different *Bacillus* species grown aerobically in LB for 5 days. Growth from – to ++++. (–) = no growth, ++++ (most turbid, largest pellicle)

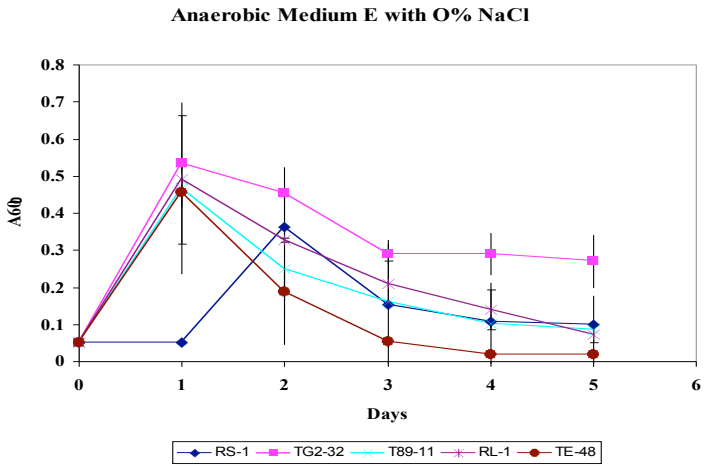
NaCl (m/V%)	*B. licheniformis* strain RS-1	*B. licheniformis* ATCC 14580	*B. sonorensis* NRRL B23154	*B. mojavensis* NRRL B14698	*B. subtilis* subsp. *subtilis* strain 168	*B. subtilis* subsp. *spizizenii* NRRL B 23049
0%	++++	+++	+++	+++	+++	++
1%	++++	+++	+++	+++	++	+++
2%	++++	+++	+++	++++	++	++++
5%	++++	+++	+++	++++	++	++++
8%	+++	+++	++	+	+	+++
10%	+++	++	+	+	+	+++
15%	++	+	–	–	–	+

Table 5-4 Biosurfactant production of different Bacillus species grown is medium with various NaCl concentrations. (The number in parenthesis is the days of incubation).

NaCl (m/V%)	*B. licheniformis* RS-1	*B. licheniformis* ATCC 14580	*B. sonorensis* NRRL B23154	*B. mojavensis* NRRL B 14698	*B. subtilis* subsp. *subtilis* strain 168	*B. subtilis subsp. spizizenii NRRL* B 23049
0%	0.65 cm (5)	**0.45 cm (5)**	0.35 cm (5)	**0.8 cm (3)**	0	0.3 cm (3)
1%	0.6 cm (3)	0.3 cm (3)	0.3 cm (4)	**0.9 cm (3)**	0	0.55 cm (3)
2%	**0.85 cm (3)**	0.5 cm (4)	0.3 cm (4)	0.35 cm (4)	0	**0.6 cm (3)**
5%	0.3 cm (3)	0.3 cm (4)	0.2 cm (4)	0.2 cm (3)	0	0.4 cm (4)
8%	0.25 cm (4)	0.1 cm (3)	**0.4 cm (3)**	0.1 cm (3)	0	0.25 cm (3)
10%	0.1 cm (5)	0.2 cm (3)	0.2 cm (3)	0	0	0.1 cm (2)
15%	0	0	0	0	0	0

Anaerobic NaCl tolerance and the effects on biosurfactant production.

Strains T89-11 and TG2-32 along with *B. licheniformis* strain RS-1 grew in medium with 5% NaCl and without NaCl added (Figure 5-2). The maximum A_{600} ranged from 0.5 to 0.6 whether NaCl was added or not, except for strain RS-1, which did not grow as well in medium without NaCl added (Figure 5-2). The group 2 strain, RL-1, grew to a maximum A_{600} of 0.5 in medium without NaCl added, but only reached an A_{600} 0.3 in medium with 5% NaCl added.



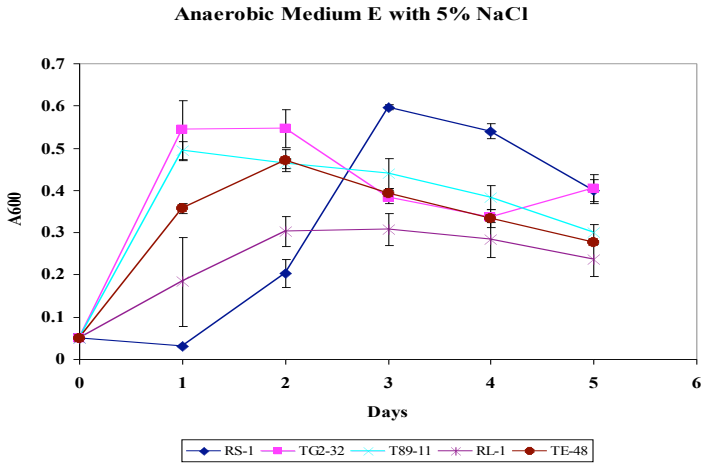


Figure 5-2 Anaerobic growth of Sonoran Desert strains and RS-1 in Medium E with 0% and 5% NaCl added.

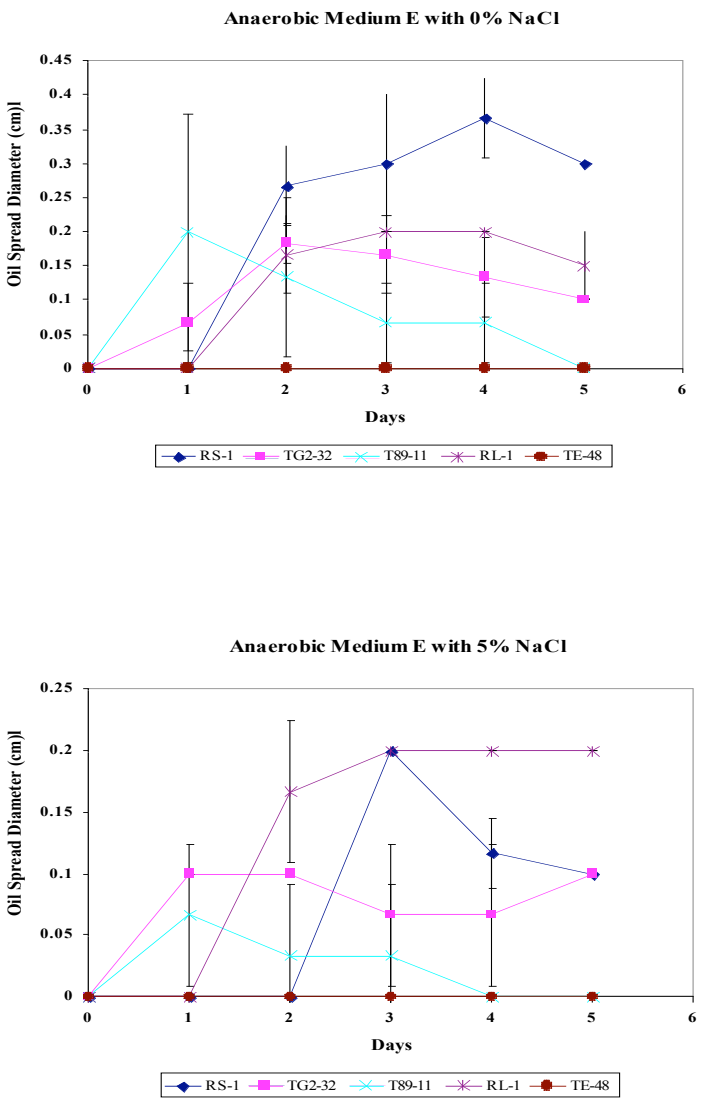


Figure 5-3 Anaerobic biosurfactant production of Sonoran Desert strains and RS-1 in Medium E with 5% NaCl

Strains RS-1, RL-1, TG2-32, and T89-11 each had biosurfactant activity when grown anaerobically in medium E with or without the addition of 5% NaCl (Figure 5-3). Strain TE-48 did not produce a biosurfactant in either medium. The Group 2 strain RL-1 and oil spread diameters of 0.2 in medium with and without the addition of 5% NaCl. The Group 1 strains TG2-32 and T89-11 had larger oil spread diameters at 0% NaCl compared to 5% NaCl. RS-1 had the largest oil spread diameter at 0% and 5% NaCl. Biosurfactant activity appeared to peak after about 3 to 4 days of incubation for both RS-1 and RL-1.

Aerobic pH Tolerance of B. licheniformis-B. subtilis *clade strains.*

B. licheniformis strain RS-1 grew in media with a pH of 2 to 10 (Table 5 -5). The other strains grew in medium with pH from 4 to 10. The optimal pH for growth for all the strains was pH 6.

Table 5-5. The pH tolerance of different *Bacillus* strains. Growth from – to ++++. no growth -, most turbid ++++

Strain	pH 2	pH 4	pH 6	pH 8	pH 10
***B. licheniformis* RS-1**	+	++	++++	++	+
***B. licheniformis* ATCC 14580**	–	+	++++	++	+
***B. sonorensis* NRRL B23154**	–	+	++++	++	+
***B. mojavensis* NRRL B 14698**	–	+	+++	+	+
***B. subtilis* subsp. *subtilis* strain 168**	–	+	+++	+	+/ –
***B. subtilis* subsp. *spizizenii* NRRL B 23049**	–	++	++	+	+

Genotypic and genetic fingerprinting analysis of B. licheniformis *strain RS-1 and Sonoran Desert isolates.*

Several *B. licheniformis*-like strains were selected from a set of isolates from the Sonoran Desert which had been previously tested by others for their salt and temperature tolerance as well as biosurfactant production ability during aerobic growth in LB medium ([116, 141].The strains represented 2 distinct subgroups of *B. licheniformis* according to 16S rRNA gene sequences. Group 1 consisted of strains TE2-G2, T89-11 and T88-14 while Group 2 consisted of TE-48 and RL-1. T88-14 was shown to be halotolerant by growth in medium with 15% sodium chloride as well as thermotolerant by its growth up to 59.5^{o}C. T89-11 grew in medium with15% NaCl and at temperatures up to 55^{o}C. TG2-32 grew in medium with up to 10% salt and at temperatures to 55^{o}C. RL-1 and TE-48 had more extensive growth at 15% salt than any of the three Group 1 strains and also grew at temperatures up to 55^{o}C.

The consensus sequences for *srfA3/licA3*, *gyrA* and the 16S rRNA genes for RS-1 and TG2-32 were 100% identical (Figures 5-4, 5, 6). Furthermore, the REP-PCR fingerprint of RS-1 and TG2-32 also appear to be identical (Figure 4-7). Based on *srfA3/licA3* sequence analysis, RL-1 and TE-48 grouped with the type strain of *B. licheniformis,* ATCC 14580. Strains TG-32, RS-1 and T89-11 group separately (Figure 5-4).

Figure 5-4 Phylogenetic tree of *srfA3/licA3* for *B. licheniformis* strain RS-1.

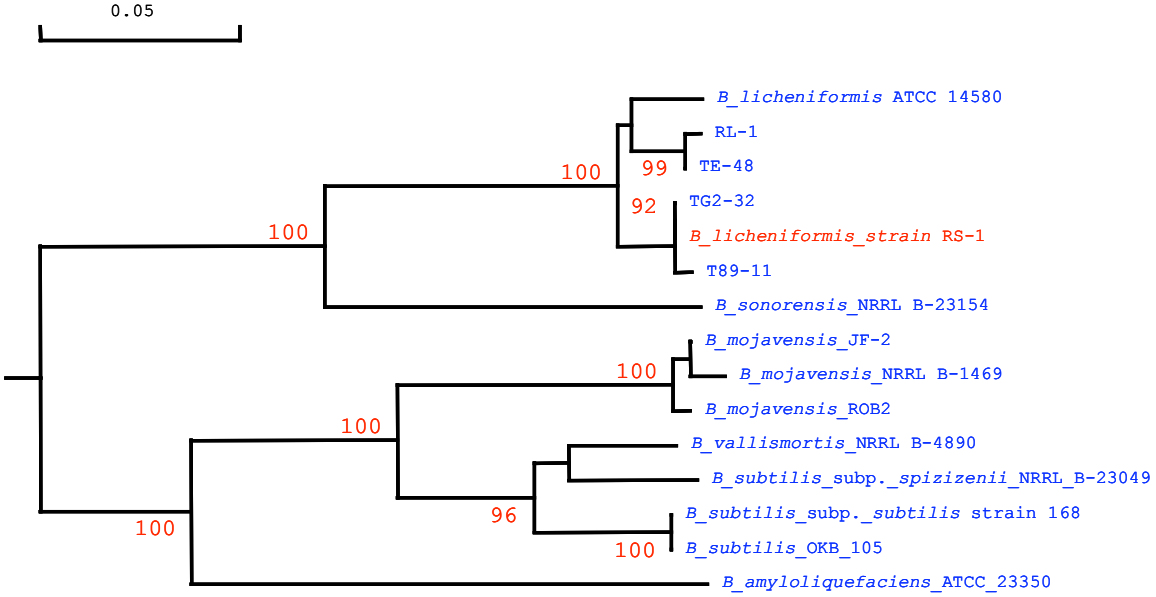


Figure 5-5 Phylogenetic tree of *gyrA* for *B. licheniformis* strain RS-1.

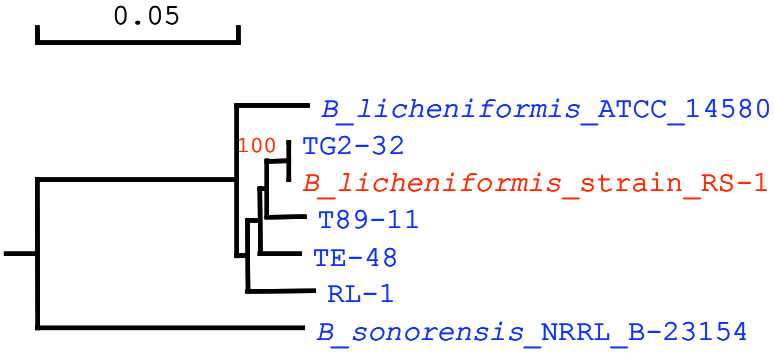
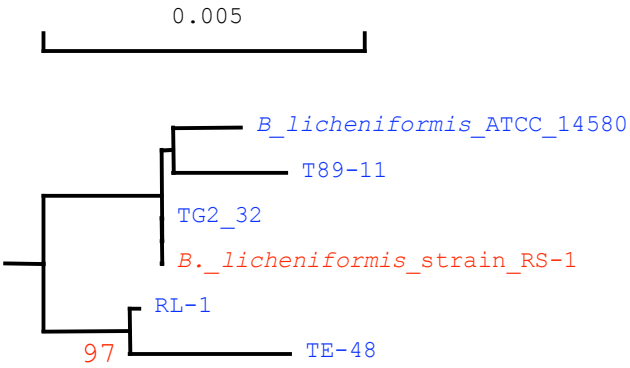


Figure 5-6 Phylogenetic tree of 16S rRNA gene for *B. licheniformis* strain RS-1.



Based on *gyrA* sequence analysis, all of the Sonoran Desert isolates and RS-1 appear to form a group distinct from *B. licheniformis* ATCC 14580 (Figure 5-5). However from16S rRNA gene sequence analysis, *B. licheniformis* ATCC 14580 groups with TG2-32, RS-1 and T89-11 while RL-1 and TE-48 group separately (Figure 5-6).

REP-PCR genetic fingerprints for *B. licheniformis* RS-1 and the Group 1 strain TG2-32 were extremely similar (Figure 5-7). Based on fingerprint patterns, T89-11 appears more closely related to the Group 2 strains TE-48 and RL-1 although T89-11 groups with Group 1 strain TG2-32 based on 16S rRNA gene sequence analysis (Figure 5-6).

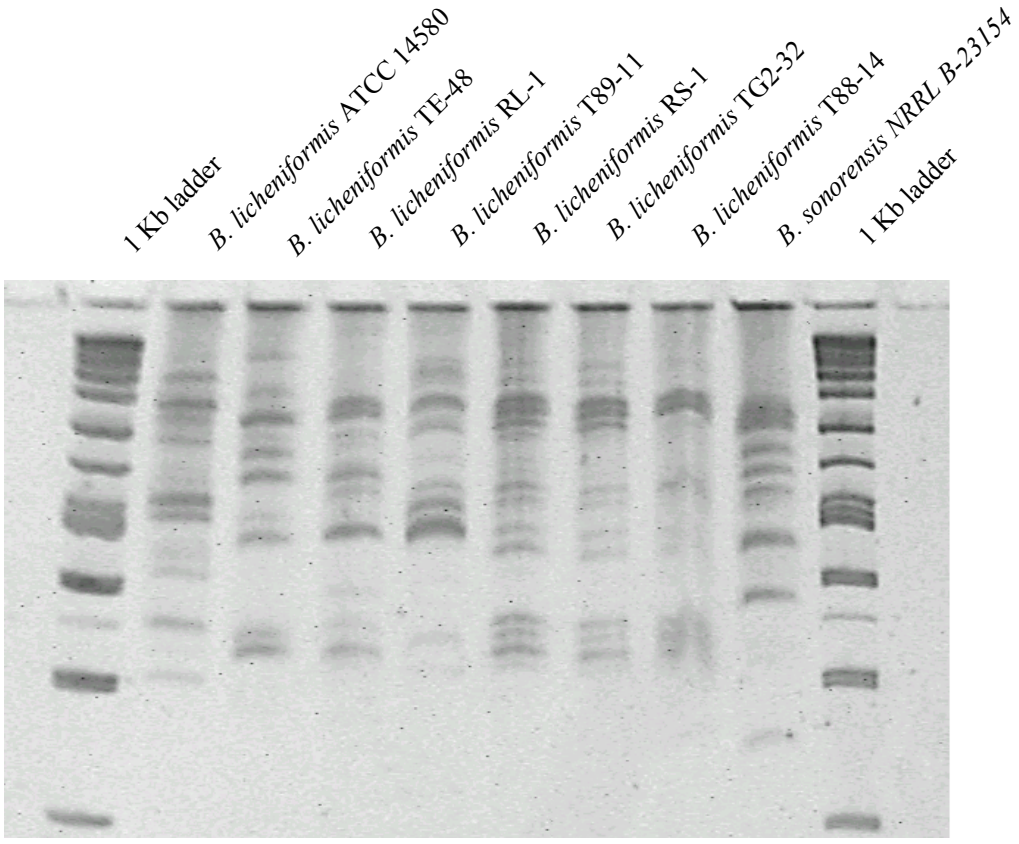


Figure 5-7 REP-PCR genetic fingerprint comparison of *B. licheniformis* Group 1 and 2 strains.

Discussion

B. licheniformis strain RS-1 grew in rich medium and minimal medium. The ability to grow in minimal medium would reduce the cost of amendment packages for MEOR biostimulation. Strain RS-1 and *B. subtilis* subsp. *spizizenii* NRRL B23049 had the broadest tolerance to NaCl with the ability to grow in medium with 0 to 15% NaCl and biosurfactant activity was detected in cultures grown aerobically in medium with 10% NaCl. In addition, RS-1 grew in medium with pH from 2 to 10. Of the four Sonoran

Desert isolates tested, three were able to produce a biosurfactant anaerobically. Protease activity of RS-1 is consistent with its placement in the *B. licheniformis/sonorensis* clade.

Genotypically*, B. licheniformis* strain RS-1 is very similar to strain TG2-32 based on 100% homology in *srfA3/licA3*, *gyrA* and 16S rRNA gene sequences as well as closely matching REP-PCR genetic fingerprints. Thus, phenotypic and genotypic results to date place RS-1 as a member of Group 1 *B. licheniformis* species.

Other strains of *B. licheniformis* have also been found to be capable of growth at high salinities [110, 116]. *B. licheniformis* RS-1 appears to be the most robust member of a generally salt tolerant group, which includes a several halotolerant, Sonoran Desert isolates, all of which produce biosurfactants both aerobically and anaerobically. This clade of microorganisms has characteristics that are useful for MEOR and could be used as an inoculum in reservoirs with differing salinities.

6. Microbial Stimulation Treatment of High Water-Cut Wells in the Viola Formation, Pontotoc County, OK

Abstract

Microbial stimulation of water-flooded reservoirs in the tertiary stage of oil recovery could be an economical technology to recover substantial quantities of entrapped oil. However, beneficial microbial metabolic activity *in-situ* has often not been rigorously correlated with the persistence and activity of injected bacterial species and oil recovery. We injected a glucose-nitrate-mineral nutrient mixture and two *Bacillus* strains into two wells to correlate *in-situ* metabolism and growth with oil recovery. Two wells producing from the same Viola formation were each inoculated with 500 bbl of tank battery brine mixed with nutrients (glucose, sodium nitrate and trace metals) as well as *Bacillus licheniformis* RS-1 and *Bacillus subtilis* subsp. *subtilis spizizenii* NRRL B-23049. Analysis of production water from the "huff and puff" treatment indicated *in-situ* metabolism of the nutrients, growth of the injected strains and other heterotrophic fermenting bacteria, and production of bacterial products including biosurfactant, carbon dioxide, acetate, lactate, succinate, ethanol and 2, 3-butanediol, many of which are potentially useful in enhancing oil recovery. A lipopeptide biosurfactant concentration of at least 11 mg/L is required to mobilize entrapped oil from sandstone cores. Both wells had a peak lipopeptide biosurfactant concentration of 20 and 28 mg/L respectively and an average carbon balance of glucose used and metabolic products and cells made of 91%. The increase in biosurfactant, acids, alcohols and carbon dioxide during the first 5 days after commencement of production corresponded directly with increasing oil recovery. Furthermore, wellhead measurements of total produced water, the water/oil ratio (WOR) and the percent oil cut as well as separation tank battery production data indicated that a corresponding net increase of 183 bbl in oil recovery occurred in during the first 100 days of sampling. Economic analysis at $60/bbl oil using the results obtained thus far showed a 55-day return on the $6000 investment, a $4980 net profit, and an average recovery cost of $33/bbl.

Introduction

When an oil reservoir reaches economic maturity during tertiary treatment by water-flooding, approximately 30% to 50% of the original oil present remains entrapped by capillary forces in the porous matrix of the formation [142-144]. In microbial stimulation processes, a well near its economic limit, is injected with beneficial bacterial culture in aqueous solution mixed with fermentable carbohydrate such as glucose or molasses [145]. In a field study of 24 microbially stimulated wells, Petrogen, Inc. determined that 75% showed an increase in oil production over a 3 to 6 month period [145]. Such microbially enhanced oil recovery (MEOR) processes employ the use of microbial metabolites such as biosurfactants, gases, acids and solvents to improve oil recovery by such mechanisms as interfacial tension reduction, reservoir pressurization, increase in porosity, viscosity reduction and wettability alteration [145-148]. Some *Bacillus* species can use glucose under nitrate-reducing conditions and produce acids such as acetate and

lactate, alcohols (solvents) such as ethanol and 2, 3-butanediol and carbon dioxide [145, 149]. A few *Bacillus* species are also able to produce detergent-like molecules called lipopeptide biosurfactants. Lipopeptide biosurfactants can create the reduction in interfacial tension between the hydrocarbon and aqueous phases required for mobilization of the entrapped oil [150, 151].

In the summer of 2005, a series of 50-bbl injections of brine mixed with nutrient (glucose, nitrate and trace metals) and two bacteria, *Bacillus licheniformis* RS-1 and *Bacillus subtilis* subsp. *subtilis spizizenii* NRRL B-23049, were conducted in a "huff and puff" style on wells producing from the same Viola formation in Pontotoc County, OK. From this field experiment, we were able to show that the injected *Bacillus* species were metabolically activity after a five day incubation period and produced the expected products lipopeptide biosurfactant, acetate, lactate, ethanol and 2, 3- butanediol. Furthermore, injected strains persisted and were recoverable from the formation. The produced fluids from the wells that received the two bacterial strains had an average lipopeptide concentration of approximately 90 mg/L, which is about 9 fold above the minimum concentration needed to mobilize entrapped oil from sandstone cores [152, 153]. Neither the biosurfactant nor the *Bacillus* strains were recovered from any of the other control wells that received only nutrients or only tank battery brine. The data established the technical feasibility of MEOR using our *Bacillus* isolates and the nutritional amendment package. As a result, in November 2007, we scaled-up the "huff and puff" treatments on the same wells to 500-bbl inoculums of brine mixed with the same nutrients and *Bacillus* species in order to determine whether the microbial process improved oil recovery.

Methodology

Baseline analysis

Robertson 13 and Robertson 15 wells, which produce from the same Viola formation in Pontotoc County, OK, were used in this study. Baseline data for Robertson 13 and Robertson 15 wells were obtained prior to the microbial treatment. Average daily WOR and percent oil cut values were determined periodically by collecting wellhead fluid samples with a graduated flask over a six-week period prior to stimulation. A flow fluid production was measured with flow totalizers installed at each wellhead pump. The average daily oil recovery from the Robertson tank battery over the 3 months prior to stimulation was obtained from office records. Baseline chemical analyses of production fluids taken just prior to treatment showed the absence of the lipopeptide biosurfactant, acetate, lactate, succinate, formate, ethanol or 2, 3-butanediol. Three-series most probable number (MPN) analyses were used to determine a background numbers of heterotrophic bacteria. Spore forming bacteria or biosurfactant-producing bacteria of which *Bacilli* would be included were not detected. Finally, an oil spread assay was also performed and established that surface active compounds such as biosurfactants were not present in the production water from each well [154, 155].

Preparation of inoculum

A total of seven, 10-liter carboys of *B. licheniformis* RS-1 and six, 10-liter carboys of *B. subtilis* subsp. *spizizenii* strain NRRL B-23049 were grown in Medium E [154, 155] at 37^{o}C for 48 hours with dispersion tubes for agitation and aeration. After concentration of the cultures by tangential flow filtration, the cells were stored at 4^{o}C in sterile, 2-liter bottles.

Microbial stimulation field experiment

A 500-bbl frac tank was placed adjacent to each of the two wellheads (Robertson 15 and Robertson 13) and filled with 50 bbl of tank battery brine free of oil by using a pump truck. Each frac tank was equipped with a 5-hp gasoline powered pump for recirculation of its contents. With the recirculation pump running, 79.5 kg of glucose, 7.9 kg of sodium nitrate, 19.9 g of magnesium sulfate, 2 g of manganese sulfate, 2 g of zinc sulfate, 2 g of iron sulfate, 0.2 g of copper sulfate, 0.2 g of aluminum potassium sulfate, 0.2 g of boric acid, 0.2 g of sodium molybdate, 0.1 g of sodium selenate and 0.6 g of nickel chloride were added to each frac tank [155]. Next, each frac tank received 3.5 liters of concentrated *B. licheniformis* RS-1 and 3 liters of *B. subtilis* subsp. *spizizenii* strain NRRL B-23049 as inoculum (total number of cells was 0.3 x 10^{13}). The frac tank contents were mixed twice per day for 5 days by using the recirculation pumps. The average outdoor ambient temperature was approximately 10^{o}C. After 5 days the inoculum was ready for injection to about 500 bbl by addition of 100-bbl increments of tank battery brine free of oil. During this time, 715.5 kg of glucose, 71.1 kg of sodium nitrate, 179.1 g of magnesium sulfate, 18 g of manganese sulfate, 18 g of zinc sulfate, 18 g of iron sulfate, 1.8 g of copper sulfate, 1.8 g of aluminum potassium sulfate, 1.8 g of boric acid, 1.8 g of sodium molybdate, 0.9 g of sodium selenate, 5.4 g of nickel chloride and 1.25 kg of sodium fluorescein were added to each frac tank and mixed by recirculation pump. The Robertson 15 production pump was stopped, and the 500-bbl inoculum with nutrients was injected into the wellhead by using the gasoline recirculation pump. Due to the lower permeability, injection into the Robertson 13 well required the use of the pump truck. The inoculum was then allowed to incubate *in-situ* for 4.5 days. Down-hole temperature was estimated to be 23^{o}C.

Both wellhead pumps were re-started on the morning of the fifth day after injection. Approximately 6 hours after production was resumed, the first daily sampling and field measurement session occurred and was repeated at approximately the same time for the next 6 days. The protocol consisted of the following for each well:

1) Record flowmeter totalizer data
2) Measure production water temperature with a thermometer
3) Measure the WOR with a graduated flask
4) Take three, 1-liter samples of production water in sterile bags for chemical and microbial analyses which were transported to laboratory on ice
5) Measure pH using indicator strip
6) Measure the alkalinity with a HACH kit, and

7) Measure the degrees of oil spreading.

Chemical analysis

The lipopeptide biosurfactant was quantified by using high performance liquid chromatography (HPLC) and standards curves prepared with purified lipopeptide from each *Bacillus* strain [154]. Oil spreading activity was measured by determining the diameter of clearing when a drop of fluid was added to a thin film of oil [154, 156]. The sugar concentration was determined by using a modified orcinol/H_2SO_4 method with glucose as the standard [157]. Acetate, succinate, formate, ethanol and 2, 3- butanediol were quantified by using gas chromatography (GC) [155, 158, 159]. Lactate was quantified by HPLC [155]. Carbon dioxide production was calculated by the amount of carbon dioxide present from the alkalinity (HACH) and pH. Glucose utilization and metabolite production was calculated by summing the product of the concentration of a compound in each sample times fluid volume produced during the interval from the last sampling. Product recoveries were corrected for the amount of product present in the inoculum.

Microbiological analysis

Heterotrophic bacteria, spore-forming bacteria and biosurfactant producing bacteria were enumerated using a three-series MPN technique in 96-well microtiter plates with production water used as inoculum [155]. Plates were incubated statically at room temperature for 48 hours. Plate count broth (PCB) with 5% sodium chloride was the medium used for total heterotrophic bacteria. Production water was incubated at 85^{o}C for 20 minutes before inoculation in PCB with 5% sodium chloride for determination of spore-forming bacteria. Dissipation of sterile crude oil added to the medium surface of PCB microtiter plates with 5% sodium chloride was used to estimate the number of biosurfactant-producing bacteria. In addition, plate count agar (PCA) with 5% sodium chloride were inoculated from positive biosurfactant-producing bacteria wells to identify colonies with morphologies identical to that of the injected *Bacillus* strains.

Oil recovery analysis

On days 5 through 20 after injection, flow totalizer and WOR measurements were recorded daily at the wellhead. Daily flow totalizer readings were used to determine daily production water volumes. WOR data determined with a graduated flask were converted to percent oil cut. After this time period, the same measurements were recorded on a weekly basis. For 100 days prior to and after the test, daily oil recovered from the Robertson separation tank battery was also obtained. The Robertson tank battery includes oil production from 7 wells. An average daily production from the Robertson tank battery prior to the microbial test was 11 bbl/day. This value was subtracted from the daily oil production data collected after the microbial test to determine the net increase in oil production due to the microbial treatment.

Results

Pre-injection, average daily WOR and percent oil cut were 39:1 and 2.5%, respectively, for each well. Average daily production rates were 150 bbl/day for Robertson 15 and 100 bbl/day for Robertson 13. The average daily oil production of the Robertson tank battery was 11 bbl/day over a 3-month interval prior to stimulation. Three-series most probable number (MPN) analyses did not detect any heterotrophic, spore forming or biosurfactant producing bacteria.

During the sampling, a total of about 5.1 and 5.8 kg of the biosurfactant was from the production fluids from the Robertson 13 and 15 wells, respectively. Previous sand-packed and sandstone core laboratory column studies indicated that about 2.2 mL of residual oil recovery was recovered per mg of lipopeptide biosurfactant used [160]. Based on the amount of biosurfactant present in the produced fluids, the laboratory derived relationship would predict that 151 bbl of oil should be recovered from the two wells. This assumes that the oil recovery is solely due to the activity of the biosurfactant. Although the data indicate oil production is still above pre-treatment levels, during the first 100 days after the microbial stimulation, the net increase in oil production is approximately 183 bbl as calculated from the Robertson separation tank battery data (Figure 1).

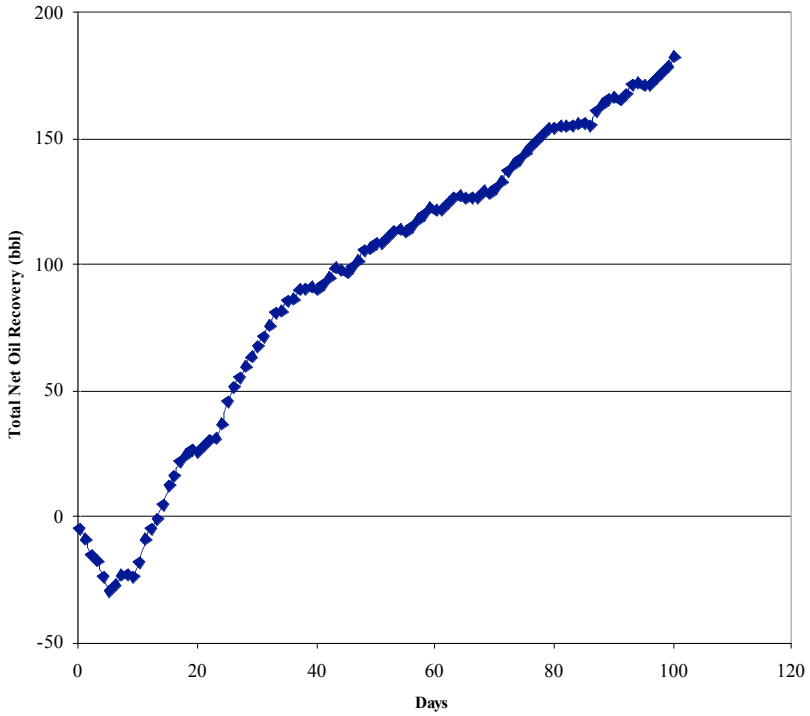


Figure 1: The total net increase in oil production from the Robertson separation tank battery

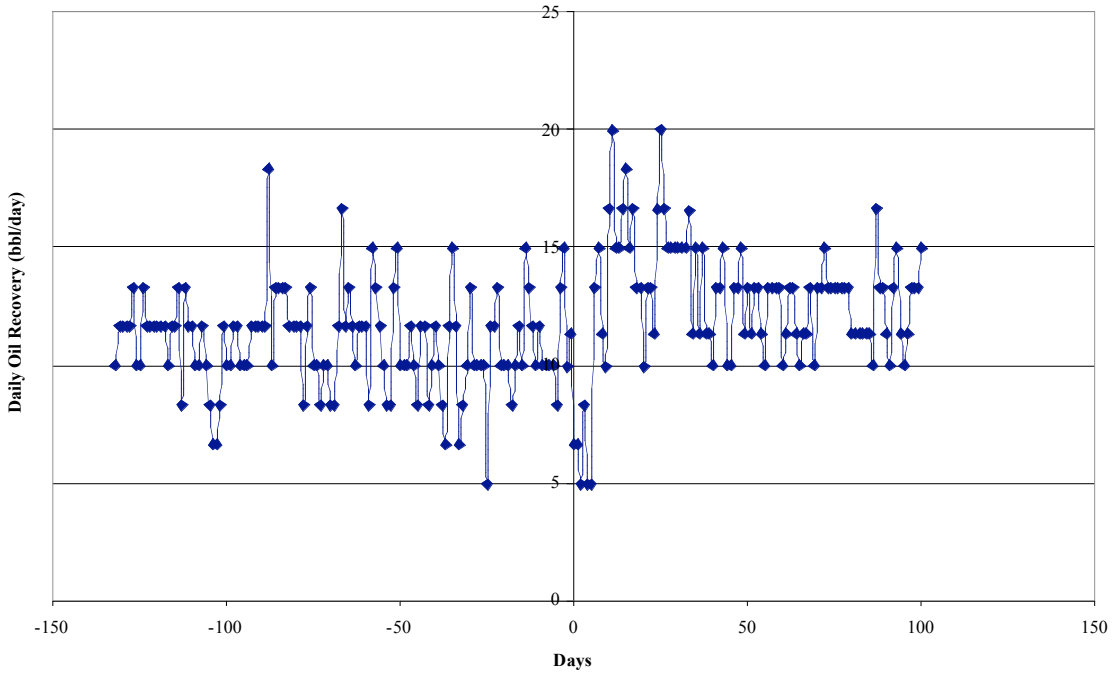


Figure 2: The daily oil recovery from the Robertson separation tank battery

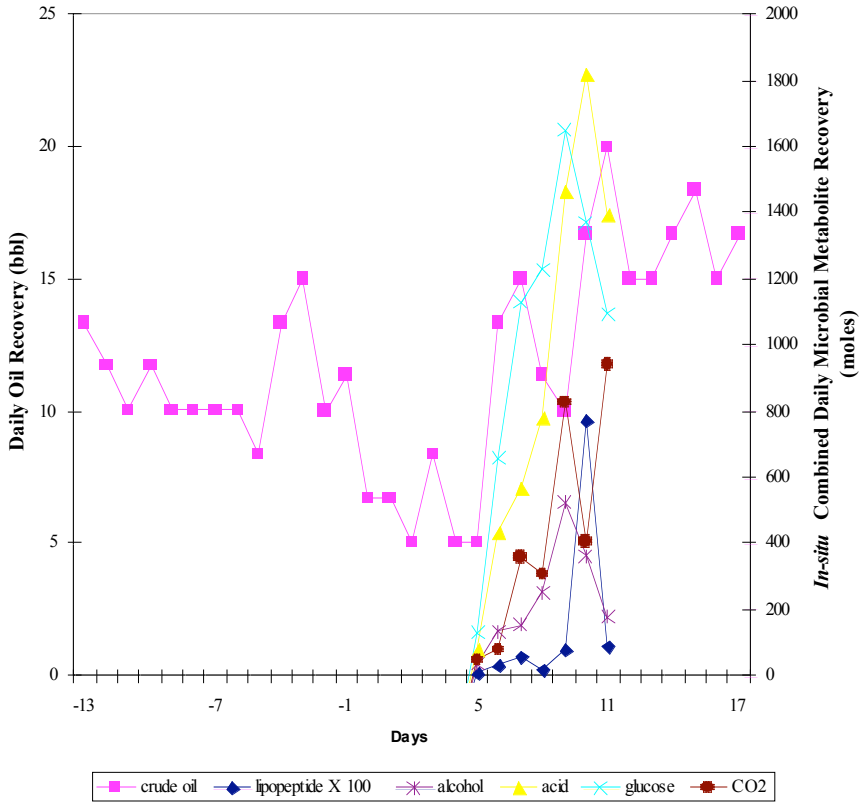


Figure 3: Comparison of the daily oil recovery with the i*n-situ* combined daily microbial metabolite recoveries for the Robertson separation tank battery

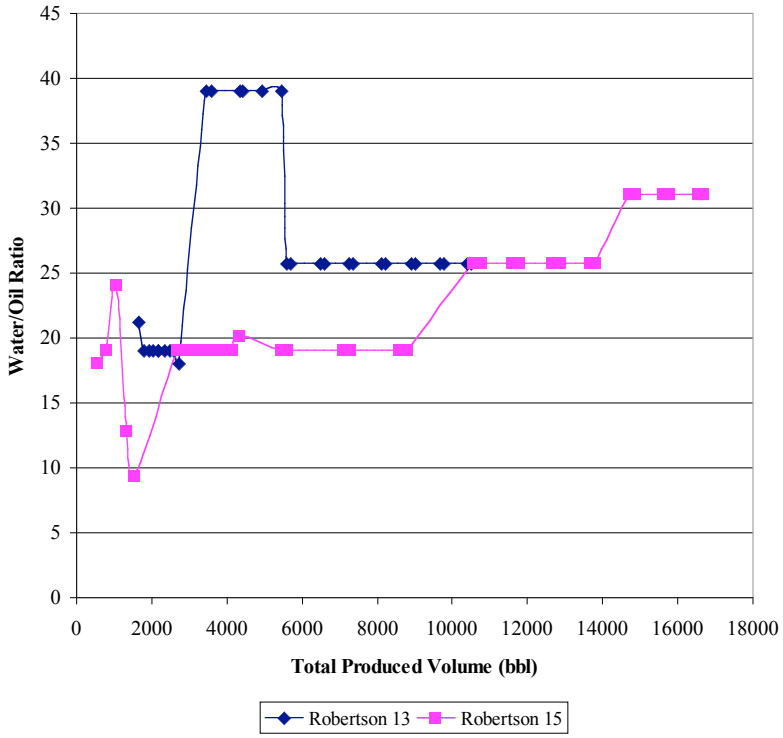


Figure 4: The time-course of WOR for Robertson 15 and 13 wellhead samples

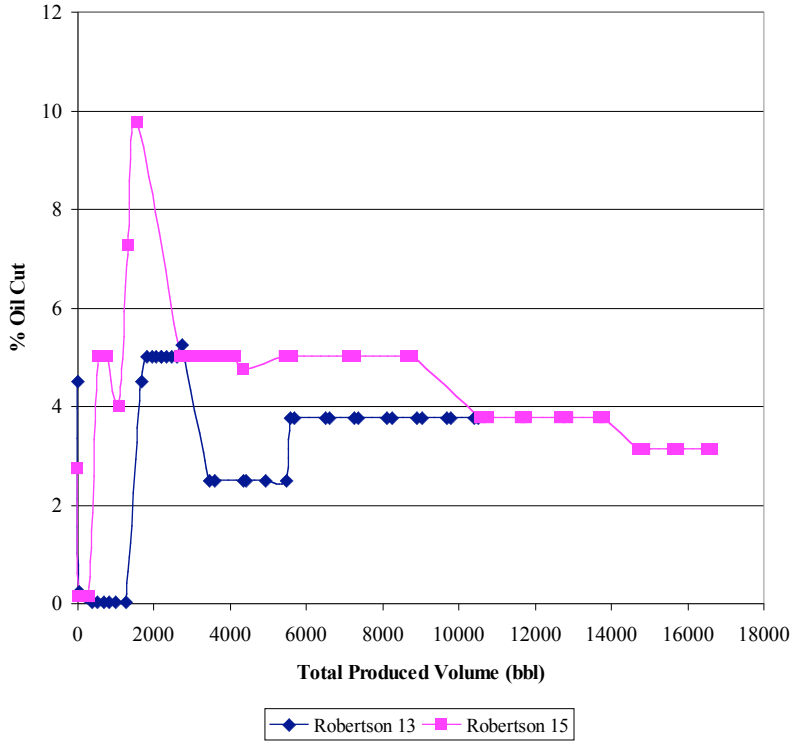


Figure 5: The time-course of percent oil cut for Robertson 15 and 13 wellhead samples

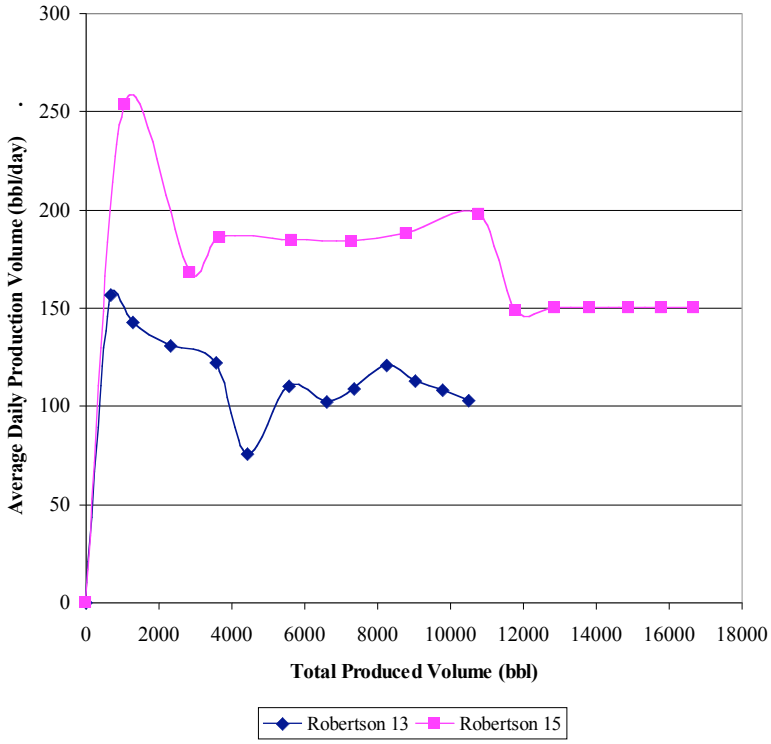


Figure 6: The average daily production volumes for Robertson 15 and 13 wellhead samples

The daily tank battery oil recovery volumes before and after stimulation are shown in Figure 2. There is a lot of scatter to the data, but oil production exceeded 15 bbl/day 27% of the time after treatment while only 7% of the time prior to treatment. The concentrations of lipopeptide biosurfactant, alcohols, acids and carbon dioxide from days 5 through 11 post-injection are shown in Figure 3. Increases in oil recovery occurred after metabolite production occurred (Figure 3). The WOR for Robertson 15 production was 39:1 prior to the microbial treatment. Immediately after the microbial treatment, the WOR decreased to a low of 9:1 and has increased to about 31:1 after 100 days, which is still below pre-treatment levels (Figure 4). Corresponding, the percent oil cut for Robertson 15 has increased due to treatment (Figure 5). A maximum of 10% was observed on post-injection day 11, which corresponded with a 9-bbl peak in net daily oil recovery from the tank battery. After 100 days, the percent oil cut is 3.1%, which is still above the 2.5% baseline value (Figure 5). Production data for the Robertson 13 well show that the WOR has decreased from the baseline value of 39:1 to near 26:1 after the first 100 days (Figure 4). The percent oil cut reached a peak of 5% and has fluctuated between the 2.5% baseline and 3.5% since then (Figure 5). The average daily production volume for Robertson 15 peaked near 250 bbl/day after 2000 bbl of total production volume and correlated with peak in percent oil cut and minimum WOR (Figure 6). Subsequently, daily production volume of Robertson 15 decreased to the baseline value of 150 bbl/day. The average daily production volume from the Robertson 13 well peaked near 160 bbl/day, but this occurred at 500-bbl total production volume before the peak in

percent oil cut and minimum WOR, which occurred at 3000 bbl total production volume. After 100 days post-stimulation, the daily production volume of Robertson 13 is at the 100 bbl/day baseline value.

The average carbon recovery for the two wells 91% indicating that almost all of the glucose carbon that was injected was accounted for in unused sugar, other metabolites and cells recovered from production fluids collected six days after production began. The microbes converted glucose into acids (acetate, lactate, formate and succinate) carbon dioxide, alcohols (ethanol and 2, 3- butanediol), the biosurfactant and cells. Both the presence of the biosurfactant and 2, 3-butanediol are characteristic of *Bacillus* species. Both wells had a peak lipopeptide biosurfactant concentration of 20 and 28 mg/L, respectively, which is above the 11 mg/L required to mobilize oil from laboratory sandstone cores (21, 31). The number of heterotrophic fermentative bacteria injected into Robertson 13 and 15 was 1×10^{13} and 3.5×10^{13}, respectively. After incubation in the formation, the total heterotrophic bacteria recovered in produced fluids was 5×10^{13} and 1×10^{14} for Robertson 13 and Robertson 15 wells, respectively. Thus, bacterial numbers increased 5-fold *in-situ* for Robertson 13 and 3-fold *in-situ* for Robertson 15. Biosurfactant-producing bacteria totaled 4.8×10^{13} for Robertson 13 and 9.7×10^{13} for Robertson 15. Spore-forming bacteria recovered from Robertson 13 totaled 1.6×10^{10} and 8.4×10^{9} for Robertson 15. Oil spread assays detected biosurfactant activity in the production fluids that were collected six days after production resumed. These quantitative results in total indicate significant, almost exclusive *in-situ* growth of our injected *Bacillus* strains occurred and corresponded with the expected metabolic activities and improved oil recovery.

Economic analysis

The total material and equipment expenses for the stimulation were approximately $3000 per well. It was estimated that the microbial process increased oil production by 183 bbl (net improvement to data). Thus, economic analysis through the first 100 days of post-stimulation production using this value indicates that additional oil was recovered at approximately $33/bbl. Assuming $60/bbl oil, a 55 day return on investment and a $4980 net profit were realized from the microbial stimulation.

Conclusion

The microbial stimulation process improved oil production, decreased the WOR, and increased the percent oil cut. The expected products of microbial metabolism were detected in the produced fluids after treatment in quantities sufficient to mobilize crude oil. Initial economics are encouraging, although optimization of the process is needed to increase biosurfactant concentrations and to reduce the incremental cost of recovery.

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Appendices

APPENDIX I
Aerobic nutrient treatment for Hunton formation fluids

Table 13. Biosurfactant production with a single nutrient component deleted for Hunton formation fluids.

	Diameter of Oil spreading (cm) for the optimal nutrient treatment package			
TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Everything added			
Day3	0.47 ± 0.06	1.30± 0.26	0.50±0.00	0.00±0.00
Day7	1.50± 0.10	2.00± 0.00	1.53± 0.06	0.00± 0.00
	Minus Glucose			
Day3	0.37 ± 0.06	1.33± 0.06	0.93± 0.31	0.00± 0.00
Day7	0.10± 0.10	1.23±0.25	1.27±0.12	0.00±0.00
	Minus Proteose peptone			
Day3	0.30±0.26	0.47±0.15	0.40±0.36	0.00±0.00
Day7	0.17±0.29	0.40±0.10	0.07±0.12	0.00±0.00
	Minus Molasses			
Day3	1.17±0.29	1.60±0.53	0.47±0.12	0.00 ±0.00
Day7	1.23±0.25	1.17±0.29	0.20±0.20	0.00±0.00
	Minus NaCl			
Day3	0.00±0.00	2.50±0.50	2.43±0.60	0.00±0.00
Day7	1.17±1.04	0.80±0.72	1.53±1.70	0.00± 0.00
	Minus Nitrate			
Day3	0.07± 0.00	0.07±0.00	1.60±0.66	0.00±0.00
Day7	0.37±0.21	0.20±0.20	0.97±1.19	0.00±0.00
	Minus Phosphate			
Day3	0.87±0.83	2.10±0.36	2.03±0.71	0.00±0.00
Day7	1.77±1.57	1.50±1.32	1.77±1.54	0.00±0.00
	Minus Glycerol			
Day3	0.00±0.00	1.17±0.29	1.10±0.36	0.00±0.00
Day7	0.80±0.35	0.77±0.40	0.63±0.32	0.00±0.00

Table 14. Biosurfactant production with double nutrient component deleted for Hunton formation fluids.

	Diameter of Oil spreading (cm) for the optimal nutrient treatment package			
TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Minus Glucose Minus Molasses			
Day3	0.30±0.14	1.00±0.00	0.47±0.15	0.00±0.00
Day7	0.27±0.06	0.90±0.10	0.27±0.06	0.00±0.00
	Minus Glucose Minus Proteose peptone			
Day3	0.00±0.00	0.53±0.12	0.43±0.51	0.00±0.00
Day7	0.03±0.06	0.53±0.12	0.53±0.21	0.00±0.00
	Minus Glucose Minus NaCl			
Day3	0.57±0.21	2.87±0.12	3.40±1.05	0.00±0.00
Day7	2.00±0.00	1.87±0.21	0.90±0.95	0.00±0.00
	Minus Glucose Minus Phosphate			
Day3	0.47±0.15	1.83±0.76	2.37±0.32	0.00±0.00
Day7	0.37±0.29	2.17±0.29	7.10±0.14	0.00±0.00
	Minus Glucose Minus Nitrate			
Day3	0.37±0.15	1.30±1.08	1.10±0.36	0.00±0.00
Day7	0.13±0.12	1.70±0.62	0.43±0.59	0.00±0.00
	Minus Glucose Minus Glycerol			
Day3	0.17±0.15	0.10±0.00	0.07±0.12	0.00±0.00
Day7	0.35±0.07	0.17±0.29	0.37±0.35	0.00±0.00
	Minus Molasses Minus Proteose peptone			
Day3	0.00±0.00	0.20±0.10	0.33±0.06	0.00±0.00
Day7	0.00±0.00	0.27±0.06	0.17±0.06	0.00±0.00
	Minus Molasses Minus NaCl			
Day3	0.20±0.10	2.00±0.50	0.37±0.06	0.00±0.00
Day7	0.23±0.06	1.83±0.29	0.13±0.12	0.00±0.00
	Minus Molasses Minus Phosphate			
Day3	0.00±0.00	0.50±0.26	0.30±0.14	0.00±0.00
Day7	1.17 ±0.67	1.97±0.57	0.13±0.12	0.00±0.00
	Minus Molasses Minus Nitrate			
Day3	0.17±0.15	0.87±0.23	0.37±0.12	0.00±0.00
Day7	0.00±0.00	0.20±0.20	0.27±0.06	0.00±0.00

TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Minus Molasses Minus Nitrate			
Day3	0.07±0.06	0.27±0.25	0.50±0.10	0.00±0.00
Day7	0.00±0.00	0.00±0.00	0.27±0.25	0.00±0.00
	Minus Proteose peptone Minus NaCl			
Day3	0.17±0.00	0.53±0.06	0.57±0.15	0.00±0.00
Day7	0.00±0.00	0.00±0.00	0.43±0.06	0.00±0.00
	Minus Proteose peptone Minus Phosphate			
Day3	0.30±0.10	0.87±0.12	0.60±0.26	0.00±0.00
Day7	0.37±0.06	0.50±0.10	0.67±0.29	0.00±0.00
	Minus Proteose peptone Minus Nitrate			
Day3	0.00±0.00	0.00±0.00	0.20±0.10	0.00±0.00
Day7	0.00±0.00	0.15±0.21	0.33±0.15	0.00±0.00
	Minus Proteose peptone Minus Glycerol			
Day3	0.00±0.00	0.53±0.23	0.93±0.42	0.00±0.00
Day7	0.00±0.00	0.20±0.17	0.53±0.06	0.00±0.00
	Minus Glycerol Minus NaCl			
Day3	0.33±0.23	0.50±0.10	0.40±0.17	0.00±0.00
Day7	0.53±0.50	0.80±0.20	2.37±0.06	0.00±0.00
	Minus Glycerol Minus Phosphate			
Day3	0.00±0.00	0.50±0.10	0.43±0.40	0.00±0.00
Day7	0.33±0.31	0.30±0.30	0.13±0.23	0.00±0.00
	Minus Glycerol Minus Nitrate			
Day3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day7	0.00±0.00	0.73±0.12	0.33±0.31	0.00±0.00

Table 15. Biosurfactant production a single nutrient component added for Hunton formation fluids.

Diameter of Oil spreading (cm) for the optimal nutrient treatment package				
TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Glucose Only			
Day3	0.35±0.05	0.00±0.00	0.00±0.00	0.00±0.00
Day7	0.00±0.00	0.30±0.26	0.13±0.23	0.00±0.00
	Molasses Only			
Day3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day7	0.30±0.26	0.43±0.45	0.93±0.86	0.00±0.00
	Proteose peptone Only			
Day3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Glycerol Only			
Day3	0.00±0.00	0.33±0.25	0.00±0.00	0.00±0.00
Day7	0.13±0.23	0.53±0.47	0.00±0.00	0.00±0.00
	Phosphate Only			
Day3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day7	0.00±0.00	0.00 ±0.00	0.30±0.26	0.00±0.00
	Nitrate Only			
Day3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day7	0.00±0.00	0.27±0.23	0.00±0.00	0.00±0.00

Table 16. Biosurfactant production with double nutrient components added for Hunton formation fluids.

Diameter of Oil spreading (cm) for the optimal nutrient treatment package				
TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUMONLY
	Plus Glucose Plus Proteose Peptone			
Day3	0.30±0.08	0.63±0.12	0.33±0.05	0.00±0.00
Day7	0.00±0.00	0.00±0.00	0.23±0.40	0.00±0.00
	Plus Glucose Plus Molasses			
Day3	0.00±0.00	0.30±0.22	0.37±0.05	0.17±0.24
Day7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Glucose Plus NaCl			
Day3	0.00±0.00	0.30±0.00	0.43±0.05	0.00±0.00
Day7	0.00±0.00	0.27±0.23	0.57±0.06	0.00±0.00
	Plus Glucose Plus Phosphate			
Day3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day7	0.00±0.00	0.13±0.23	0.73±0.31	0.00±0.00
	Plus Glucose Plus Nitrate			
Day3	0.00±0.00	0.63±0.05	0.50±0.08	0.00±0.00
Day7	0.00±0.00	1.30±0.42	0.55±0.07	0.00±0.00
	Plus Glucose Plus Glycerol			
Day3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day7	0.13±0.23	0.67±0.06	0.40±0.00	0.00±0.00
	Plus Molasses Plus Proteose Peptone			
Day3	1.40±0.57	0.53±0.75	3.00±0.29	0.00±0.00
Day7	1.23±0.31	0.00±0.00	0.87±0.31	0.00±0.00
	Plus Molasses Plus NaCl			
Day3	0.40±0.08	1.10±0.08	2.00±0.43	0.00±0.00
Day7	0.00±0.00	1.26±0.20	3.53±0.45	0.00±0.00
	Plus Molasses Plus Nitrate			
Day3	0.40±0.08	1.10±0.08	2.0±0.43	0.00±0.00
Day7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Molasses Plus Phosphate			
Day3	0.23±0.17	0.33±0.05	0.30±0.00	0.00±0.00
Day7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Plus Molasses Plus Glycerol			
Day3	0.57±0.12	0.27±0.19	0.57±0.09	0.00±0.00
Day7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Proteose peptone Plus NaCl			
Day3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Proteose peptone Plus Nitrate			
Day3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Proteose peptone Plus Phosphate			
Day3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Proteose peptone Plus Glycerol			
Day3	1.50±0.78	0.50±0.16	0.23±0.17	0.00±0.00
Day7	1.40±0.36	2.00±0.00	0.20±0.00	0.00±0.00
	Plus Glycerol Plus NaCl			
Day3	0.00±0.00	0.40±0.08	0.10±0.14	0.00±0.00
Day7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Glycerol Plus Nitrate			
Day3	0.37±0.05	1.60±0.43	0.27±0.19	0.00±0.00
Day7	0.00±0.00	2.53±1.05	0.40±0.69	0.00±0.00
	Plus Glycerol Plus Phosphate			
Day3	0.00±0.00	0.23±0.17	0.10±0.14	0.00±0.00
Day7	0.00±0.00	0.00±0.00	0.37±0.32	0.00±0.00

APPENDIX II
Aerobic nutrient treatment for Wewoka formation fluids

Table 17. Biosurfactant production with a single nutrient component deleted for Wewoka formation fluids.

Diameter of Oil Spreading (cm) for optimal nutrient treatment package.				
TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Everything added			
Day 3	0.00±0.00	2.53±0.06	2.70±2.40	0.00±0.00
Day 5	0.00±0.00	3.33±0.29	6.63±0.32	0.00±0.00
Day 7	0.00±0.00	4.20±0.20	6.50±0.10	0.00±0.00
	Minus Glucose			
Day 3	0.00±0.00	1.80±0.20	1.20±0.52	0.00±0.00
Day 5	0.00±0.00	1.63±0.29	0.47±0.12	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.73±0.21	0.00±0.00
	Minus Protese peptone			
Day 3	0.00±0.00	0.33±0.06	0.37±0.15	0.00±0.00
Day 5	0.00±0.00	0.13±0.15	0.30±0.26	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Minus Molasses			
Day 3	0.00±0.00	0.23±0.21	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	1.70±0.17	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Minus NaCl			
Day 3	0.00±0.00	3.00±0.20	3.43±0.12	0.00±0.00
Day 5	0.00±0.00	3.03±0.06	3.40±0.06	0.00±0.35
Day 7	0.00±0.00	0.37±0.06	3.30±0.26	0.00±0.00
	Minus Nitrate			
Day 3	0.00±0.00	0.10±0.17	0.20±0.17	0.00±0.00
Day 5	0.00±0.00	0.17±0.15	0.03±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Minus Phosphate			
Day 3	0.00±0.00	3.70±0.75	3.53±0.23	0.00±0.00
Day 5	0.00±0.00	4.30±0.66	4.73±0.12	0.00±0.00
Day 7	0.00±0.00	2.77±0.25	2.50±0.10	0.00±0.00

TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Minus Glycerol			
Day 3	0.00±0.00	1.40±0.35	0.77±0.06	0.00±0.00
Day 5	0.00±0.00	0.83±0.15	0.73±0.06	0.00±0.00
Day 7	0.00±0.00	0.53±0.06	0.43±0.06	0.00±0.00

Table 18. Biosurfactant production with double nutrient components deleted for Wewoka formation fluids.

Diameter of Oil Spreading (cm) for optimal nutrient treatment package.				
Time (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Minus Glucose Minus Molasses			
Day 3	0.00±0.00	0.80±0.35	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.73±0.31	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.47±0.06	0.00±0.00	0.00±0.00
	Minus Glucose Minus Proteose peptone			
Day 3	0.00±0.00	0.00±0.00	0.33±0.06	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.13±0.15	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	1.90±0.17	0.00±0.00
	Minus Glucose Minus NaCl			
Day 3	0.00±0.00	2.93±0.06	2.87±0.06	0.00±0.00
Day 5	0.00±0.00	2.07±0.31	2.93±0.06	0.00±0.00
Day 7	0.00±0.00	1.87±0.12	3.47±0.06	0.00±0.00
	Minus Glucose Minus Phosphate			
Day 3	0.00±0.00	2.63±0.23	4.27±0.06	0.00±0.00
Day 5	0.00±0.00	2.17±0.06	4.33±0.00	0.00±0.00
Day 7	0.00±0.00	0.83±0.00	6.97±0.15	0.00±0.00
	Minus Glucose Minus Nitrate			
Day 3	0.00±0.00	1.13±0.23	1.03±0.06	0.00±0.00
Day 5	0.00±0.00	0.87±0.06	1.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.17±0.15	0.00±0.00
	Minus Glucose Minus Glycerol			
Day 3	0.00±0.00	0.70±0.10	0.60±0.10	0.00±0.00
Day 5	0.00±0.00	0.63±0.15	0.37±0.06	0.00±0.00
Day 7	0.00±0.00	0.30±0.10	0.20±0.10	0.00±0.00

TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Minus Molasses Minus Proteose peptone			
Day 3	0.00±0.00	0.10±0.10	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.13±0.06	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.07±0.06	0.00±0.00	0.00±0.00
	Minus Molasses Minus NaCl			
Day 3	0.00±0.00	2.27±0.12	0.57±0.25	0.00±0.00
Day 5	0.00±0.00	1.40±0.40	0.10±0.10	0.00±0.00
Day 7	0.00±0.00	0.83±0.15	0.17±0.06	0.00±0.00
	Minus Molasses Minus Phosphate			
Day 3	0.00±0.00	3.27±0.31	0.50±0.10	0.00±0.00
Day 5	0.00±0.00	0.73±0.64	0.53±0.06	0.00±0.00
Day 7	0.00±0.00	0.23±0.21	0.47±0.06	0.00±0.00
	Minus Molasses Minus Nitrate			
Day 3	0.00±0.00	0.90±0.31	0.53±0.10	0.00±0.00
Day 5	0.00±0.00	0.73±0.64	0.53±0.06	0.00±0.00
Day 7	0.00±0.00	0.23±0.21	0.47±0.06	0.00±0.00
	Minus Molasses Minus Glycerol			
Day 3	0.00±0.00	0.50±0.10	0.33±0.06	0.00±0.00
Day 5	0.00±0.00	0.33±0.06	0.37±0.06	0.00±0.00
Day 7	0.00±0.00	0.10±0.12	0.00±0.06	0.00±0.00
	Minus Proteose peptone Minus NaCl			
Day 3	0.00±0.00	0.33±0.06	1.60±2.08	0.00±0.00
Day 5	0.00±0.00	0.37±0.00	0.33±0.00	0.00±0.00
Day 7	0.00±0.00	0.67±0.00	0.27±0.00	0.00±0.00
	Minus Proteose peptone Minus Phosphate			
Day 3	0.00±0.00	0.27±0.06	0.43±0.06	0.00±0.00
Day 5	0.00±0.00	0.00±0.06	0.00±0.29	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.06	0.00±0.00
	Minus Proteose peptone Minus Nitrate			
Day 3	0.00±0.00	0.20±0.10	0.20±0.10	0.00±0.00
Day 5	0.00±0.00	0.07±0.06	0.33±0.06	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.47±0.00	0.00±0.00

TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Minus Proteose peptone Minus Glycerol			
Day 3	0.00±0.00	0.53±0.06	0.60±0.00	0.00±0.00
Day 5	0.00±0.00	0.07±0.06	0.13±0.10	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.06	0.00±0.00
	Minus Glycerol Minus NaCl			
Day 3	0.00±0.00	1.67±0.15	2.47±0.06	0.00±0.00
Day 5	0.00±0.00	0.13±0.10	0.30±0.06	0.00±0.00
Day 7	0.00±0.00	0.00±0.10	0.17±0.12	0.00±0.00
	Minus Glycerol Minus Phosphate			
Day 3	0.00±0.00	1.37±0.15	2.47±0.06	0.00±0.00
Day 5	0.00±0.00	1.10±0.10	1.17±0.06	0.00±0.00
Day 7	0.00±0.00	0.50±0.06	0.67±0.12	0.00±0.00
	Minus Glycerol Minus Nitrate			
Day 3	0.00±0.00	0.10±0.10	0.13±0.06	0.00±0.00
Day 5	0.00±0.00	0.23±0.06	0.03±0.10	0.10±0.10
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Table 19. Biosurfactant production with a single nutrient component added for Wewoka formation fluids.

Diameter of Oil Spreading (cm) for optimal nutrient treatment package.				
Time (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Glucose only			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Molasses only			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Proteose peptone only			
Day 3	0.00±0.00	0.00±0.00	0.13±0.23	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Glycerol only			
Day 3	0.00±0.00	0.00±0.00	0.13±0.23	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Phosphate only			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Nitrate only			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Table 20. Biosurfactant production with double nutrient component added for Wewoka formation fluids.

Diameter of Oil Spreading (cm) for optimal nutrient treatment package.				
TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Plus Glucose Plus Proteose peptone			
Day 3	0.00±0.00	3.07±0.38	0.23±0.21	0.00±0.00
Day 5	0.00±0.00	2.97±0.40	0.90±0.17	0.00±0.00
Day 7	0.00±0.00	0.03±0.06	0.83±0.15	0.00±0.00
	Plus Glucose Plus Molasses			
Day 3	0.00±0.00	0.53±0.06	0.40±0.00	0.00±0.00
Day 5	0.00±0.00	0.27±0.06	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.33±0.06	0.00±0.00	0.00±0.00
	Plus Glucose Plus NaCl			
Day 3	0.00±0.00	0.27±0.06	0.40±0.00	0.00±0.00
Day 5	0.00±0.00	0.53±0.06	0.37±0.06	0.00±0.00
Day 7	0.00±0.00	0.03±0.06	0.13±0.23	0.00±0.00
	Plus Glucose Plus Phosphate			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Glucose Plus Nitrate			
Day 3	0.00±0.00	0.20±0.10	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.50±0.10	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.30±0.06	0.47±0.06	0.00±0.00
	Plus Glucose Plus Glycerol			
Day 3	0.00±0.00	0.17±0.06	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.17±0.06	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Molasses Plus Proteose peptone			
Day 3	0.00±0.00	0.57±0.06	0.57±0.15	0.00±0.00
Day 5	0.00±0.00	0.80±0.20	4.83±1.04	0.00±0.00
Day 7	0.00±0.00	0.63±0.15	1.20±0.30	0.00±0.00

TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Plus Molasses Plus NaCl			
Day 3	0.00±0.00	0.37±0.06	0.57±0.06	0.00±0.00
Day 5	0.00±0.00	1.43±0.40	0.87±0.32	0.00±0.00
Day 7	0.00±0.00	0.50±0.10	0.50±0.10	0.00±0.00
	Plus Molasses Plus Nitrate			
Day 3	0.00±0.00	1.10±0.10	3.53±0.45	0.00±0.00
Day 5	0.00±0.00	0.73±0.32	2.93±0.21	0.00±0.00
Day 7	0.00±0.00	0.53±0.06	3.53±0.45	0.00±0.00
	Plus Molasses Plus Phosphate			
Day 3	0.00±0.00	0.17±0.06	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.80±0.20	1.57±0.86	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	1.80±0.36	0.00±0.00
	Plus Molasses Plus Glycerol			
Day 3	0.00±0.00	0.20±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.47±0.06	3.30±0.82	0.00±0.00
Day 7	0.00±0.00	0.37±0.06	3.47±1.08	0.00±0.00
	Plus Proteose peptone Plus NaCl			
Day 3	0.00±0.00	0.15±0.07	0.53±0.06	0.00±0.00
Day 5	0.00±0.00	0.07±0.06	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Proteose peptone Plus Nitrate			
Day 3	0.00±0.00	0.10±0.17	0.50±0.17	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Proteose peptone Plus Phosphate			
Day 3	0.00±0.00	0.00±0.00	0.43±0.06	0.00±0.00
Day 5	0.00±0.00	0.000.00	0.43±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Proteose peptone Plus Glycerol			
Day 3	0.00±0.00	0.17±0.06	0.37±0.32	0.00±0.00
Day 5	0.00±0.00	2.63±0.15	3.300.82	0.00±0.00
Day 7	0.00±0.00	1.30±0.17	0.00±0.00	0.00±0.00

TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Plus Glycerol Plus NaCl			
Day 3	0.00±0.00	0.10±0.10	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.93±0.12	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.53±0.06	0.00±0.00	0.00±0.00
	Plus Glycerol Plus Nitrate			
Day 3	0.00±0.00	0.03±0.06	0.27±0.23	0.00±0.00
Day 5	0.00±0.00	0.57±0.06	0.50±0.10	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Glycerol Plus Phosphate			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Appendix III
Aerobic Nutrient treatment for Earlsboro formation

Table 21. Biosurfactant production with a single nutrient component deleted for Earlsboro formation fluids.

Oil Spreading (cm) for the optimal aerobic nutrient treatment package				
TIME (Days)	MEDIUM + BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Everything added			
Day 3	0.00±0.00	0.20±0.10	2.53±0.06	0.00±0.00
Day 5	0.00±0.00	0.60±0.20	4.53±0.42	0.00±0.00
Day 7	0.00±0.00	0.33±0.06	4.50±0.10	0.00±0.00
	Minus Glucose			
Day 3	0.00±0.00	0.33±0.06	1.27±0.40	0.00±0.00
Day 5	0.00±0.00	0.40±0.10	0.47±0.12	0.00±0.00
Day 7	0.00±0.00	0.33±0.06	0.73±0.21	0.00±0.00
	Minus Proteose peptone			
Day 3	0.00±0.00	0.33±0.06	0.37±0.15	0.00±0.00
Day 5	0.00±0.00	0.13±0.15	0.30±0.26	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Minus Molasses			
Day 3	0.00±0.00	0.30±0.00	0.27±0.06	0.00±v
Day 5	0.00±0.00	0.63±0.06	0.17±0.06	0.00±0.00
Day 7	0.00±0.00	0.20±0.10	0.00±0.00	0.00±0.00
	Minus NaCl			
Day 3	0.00±0.00	0.47±0.06	0.67±0.21	0.00±0.00
Day 5	0.00±0.00	2.40±0.10	2.57±0.06	0.00±0.00
Day 7	0.00±0.00	0.80±0.20	1.00±0.10	0.00±0.00
	Minus Nitrate			
Day 3	0.00±0.00	0.53±0.06	0.53±0.06	0.00±0.00
Day 5	0.00±0.00	0.63±0.06	0.20±0.10	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Minus Phosphate			
Day 3	0.00±0.00	0.43±0.12	0.43±0.06	0.00±0.00
Day 5	0.00±0.00	2.77±0.06	3.17±0.15	0.00±0.00
Day 7	0.00±0.00	1.03±0.06	2.40±0.17	0.00±0.00

TIME (Days)	MEDIUM + BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Minus Glycerol			
Day 3	0.00±0.00	0.43±0.06	0.57±0.06	0.00±0.00
Day 5	0.00±0.00	0.50±0.10	1.00±0.10	0.00±0.00
Day 7	0.00±0.00	0.37±0.12	0.47±0.06	0.00±0.06

Table 22. Biosurfactant production with double nutrient components deleted for Earlsboro formation fluids.

Oil Spreading (cm) for the optimal aerobic nutrient treatment package for the Earlsboro formation fluids				
TIME (Days)	MEDIUM + BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Minus Glucose Minus Molasses			
Day 3	0.00±0.00	0.10±0.10	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.67±0.12	0.27±0.06	0.00±0.00
Day 7	0.00±0.00	0.43±0.06	0.20±0.10	0.00±0.00
	Minus Glucose Minus Proteose peptone			
Day 3	0.00±0.00	0.03±0.06	0.63±0.06	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.70±0.10	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.80±0.26	0.00±0.00
	Minus Glucose Minus NaCl			
Day 3	0.00±0.00	0.90±0.10	2.50±0.10	0.00±0.00
Day 5	0.00±0.00	0.77±0.15	0.37±0.06	0.00±0.00
Day 7	0.00±0.00	0.47±0.06	0.00±0.06	0.00±0.00
	Minus Glucose Minus Phosphate			
Day 3	0.00±0.00	3.13±0.15	4.53±0.06	0.00±0.00
Day 5	0.00±0.00	2.53±0.46	4.53±0.06	0.00±0.00
Day 7	0.00±0.00	1.70±0.26	3.97±0.15	0.00±0.00
	Minus Glucose Minus Nitrate			
Day 3	0.00±0.00	0.37±0.06	0.67±0.21	0.00±0.00
Day 5	0.00±0.00	0.57±0.06	0.57±0.06	0.00±0.00
Day 7	0.00±0.00	0.20±0.10	0.20±0.10	0.00±0.00
	Minus Glucose Minus Glycerol			
Day 3	0.00±0.00	0.90±0.10	1.00±0.00	0.00±0.00
Day 5	0.00±0.00	1.63±0.15	0.63±0.25	0.00±0.00
Day 7	0.00±0.00	0.50±0.10	0.37±0.32	0.00±0.00

TIME (Days)	MEDIUM + BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Minus Molasses Minus Proteose peptone			
Day 3	0.00±0.00	0.47±0.06	0.30±0.10	0.00±0.00
Day 5	0.00±0.00	0.27±0.06	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Minus Molasses Minus NaCl			
Day 3	0.00±0.00	0.27±0.06	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.57±0.06	0.10±0.10	0.00±0.00
Day 7	0.00±0.00	0.20±0.10	0.27±0.06	0.00±0.00
	Minus Molasses Minus Phosphate			
Day 3	0.00±0.00	1.03±0.06	0.80±0.10	0.00±0.00
Day 5	0.00±0.00	1.07±0.12	0.67±0.21	0.00±0.00
Day 7	0.00±0.00	0.47±0.15	0.27±0.23	0.00±0.00
	Minus Molasses Minus Nitrate			
Day 3	0.00±0.00	0.47±0.06	1.20±0.10	0.00±0.00
Day 5	0.00±0.00	0.67±0.12	0.40±0.10	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Minus Molasses Minus Glycerol			
Day 3	0.00±0.00	0.33±0.06	0.33±0.06	0.00±0.00
Day 5	0.00±0.00	0.43±0.06	0.23±0.15	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Minus Proteose peptone Minus NaCl			
Day 3	0.00±0.00	4.30±0.26	2.53±0.06	0.00±0.00
Day 5	0.00±0.00	2.63±0.15	2.20±0.10	0.00±0.00
Day 7	0.00±0.00	0.77±0.15	0.53±0.12	0.00±0.00
	Minus Proteose peptone Minus Phosphate			
Day 3	0.00±0.00	0.00±0.00	0.50±0.10	0.00±0.00
Day 5	0.00±0.00	0.60±0.00	0.53±0.06	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.23±0.06	0.00±0.00
	Minus Proteose peptone Minus Nitrate			
Day 3	0.00±0.00	0.20±0.00	0.20±0.10	0.00±0.00
Day 5	0.00±0.00	0.20±0.10	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

TIME (Days)	MEDIUM + BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Minus Proteose peptone Minus Glycerol			
Day 3	0.00±0.00	0.13±0.06	0.30±0.10	0.00±0.00
Day 5	0.00±0.00	0.20±0.00	0.33±0.15	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.17±0.06	0.00±0.00
	Minus Glycerol Minus NaCl			
Day 3	0.00±0.00	0.30±0.10	1.43±0.21	0.00±0.00
Day 5	0.00±0.00	0.47±0.15	0.80±0.10	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Minus Glycerol Minus Phosphate			
Day 3	0.00±0.00	1.30±0.17	2.47±0.12	0.00±0.00
Day 5	0.00±0.00	1.53±0.06	1.50±0.30	0.00±0.00
Day 7	0.00±0.00	0.67±0.21	0.53±0.06	0.00±0.00
	Minus Glycerol Minus Nitrate			
Day 3	0.00±0.00	0.30±0.10	0.50±0.10	0.00±0.00
Day 5	0.00±0.00	0.33±0.15	0.40±0.10	0.00±0.00
Day 7	0.00±0.00	0.50±0.10	0.00±0.00	0.00±0.00

Table 23. Biosurfactant production a single nutrient component added for Earlsboro formation fluids.

Oil Spreading (cm) for the optimal aerobic nutrient treatment package				
TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Glucose only			
Day 3	0.00±0.00	0.00±0.00	0.13±0.06	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.13±0.12	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.10±0.00	0.00±0.00
	Molasses only			
Day 3	0.00±0.00	0.00±0.00	0.20±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Protoese peptone only			
Day 3	0.00±0.00	0.60±0.17	0.53±0.06	0.00±0.00
Day 5	0.00±0.00	0.53±0.06	0.30±0.10	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Glycerol only			
Day 3	0.00±0.00	0.20±0.10	0.33±0.06	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.20±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Phosphate only			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Nitrate only			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Table 24. Biosurfactant production with double nutrient components added for Earlsboro formation fluids.

Oil Spreading (cm) for the optimal aerobic nutrient treatment package				
TIME (Days)	MEDIUM + BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Plus Glucose Plus Proteose peptone			
Day 3	0.00±0.00	0.63±0.06	0.60±0.10	0.00±0.00
Day 5	0.00±0.00	2.10±0.10	0.90±0.17	0.00±0.00
Day 7	0.00±0.00	1.00±0.10	0.17±0.06	0.00±0.00
	Plus Glucose Plus Molasses			
Day 3	0.00±0.00	0.00±v	0.40±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Glucose Plus NaCl			
Day 3	0.00±0.00	0.37±0.06	0.30±0.26	0.00±0.00
Day 5	0.00±0.00	0.43±0.12	0.20±0.10	0.00±0.00
Day 7	0.00±0.00	0.13±0.06	0.00±0.00	0.00±0.00
	Plus Glucose Plus Phosphate			
Day 3	0.00±0.00	0.17±0.06	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.20±0.10	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Glucose Plus Nitrate			
Day 3	0.00±0.00	0.23±0.06	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.17±0.15	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Glucose Plus Glycerol			
Day 3	0.00±0.00	0.13±0.06	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.27±0.06	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Molasses Plus Proteose peptone			
Day 3	0.00±0.00	0.60±0.10	0.53±0.06	0.00±0.00
Day 5	0.00±0.00	0.50±0.10	4.77±0.74	0.00±0.00
Day 7	0.00±0.00	0.23±0.15	1.20±0.40	0.00±0.00

TIME (Days)	MEDIUM + BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Plus Molasses Plus NaCl			
Day 3	0.00±0.00	0.00±0.00	0.57±0.06	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.83±0.21	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.50±0.10	0.00±0.00
	Plus Molasses Plus Nitrate			
Day 3	0.00±0.00	1.63±0.15	2.43±0.80	0.00±0.00
Day 5	0.00±0.00	2.17±0.06	2.93±0.21	0.00±0.00
Day 7	0.00±0.00	1.27±0.21	3.53±0.45	0.00±0.00
	Plus Molasses Plus Phosphate			
Day 3	0.00±0.00	0.10±0.10	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Molasses Plus Glycerol			
Day 3	0.00±0.00	0.37±0.06	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.17±0.06	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Proteose peptone Plus NaCl			
Day 3	0.00±0.00	0.47±0.15	0.53±0.06	0.00±0.00
Day 5	0.00±0.00	0.30±0.10	0.20±0.10	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Proteose peptone Plus Nitrate			
Day 3	0.00±0.00	0.53±0.06	0.47±0.06	0.00±0.00
Day 5	0.00±0.00	0.60±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.30±0.00	0.00±0.00	0.00±0.00
	Plus Proteose peptone Plus Phosphate			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Proteose peptone Plus Glycerol			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

TIME (Days)	MEDIUM + BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Plus Glycerol Plus NaCl			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Glycerol Plus Nitrate			
Day 3	0.00±0.00	0.00±0.00	0.27±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.17±0.06	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.130.06	0.00±0.00
	Plus Glycerol Plus Phosphate			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

APPENDIX IV
Aerobic Nutrient Treatment for Gilcrease Formation

Table 25. Biosurfactant production with a single nutrient component deleted for Gilcrease formation fluids.

Oil Spreading (cm) for aerobic optimal nutrient treatment package				
TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Everything			
Day 3	0.00±0.00	0.53±0.06	1.27±0.12	0.00±0.00
Day 5	0.00±0.00	1.53±0.06	3.10±0.10	0.00±0.00
Day 7	0.00±0.00	2.13±0.06	2.27±0.21	0.00±0.00
	Minus Glucose			
Day 3	0.00±0.00	0.60±0.00	2.20±0.10	0.00±0.00
Day 5	0.00±0.00	1.47±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.47±0.06	0.00±0.00	0.00±0.00
	Minus Proteose peptone			
Day 3	0.00±0.00	0.27±0.06	1.23±0.15	0.00±0.00
Day 5	0.00±0.00	0.53±0.06	0.57±0.06	0.00±0.00
Day 7	0.00±0.00	0.37±0.06	0.57±0.06	0.00±0.00
	Minus Molasses			
Day 3	0.00±0.00	0.23±0.06	1.03±0.15	0.00±0.00
Day 5	0.00±0.00	0.53±0.06	0.47±0.06	0.00±0.00
Day 7	0.00±0.00	0.20±0.10	0.30±0.00	0.00±0.00
	Minus NaCl			
Day 3	0.00±0.00	2.33±0.15	3.20±0.10	0.00±0.00
Day 5	0.00±0.00	1.90±0.10	3.00±0.20	0.00±0.00
Day 7	0.00±0.00	1.10±0.10	1.17±0.29	0.00±0.00
	Minus Nitrate			
Day 3	0.00±0.00	1.97±0.15	2.53±0.06	0.00±0.00
Day 5	0.00±0.00	1.40±0.17	1.65±1.22	0.00±0.00
Day 7	0.00±0.00	0.57±0.06	0.73±0.21	0.00±0.00
	Minus Phosphate			
Day 3	0.00±0.00	0.70±0.10	2.20±0.52	0.00±0.00
Day 5	0.00±0.00	1.63±0.15	3.63±0.15	0.00±0.00
Day 7	0.00±0.00	2.37±0.32	2.50±0.10	0.00±0.00

TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Minus Glycerol			
Day 3	0.00±0.00	0.63±0.06	1.53±0.06	0.00±0.00
Day 5	0.00±0.00	0.70±0.10	1.80±0.20	0.00±0.00
Day 7	0.00±0.00	0.40±0.10	0.60±0.10	0.00±0.00

Table 26. Biosurfactant production with double nutrient components deleted for the Gilcrease formation fluids.

Oil Spreading (cm) for aerobic optimal nutrient treatment package for Gilcrease formation fluids				
TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Minus Glucose Minus Molasses			
Day 3	0.00±0.00	0.20±0.10	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.20±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Minus Glucose Minus Proteose peptone			
Day 3	0.00±0.00	0.53±0.06	0.63±0.06	0.00±0.00
Day 5	0.00±0.00	0.27±0.06	0.90±0.10	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.43±0.06	0.00±0.00
	Minus Glucose Minus NaCl			
Day 3	0.00±0.00	0.60±0.10	2.77±0.38	0.00±0.00
Day 5	0.00±0.00	1.63±0.15	3.27±0.06	0.00±0.00
Day 7	0.00±0.00	0.23±0.21	0.67±0.12	0.00±0.00
	Minus Glucose Minus Phosphate			
Day 3	0.00±0.00	0.63±0.06	3.23±0.06	0.00±0.00
Day 5	0.00±0.00	1.43±0.21	4.63±0.15	0.00±0.00
Day 7	0.00±0.00	0.63±0.15	3.27±0.06	0.00±0.00
	Minus Glucose Minus Nitrate			
Day 3	0.00±0.00	0.37±0.32	0.53±0.06	0.00±0.00
Day 5	0.00±0.00	1.30±0.17	1.10±0.10	0.00±0.00
Day 7	0.00±0.00	0.57±0.06	0.50±0.10	0.00±0.00
	Minus Glucose Minus Glycerol			
Day 3	0.00±0.00	2.60±2.00	0.77±0.15	0.00±0.00
Day 5	0.00±0.00	1.10±0.10	1.03±0.06	0.00±0.00
Day 7	0.00±0.00	0.47±0.12	0.50±0.00	0.00±0.00

TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Minus Molasses Minus Proteose peptone			
Day 3	0.00±0.00	0.17±0.06	0.53±0.06	0.00±0.00
Day 5	0.00±0.00	0.10±0.10	0.30±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.10±0.10	0.00±0.00
	Minus Molasses Minus NaCl			
Day 3	0.00±0.00	0.37±0.06	0.63±0.15	0.00±0.00
Day 5	0.00±0.00	0.83±0.06	1.37±0.32	0.00±0.00
Day 7	0.00±0.00	0.37±0.06	0.53±0.06	0.00±0.00
	Minus Molasses Minus Phosphate			
Day 3	0.00±0.00	0.30±0.10	2.33±0.12	0.00±0.00
Day 5	0.00±0.00	0.63±0.15	2.00±0.20	0.00±0.00
Day 7	0.00±0.00	0.30±0.00	0.33±0.15	0.00±0.00
	Minus Molasses Minus Nitrate			
Day 3	0.00±0.00	0.20±0.00	0.30±0.00	0.00±0.00
Day 5	0.00±0.00	0.30±0.00	0.23±0.06	0.00±0.00
Day 7	0.00±0.00	0.17±0.06	0.00±0.00	0.00±0.00
	Minus Molasses Minus Glycerol			
Day 3	0.00±0.00	0.53±0.06	0.70±0.00	0.00±0.00
Day 5	0.00±0.00	0.43±0.06	0.63±0.06	0.00±0.00
Day 7	0.00±0.00	0.27±0.06	0.33±0.15	0.00±0.00
	Minus Proteose peptone Minus NaCl			
Day 3	0.00±0.00	0.63±0.06	0.87±0.06	0.00±0.00
Day 5	0.00±0.00	0.70±0.10	0.80±0.00	0.00±0.00
Day 7	0.00±0.00	0.23±0.06	0.13±0.12	0.00±0.00
	Minus Proteose peptone Minus Phosphate			
Day 3	0.00±0.00	0.00±0.00	0.30±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.47±0.12	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.40±0.00	0.00±0.00
	Minus Proteose peptone Minus Nitrate			
Day 3	0.00±0.00	0.37±0.06	0.77±0.06	0.00±0.00
Day 5	0.00±0.00	0.77±0.15	0.63±0.15	0.00±0.00
Day 7	0.00±0.00	0.40±0.10	0.33±0.06	0.00±0.00

TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Minus Proteose peptone Minus Glycerol			
Day 3	0.00±0.00	0.37±0.06	0.80±0.00	0.00±0.00
Day 5	0.00±0.00	0.47±0.12	1.03±0.00	0.00±0.00
Day 7	0.00±0.00	0.57±0.06	0.33±0.15	0.00±0.00
	Minus Glycerol Minus NaCl			
Day 3	0.00±0.00	0.53±0.00	2.53±0.64	0.00±0.00
Day 5	0.00±0.00	1.00±0.00	2.80±0.20	0.00±0.00
Day 7	0.00±0.00	0.20±0.10	0.00±0.00	0.00±0.00
	Minus Glycerol Minus PO4			
Day 3	0.00±0.00	0.43±0.06	1.87±0.12	0.00±0.00
Day 5	0.00±0.00	0.77±0.15	1.63±0.15	0.00±0.00
Day 7	0.00±0.00	0.53±0.06	1.10±0.10	0.00±0.00

Table 27. Biosurfactant production with a single nutrient component added for the Gilcrease formation fluids.

Oil Spreading (cm) for aerobic optimal nutrient treatment package for Gilcrease formation fluids				
TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+ NOCULUM	MEDIUM ONLY
	Glucose only			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Molasses only			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Proteose peptone only			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Glycerol only			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Phosphate only			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Nitrate only			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Table 28. Biosurfactant production with double nutrient components added for the Gilcrease formation fluids.

Oil Spreading (cm) for aerobic optimal nutrient treatment package				
TIME (Days)	MEDIUM + BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Plus Glucose Plus Proteose peptone			
Day 3	0.00±0.00	0.67±0.06	0.43±0.06	0.00±0.00
Day 5	0.00±0.00	2.00±0.20	1.03±0.21	0.00±0.00
Day 7	0.00±0.00	0.63±0.15	0.83±0.15	0.00±0.00
	Plus Glucose Plus Molasses			
Day 3	0.00±0.00	0.20±0.10	0.27±0.06	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Glucose Plus NaCl			
Day 3	0.00±0.00	0.33±0.29	0.33±0.06	0.00±0.00
Day 5	0.00±0.00	0.23±0.06	0.37±0.06	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.20±0.10	0.00±0.00
	Plus Glucose Plus Phosphate			
Day 3	0.00±0.00	0.20±0.10	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.20±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.17±0.12	0.00±0.00	0.00±0.00
	Plus Glucose Plus Nitrate			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Glucose Plus Glycerol			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Molasses Plus Proteose peptone			
Day 3	0.00±0.00	0.77±0.15	0.90±0.10	0.00±0.00
Day 5	0.00±0.00	1.07±0.06	1.27±0.21	0.00±0.00
Day 7	0.00±0.00	0.57±0.06	0.97±0.21	0.00±0.00

TIME (Days)	MEDIUM + BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Plus Molasses Plus NaCl			
Day 3	0.00±0.00	0.53±0.06	0.83±0.06	0.00±0.00
Day 5	0.00±0.00	1.07±0.06	1.40±0.17	0.00±0.00
Day 7	0.00±0.00	0.43±0.06	0.53±0.00	0.00±0.00
	Plus Molasses Plus Nitrate			
Day 3	0.00±0.00	1.80±0.20	1.87±0.12	0.00±0.00
Day 5	0.00±0.00	2.97±0.25	3.27±0.06	0.00±0.00
Day 7	0.00±0.00	3.17±0.15	3.37±0.23	0.00±0.00
	Plus Molasses Plus Phosphate			
Day 3	0.00±0.00	0.40±0.10	0.60±0.20	0.00±0.00
Day 5	0.00±0.00	0.53±0.06	0.70±0.17	0.00±0.00
Day 7	0.00±0.00	0.30±0.10	0.20±0.00	0.00±0.00
	Plus Molasses Plus Glycerol			
Day 3	0.00±0.00	1.53±0.06	1.33±0.12	0.00±0.00
Day 5	0.00±0.00	1.13±0.12	2.37±0.32	0.00±0.00
Day 7	0.00±0.00	1.40±0.35	1.93±0.12	0.00±0.00
	Plus Proteose peptone Plus NaCl			
Day 3	0.00±0.00	0.17	0.27	0.00
Day 5	0.00±0.00	0.10	0.20	0.00
Day 7	0.00±0.00	0.00	0.00	0.00
	Plus Proteose peptone Plus Nitrate			
Day 3	0.00±0.00	0.00±0.00	0.37±0.06	0.00±0.00
Day 5	0.00±0.00	0.20±0.00	0.17±0.06	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Proteose peptone Plus Phosphate			
Day 3	0.00±0.00	0.00±0.00	0.13±0.06	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.10±0.10	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Proteose peptone Plus Glycerol			
DAY3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
DAY5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
DAY7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

TIME (Days)	MEDIUM + BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Plus Glycerol Plus NaCl			
DAY3	0.00±0.00	0.30±0.10	0.00±0.00	0.00±0.00
DAY5	0.00±0.00	0.50±0.10	0.00±0.00	0.00±0.00
DAY7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Glycerol Plus NaNO3			
DAY3	0.00±0.00	0.30±0.10	0.33±0.06	0.00±0.00
DAY5	0.00±0.00	0.50±0.00	0.43±0.15	0.00±0.00
DAY7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Glycerol Plus PO4			
DAY3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
DAY5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
DAY7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

APPENDIX V
Aerobic Nutrient Treatment for the Skinner flood formation

Table 29. Biosurfactant production with a single nutrient component deleted for Skinner flood formation fluids

Oil Spreading (cm) for anaerobic optimal nutrient treatment package				
TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Everything			
Day 3	0.23±0.12	0.07±0.05	0.43±0.05	0.00±0.00
Day 5	0.37±0.12	0.27±0.12	0.73±0.05	0.00±0.00
Day 7	0.23±0.05	0.03±0.05	0.60±0.08	0.00±0.00
	Minus Glucose			
Day 3	0.20±0.00	0.13±0.05	0.50±0.08	0.00±0.00
Day 5	0.10±0.00	0.30±0.08	0.67±0.12	0.00±0.00
Day 7	0.10±0.08	0.00±0.00	2.53±0.42	0.00±0.00
	Minus Molasses			
Day 3	0.00±0.00	0.00±0.00	0.40±0.00	0.00±0.00
Day 5	0.13±0.05	0.40±0.08	0.63±0.05	0.00±0.00
Day 7	0.00±0.00	0.03±0.05	0.43±0.05	0.00±0.00
	Minus Nitrate			
Day 3	0.17±0.05	0.17±0.05	0.47±0.12	0.00±0.00
Day 5	0.17±0.05	0.40±0.08	0.63±0.09	0.00±0.00
Day 7	0.20±0.00	0.17±0.05	0.33±0.12	0.00±0.00

Table 30. Biosurfactant production with a single nutrient component added for Skinner flood formation fluids

Oil Spreading (cm) for anaerobic optimal nutrient treatment package				
TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Molasses only			
Day 3	0.13±0.05	0.10±0.00	0.33±0.05	0.00±0.00
Day 5	0.10±0.00	0.37±0.05	0.53±0.05	0.00±0.00
Day 7	0.20±0.00	0.13±0.05	0.40±0.00	0.00±0.00
	Glucose only			
Day 3	0.03±0.05	0.10±0.14	0.23±0.05	0.00±0.00
Day 5	0.10±0.08	0.13±0.19	0.47±0.05	0.00±0.00
Day 7	0.00±0.00	0.07±0.09	0.37±0.09	0.00±0.00

APPENDIX VI
Anaerobic Nutrient Treatment for Hunton Formation

Table 31. Biosurfactant production with a single nutrient component deleted for Hunton formation fluids.

Oil Spreading (cm) for anaerobic optimal nutrient treatment package				
TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Everything			
Day 3	0.13±0.06	0.47±0.06	0.53±0.06	0.00±0.00
Day 5	0.10±0.00	0.57±0.06	0.63±0.06	0.00±0.00
Day 7	0.00±0.00	0.40±0.00	0.37±0.06	0.00±0.00
Day 14	0.00±0.00	0.30±0.00	0.33±0.06	0.00±0.00
	Minus Glucose			
Day 3	0.20±0.00	0.43±0.05	0.47±0.09	0.00±0.00
Day 5	0.27±0.09	0.30±0.00	0.10±0.14	0.00±0.00
Day 7	0.13±0.09	0.37±0.05	0.33±0.12	0.00±0.00
Day 14	0.23±0.15	1.00±0.20	0.67±0.12	0.00±0.00
	Minus Molasses			
Day 3	0.03±0.05	0.20±0.00	0.47±0.05	0.00±0.00
Day 5	0.07±0.05	0.13±0.05	0.07±0.09	0.00±0.00
Day 7	0.10±0.00	0.03±0.05	0.03±0.05	0.00±0.00
Day 14	0.20±0.00	0.53±0.12	0.70±0.10	0.00±0.00
	Minus Nitrate			
Day 3	0.00±0.00	0.63±0.05	0.73±0.05	0.00±0.00
Day 5	0.20±0.16	0.20±0.08	0.07±0.05	0.00±0.00
Day 7	0.17±0.09	0.13±0.05	0.17±0.05	0.00±0.00
Day 14	0.13±0.06	0.57±0.06	0.47±0.06	0.00±0.00
	Minus Metals			
Day 3	0.00±0.00	0.57±0.26	0.20±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.23±0.05	0.00±0.00
Day 7	0.10±0.14	0.17±0.05	0.10±0.00	0.00±0.00
Day 14	0.23±0.06	0.40±0.00	0.57±0.06	0.00±0.00

Table 32. Biosurfactant production with a single nutrient component added for Hunton formation fluids.

Oil Spreading (cm) for anaerobic optimal nutrient treatment package for Hunton formation fluids				
TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Plus Glucose Only			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 14	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Molasses Only			
Day 3	0.17±0.12	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.10±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.13±0.05	0.00±0.00	0.00±0.00
Day 14	0.10±0.00	0.03±0.06	0.00±0.00	0.00±0.00
	Plus Nitrate Only			
Day 3	0.10±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.10±0.00	0.10±0.00	0.00±0.00	0.00±0.00
Day 14	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Metals Only			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.10±0.00	0.10±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 14	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Table 33. Biosurfactant production with double nutrient components added for Hunton formation fluids.

Oil Spreading (cm) for anaerobic optimal nutrient treatment package				
TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Plus Glucose Plus Molasses			
Day 3	0.07±0.09	0.13±0.09	0.08±0.02	0.00±0.00
Day 5	0.00±0.00	0.30±0.00	0.60±0.00	0.00±0.00
Day 7	0.07±0.09	0.27±0.05	0.20±0.00	0.00±0.00
Day 14	0.13±0.12	0.47±0.06	0.57±0.06	0.00±0.00
	Plus Glucose Plus NaNO3			
Day 3	0.03±0.05	0.07±0.05	0.03±0.05	0.00±0.00
Day 5	0.10±0.00	0.23±0.05	0.50±0.00	0.00±0.00
Day 7	0.00±0.00	0.07±0.05	0.03±0.05	0.00±0.00
Day 14	0.20±0.00	0.40±0.00	0.37±0.06	0.00±0.00
	Plus Glucose Plus Metals			
Day 3	0.00±0.00	0.03±0.05	0.07±0.05	0.00±0.00
Day 5	0.10±0.00	0.43±0.09	0.67±0.09	0.00±0.00
Day 7	0.17±0.05	0.13±0.05	0.10±0.08	0.00±0.00
Day 14	0.20±0.10	0.60±0.10	0.43±0.06	0.00±0.00
	Plus Molasses Plus Nitrate			
Day 3	0.10±0.08	0.23±0.12	0.27±0.05	0.00±0.00
Day 5	0.00±±0.00	1.17±0.05	1.07±0.05	0.00±0.00
Day 7	0.20±0.08	0.23±0.12	0.33±0.09	0.00±0.00
Day 14	0.23±0.06	1.27±0.06	1.53±0.06	0.00±0.00
	Plus Molasses Plus Metals			
Day 3	0.23±0.00	0.57±0.06	0.57±0.06	0.00±0.00
Day 5	0.23±0.09	0.37±0.05	0.43±0.05	0.00±0.00
Day 7	0.00±0.00	0.57±0.05	0.20±0.00	0.00±0.00
Day 14	0.07±0.05	0.05±0.04	0.17±0.05	0.00±0.00

APPENDIX VII
Anaerobic Nutrient Treatment for Wewoka Formation

Table 34. Biosurfactant production with a single nutrient component deleted for Wewoka formation fluids.

Oil Spreading (cm) for anaerobic optimal nutrient treatment package				
TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Everything			
Day 3	0.20±0.17	0.53±0.06	0.53±0.06	0.00±0.00
Day 5	0.10±0.00	0.63±0.06	0.80±0.00	0.00±0.00
Day 7	0.13±0.12	0.30±0.10	0.50±0.00	0.00±0.00
Day 14	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Minus Glucose			
Day 3	0.00±0.00	0.57±0.32	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.80±0.00	0.00±0.00	0.00±0.00
Day 7	0.07±0.06	0.57±0.06	0.00±0.00	0.00±0.00
Day 14	0.00±0.06	0.10±0.00	0.10±0.10	0.00±0.00
	Minus Molasses			
Day 3	0.00±0.00	0.73±0.12	0.53±0.12	0.00±0.00
Day 5	0.13±0.06	0.57±0.06	0.73±0.12	0.00±0.00
Day 7	0.00±0.00	0.37±0.06	0.50±0.00	0.00±0.00
Day 14	0.03±0.06	0.10±0.10	0.07±0.12	0.00±0.00
	Minus Nitrate			
Day 3	0.13±0.06	0.60±0.10	0.30±0.10	0.00±0.00
Day 5	0.10±0.00	0.53±0.06	0.53±0.06	0.00±0.00
Day 7	0.07±0.06	0.33±0.06	0.30±0.00	0.00±0.00
Day 14	0.00±0.00	0.03±0.06	0.07±0.06	0.00±0.00
	Minus Metals			
Day 3	0.17±0.06	0.47±0.06	0.57±0.06	0.00±0.00
Day 5	0.10±0.00	0.53±0.06	0.63±0.06	0.00±0.00
Day 7	0.00±0.00	0.47±0.06	0.47±0.06	0.00±0.00
Day 14	0.00±0.00	0.10±0.00	0.10±0.00	0.00±0.00

Table 35. Biosurfactant production with a single nutrient component added for Wewoka formation fluids.

Oil Spreading (cm) for anaerobic optimal nutrient treatment package				
TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Glucose only			
Day 3	0.00±0.00	0.20±0.10	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.13±0.06	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.17±0.06	0.00±0.00	0.00±0.00
Day 14	0.00±0.00	0.10±0.00	0.00±0.00	0.00±0.00
	Molasses only			
Day 3	0.20±0.00	0.20±0.00	0.40±0.00	0.00±0.00
Day 5	0.20±0.00	0.20±0.00	0.33±0.06	0.00±0.00
Day 7	0.10±0.00	0.20±0.00	0.23±0.12	0.00±0.00
Day 14	0.00±0.00	0.00±0.00	0.10±0.00	0.00±0.00
	Nitrate only			
Day 3	0.00±0.00	0.17±0.06	0.07±0.06	0.00±0.00
Day 5	0.00±0.00	0.20±0.10	0.20±0.10	0.00±0.00
Day 7	0.00±0.00	0.13±0.06	0.13±0.06	0.00±0.00
Day 14	0.10±0.00	0.20±0.00	0.10±0.00	0.00±0.00
	Metals only			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 14	0.00±0.00	0.00±0.00	0.00±0.00	0.10±0.00

Table 36. Biosurfactant production with double nutrient components added for Wewoka formation fluids.

Oil Spreading (cm) for anaerobic optimal nutrient treatment package				
TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM + INOCULUM	MEDIUM ONLY
	Plus Glucose Plus Molasses			
Day 3	0.00±0.00	0.17±0.06	0.33±0.06	0.00±0.00
Day 5	0.00±0.00	0.13±0.06	0.37±0.06	0.00±0.00
Day 7	0.00±0.00	0.13±0.12	0.30±0.10	0.00±0.00
Day 14	0.03±0.06	0.10±0.17	0.00±0.00	0.00±0.00
	Plus Glucose Plus Nitrate			
Day 3	0.17±0.06	0.33±0.06	0.37±0.06	0.00±0.00
Day 5	0.10±0.00	0.57±0.06	0.53±0.06	0.00±0.00
Day 7	0.17±0.06	0.47±0.06	0.57±0.06	0.00±0.00
Day 14	0.10±0.10	0.07±0.06	0.00±0.00	0.00±0.00
	Plus Glucose Plus Nitrate			
Day 3	0.17±0.06	0.33±0.06	0.37±v	0.00±0.00
Day 5	0.10±0.00	0.57±0.06	0.53±0.06	0.00±0.00
Day 7	0.17±0.06	0.47±v	0.57±0.06	0.00±0.00
Day 14	0.10±0.10	0.07±0.06	0.00±0.00	0.00±0.00
	Plus Glucose Plus Metals			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.53±0.06	0.50±0.10	0.00±0.00
Day 7	0.00±0.00	0.43±0.06	0.37±0.12	0.00±0.00
Day 14	0.00±0.00	0.00±0.00	0.00±0.00	0.07±0.00
	Plus Molasses Plus Nitrate			
Day 3	0.00±0.00	0.63±0.06	0.60±0.00	0.00±0.00
Day 5	0.00±0.00	0.83±0.06	0.67±0.06	0.00±0.00
Day 7	0.00±0.00	0.57±0.06	0.60±0.00	0.00±0.00
Day 14	0.17±0.15	0.20±0.20	0.00±0.00	0.00±0.00
	Plus Molasses Plus Metals			
Day 3	0.00±0.00	0.60±0.00	0.70±0.10	0.00±0.00
Day 5	0.00±0.00	0.57±0.06	0.50±0.10	0.00±0.00
Day 7	0.00±0.00	0.47±0.06	0.47±0.06	0.00±0.00
Day 14	0.03±0.06	0.10±0.00	0.00±0.00	0.00±0.00

APPENDIX VIII
Anaerobic Nutrient Treatment for Earlsboro Formation

TABLE 37. Biosurfactant production with a single nutrient component deleted for Earlsboro formation fluids.

Oil Spreading (cm) for anaerobic optimal nutrient treatment package				
TIME (Days)	MEDIUM+BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Everything			
Day 3	0.27±0.06	0.15±0.07	0.13±0.06	0.00±0.00
Day 5	0.13±0.06	0.10±0.00	0.07±0.06	0.00±0.00
Day 7	0.07±0.00	0.20±0.00	0.00±0.00	0.00±0.00
Day 14	0.08±0.03	0.07±0.12	0.02±0.03	0.00±0.00
	Minus Glucose			
Day 3	0.20±0.10	0.20±0.10	0.17±0.06	0.00±0.00
Day 5	0.15±0.07	0.15±0.07	0.11±0.08	0.00±0.00
Day 7	0.11±0.04	0.11±0.04	0.08±0.03	0.00±0.00
Day 14	0.07±0.03	0.07±0.03	0.06±0.03	0.00±0.00
	Minus Molasses			
Day 3	0.03±0.06	0.07±0.06	0.17±0.15	0.00±0.00
Day 5	0.05±0.02	0.06±0.01	0.16±0.01	0.00±0.00
Day 7	0.04±0.02	0.04±0.03	0.11±0.08	0.00±0.00
Day 14	0.03±0.01	0.03±0.02	0.07±0.05	0.00±0.00
	Minus Nitrate			
Day 3	0.20±0.00	0.23±0.06	0.20±0.20	0.00±0.00
Day 5	0.10±0.14	0.15±0.12	0.20±0.00	0.00±0.00
Day 7	0.08±0.07	0.11±0.05	0.13±0.12	0.00±0.00
Day 14	0.10±0.04	0.09±0.04	0.08±0.07	0.00±0.00
)	**Minus Metals**			
Day 3	0.30±0.17	0.10±0.00	0.10±0.00	0.00±0.00
Day 5	0.24±0.09	0.05±0.07	0.05±0.07	0.00±0.00
Day 7	0.17±0.07	0.04±0.04	0.04±0.04	0.00±0.00
Day 14	0.11±0.05	0.05±0.02	0.05±0.02	0.00±0.00

Table 38. Biosurfactant production with a single nutrient component added for Earlsboro formation fluids.

Oil Spreading (cm) for anaerobic optimal nutrient treatment package				
TIME (Days)	MEDIUM + BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Glucose only			
Day 3	0.00±0.00	0.10±0.00	0.00±0.00	0.00±0.00
Day 5	0.10±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 14	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Molasses Only			
Day 3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 5	0.10±0.00	0.20±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 14	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Nitrate Only			
Day 3	0.00±0.00	0.20±0.00	0.10±0.00	0.00±0.00
Day 5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 14	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Metals Only			
Day 3	0.00±0.00	0.10±0.00	0.00±0.00	0.00±0.00
Day 5	0.00±0.00	0.20±0.00	0.10±0.00	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.03±0.06	0.00±0.00
Day 14	0.00±0.00	0.00±0.00	0.03±0.03	0.00±0.00

Table 39. Biosurfactant production with double nutrient components added for Earlsboro formation fluids.

Oil Spreading (cm) for anaerobic optimal nutrient treatment package				
TIME (Days)	MEDIUM + BRINE	MEDIUM+BRINE+INOCULUM	MEDIUM+INOCULUM	MEDIUM ONLY
	Plus Glucose Plus Molasses			
Day 3	0.13±0.06	0.20±0.10	0.20±0.00	0.00±0.00
Day 5	0.20±0.00	0.07±0.06	0.20±0.00	0.00±0.00
Day 7	0.23±0.06	0.23±0.12	0.10±0.00	0.00±0.00
Day 14	0.10±0.12	0.14±0.09	0.03±0.06	0.00±0.00
	Plus Glucose Plus Nitrate			
Day 3	0.20±0.10	0.17±0.06	0.03±0.06	0.00±0.00
Day 5	0.03±0.06	0.17±0.06	0.07±0.06	0.00±0.00
Day 7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day 14	0.02±0.03	0.02±0.03	0.02±0.03	0.00±0.00
	Plus Glucose Plus Metals			
Day 3	0.00±0.00	0.07±0.06	0.07±0.06	0.00±0.00
Day 5	0.00±0.00	0.10±0.10	0.10±0.00	0.00±0.00
Day 7	0.07±0.06	0.07±0.06	0.03±0.06	0.00±0.00
Day 14	0.04±0.06	0.07±0.02	0.03±0.03	0.00±0.00
	Plus Molasses Plus Nitrate			
Day 3	0.13±0.06	0.40±0.10	0.30±0.10	0.00±0.00
Day 5	0.23±0.06	0.40±0.17	0.30±0.00	0.00±0.00
Day 7	0.23±0.06	0.50±0.10	0.30±0.10	0.00±0.00
Day 14	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Plus Molasses Plus Metals			
Day 3	0.03±0.06	0.13±0.06	0.10±0.00	0.00±0.00
Day 5	0.00±0.00	0.17±0.06	0.10±0.00	0.00±0.00
Day 7	0.00±0.00	0.13±0.06	0.13±0.06	0.00±0.00
Day 14	0.00±0.00	0.08±0.04	0.06±0.07	0.00±0.00